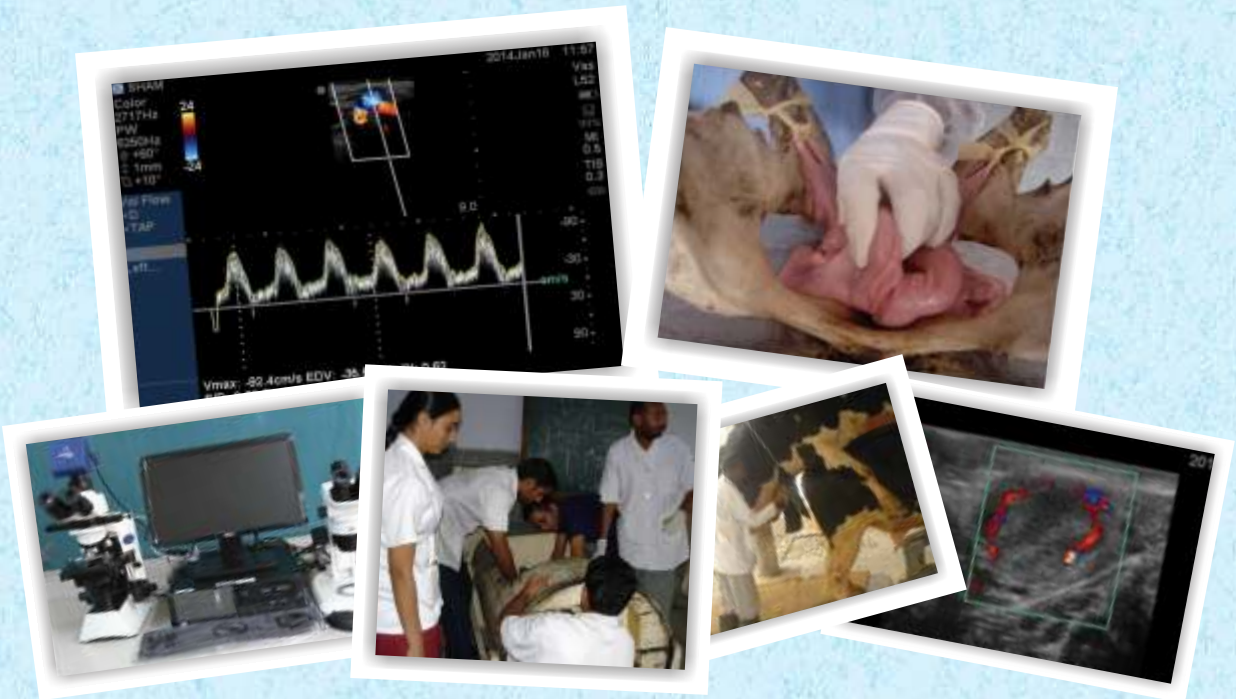
Adoption of Newer Reproductive Techniques in Education, Diagnostics and Research



Editors
SPS Ghuman, VK Gandotra, PS Brar

CENTRE OF ADVANCED FACULTY TRAINING IN
VETERINARY GYNAECOLOGY AND REPRODUCTION

(Advanced Training Course, September 10-30, 2014)

Department of Veterinary Gynaecology and Obstetrics
College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University,
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Preface

In the recent past, considerable developments have occurred in animal reproduction that has lead to understanding of the mechanisms controlling folliculogenesis, fertilization, maintenance of pregnancy and functioning of corpus luteum, along with exogenous control of ovulation and hormonal interventions for infertility. Many advanced techniques have come up like *in vitro* fertilization and sperm/embryo sexing. Ultrasonography, RIA/ELISA and bull fertility markers are making it easier to identify and resolve reproductive problems for the benefit of dairy farming community. Therefore, teachers and scientists shall learn and adopt the current know-how for the benefit of teaching and research community in their institutes.

The present course on "Adoption of Newer Reproductive Techniques in Education, Diagnostics and Research" from September 10 to 30, 2014, put emphasis on understanding the etiology and diagnosis of various reproductive problems through modern techniques and regulating fertility through appropriate interventions.

The format of this training course consisted of theoretical as well as practical training covering recent advances in most aspects related to newer reproductive techniques in education, diagnostics and research. The scientists delivered the expert lectures from this Centre of Advanced Faculty Training and from other departments of the College of Veterinary Science, Ludhiana. In addition to this, eminent scientists from National Institutes also interacted with the participants.

The lectures delivered during the training course have been compiled in the form of compendium, which will be a useful document for the participants and all other workers in the area of animal reproduction.

The requisite funds provided by the Indian Council of Agricultural Research, New Delhi for conducting the training programme are duly acknowledged. We thank for the timely help provided by Dean, Postgraduate Studies-cum-Coordinator of CAFT and Dean, College of Veterinary Science.

Last but not least, we gratefully acknowledge the contributions made by the organizing faculty and the non-teaching staff.

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Contents

Endocrine disruptors: a risk to fertility

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Increasing endocrine imbalance, genetic improvements for milk production, diversion of nutrients for growth and environmental factors have been depicted in the continuously increasing number of dairy animals suffering from reduced fertility. Pollutants viz, pesticides in the environment may play an important role, contributing at least to some extent to underlying causes of fertility problems in dairy livestock. Animals are exposed to a much larger amount and variety of pesticides. Although an individual component of a cocktail of pesticide residues may have no observable biological effect, but additive or synergistic impact of each residue in the mixture can exert significant effects. This makes critically important to assess the biological responses of the pesticide residues.

There is growing evidence regarding the adverse impact of certain pesticides on reproductive system, and such pesticides are known as 'reproductive toxicants' or 'endocrine disrupters'. These toxicants modulate and/or disrupt reproductive hormone milieu by acting at a variety of sites including hypothalamus, pituitary and reproductive organs. Published data in female of a species indicate that the reproductive functions can be compromised by exposure to pesticides resulting in infertility. The exposure of females to pesticides may cause alterations in reproductive behavior and contribute to sub-fecundity, infertility, pregnancy loss, growth retardation, intra-uterine fetal demise and ovarian failure. Higher concentrations of organochlorine pesticide residues were detected in the milk of women undergoing premature delivery and stillbirths than those undergoing full-term normal deliveries.

Understanding endocrine disruptors

The half lives of endocrine-disrupting pesticides in the environment range from days to years. Endocrine-disrupting organochlorine or organophosphate pesticides (OCPs or OPPs) may interfere with the synthesis, secretion, transport, metabolism, binding, action or elimination of endogenous hormones that are responsible for homeostasis, reproduction and developmental processes. These endocrine disruptors may act like endogenous steroids viz, estrogenic, anti-androgenic, androgenic or anti-estrogenic, through their interaction with receptors. The degree of affinity of endocrine disruptors for the hormone receptors is variable but is always much lower than the affinity of natural steroids by a factor of 100-1000. Some endocrine disruptors can get concentrated in glandular tissue to cause cell death and necrosis, thus the tissue responsible for hormone production is destroyed or

significantly reduced. Endocrine disruptors are biologically significant as they can exert effects on physiological systems at very low concentrations with orders of magnitude lower than those known to have acute toxic effects.

Fetus, a preferential site for endocrine disruptors

As the growth and development of fetus is highly controlled by the endocrine system, they are more vulnerable to pesticide exposure and may suffer overt or subtle lifelong health and reproductive abnormalities. The offspring at birth had dioxins concentrations that were 25% higher than those circulating in the mother. Following a low dose exposure to pesticides throughout life of mother, there is accumulation of lipophilic pesticides in the fat stores. These fat stores are rapidly mobilized during the energetically expensive periods of pregnancy and in particular lactation, and exert endocrine disrupting effects in late pregnancy and post-natal life. The underdeveloped reproductive and immune axes during fetal and post-natal life are exposed to unusually high concentrations of pesticides that cannot be easily metabolized or excreted at these stages of development. Moreover, these underdeveloped systems in the developing fetus are more prone to adverse impact of pesticides. The risk of pesticide exposure through feed for young animals is different from the adult because they consume milk and owing to the high fat content of milk the rates of pesticide exposure are relatively high.

Endocrine disruption at Hypothalamic-Pituitary level

On reproductive system, the endocrine disrupting effects of pesticides could be through their binding to estrogen, androgen or other receptors. Thus, at hypothalamus, pituitary and at reproductive organs, various endocrine disruptors may act either like endogenous steroids or can block the action of endogenous steroids. In females, an extensive damage to ovarian follicles disrupts the endocrine balance leading to a reduction in circulating estrogen and progesterone and an increase in follicle stimulating hormone and luteinizing hormone. In an *in vitro* study, progesterone and estrogen release was decreased after the exposure of bovine granulosa cells to OCPs at 0.0001 to 1.0 parts per billion (ppb) Various endocrine disrupting pesticides can indirectly change the balance of feedback control of the hypothalamus-pituitary-ovarian system. In males, an inverse correlation was reported between the presence of OCPs and OPPs in the blood or seminal plasma in one hand and blood testosterone concentrations or semen characteristics on the other. The adult ewes were maintained on sewage sludge irrigated pastures to address the real world exposure. In the pituitary glands of these ewes, the population and phenotype of gonadotrophes was altered. The alterations caused by EDCs to the hypothalamus-pituitary axis may have deleterious consequences for ovarian or testicular function and thus reproductive function and fertility.

Disruption in males

Pesticide residues can be detrimental to male reproductive system by causing toxicity to sperm plasma membrane as many lipophilic OCPs and PCBs have the ability to concentrate in seminal plasma and were detected in the seminal plasma of farm animals and humans. The presence of pesticide residues in the fluids surrounding spermatozoa could negatively influence spermatozoa cell function viz, spermatozoa density, motility and morphology. Organophosphorus pesticides can alter spermatozoa chromatin structure and DNA quality at different stages of spermatogenesis, and by disrupting the hypothalamic-pituitary-gonadal axis. The exposure of buffalo spermatozoa to pesticides (chlorpyrifos and endosulfan) negatively affected the spermatozoa plasma membrane integrity, mitochondrial membrane potential and fertilization competence. The pesticide residues, classified as epididymal toxicants, can alter the time required for sperm transport through the epididymis. During the course of fetal or early neonatal life, any disruption in the differentiation/multiplication of Sertoli cells in fetal testis by the environmental estrogens is detrimental for the adult reproductive potential because the capacity of an adult to produce sperm is determined by the Sertoli cells. The offspring born from the pregnant ewes reared on pasture fertilised with sewage sludge exhibited decrease in foetal blood testosterone levels alongside of reductions in Leydig, Sertoli and germ cell numbers. Male fertility is declining in many countries and perinatal hypospadias and cryptorchidism are associated risk factors for reduced sperm quality and testicular cancer in adulthood.

Disruption in females

The ability of lipophilic OCPs to concentrate in the bovine ovary and in follicular fluid makes them detrimental for granulosa cells. As the follicular cells surround oocytes at all stages of development, the pesticide residues may cause alterations within the follicular wall, which compromises its ability to maintain oocyte viability. The exposure of ovary to pesticides can have temporary or permanent impact on fertility depending upon the developmental stage at which damage occurs. However, the pesticides that extensively destroy oocytes contained in the primordial and primary follicles have a permanent effect on reproduction. A mixture of pesticides (1.0 ppb) exerted adverse impact on bovine oocyte maturation and embryonic development. The maturation stages of oocyte and early embryo have susceptibility even towards background doses of pesticide residues. The pesticide exposure during neonatal period causes damage to primordial follicles (germ cells) in ovaries thus producing irreversible form of infertility (sterility) in the adult dairy animals.

The disruptive impact of pesticides on estrus cycle activity of dairy cattle was shown in a study where transient exposure of an OCP, malathion at the onset of estrus led to inhibition of progesterone secretion and poor conception rate. From ovary, progesterone and estrogen release was decreased after exposure of bovine granulosa cells to OCPs at 0.0001 to 1.0

parts per billion (PPB). During luteolysis, a positive feedback loop existing between $PGF_{2\alpha}$ and oxytocin was altered by DDT and its metabolites.

In a field study in Punjab state, higher proportion of dairy animals reared in low-pesticide usage area were showing regular estrus cycles followed by successful conception, whereas, the proportion of animals exhibiting irregular estrous, anestrous or repeat breeding were more in high-pesticide usage area. Moreover, the proportion of dairy animals positive for pesticide residues as well as suffering from repeat breeding syndrome was high (24%) along with high serum pesticide residues (70.1±82.8 ng/ml). In contrast, the proportion of animals positive for a pesticide residue and exhibiting pregnancy was less (4%), and their serum pesticide residues were low (11.8±0.5 ng/ml). Furthermore, the major proportion of pesticide residue positive estrus cyclic, anestrous and repeat breeder animals were present in the high-pesticide usage area and had higher serum pesticide residues compared to their counterparts in low-pesticide usage area. These findings in pregnant animals in comparison to repeat breeder animals suggested that serum pesticide residues might play a role in the occurrence of reproductive disorders.

Embryo implantation is highly vulnerable to pesticide residue induced endocrine disruption even for an exposure period as short as 4 h at concentrations as low as 10nM or 2 ng/kg. Pesticides can interfere with the actions of many hormones and receptors essential for embryo implantation and embryo development. It was suggested that any event that delays ovulation results in chromosomal abnormalities and early embryonic death. Pesticide residues that delay the LH surge were associated with increased fetal loss in rats. There is also the potential for pesticide residues to speed up the rate of embryo transport through the oviduct, thus preventing implantation because of insufficient time for uterine preparation. In cattle, an environmentally relevant mixture of over 60 PCB congeners affected oocyte maturation, fertilization and embryo development at doses that ranged between 0.001 and 1 mg/ml, the minimum effective dose (0.001mg/ml) being approximately 10-fold lower than the mean level found in human follicular fluid in non-exposed women.

Organophosphorus pesticides have the potential to cause fetal death and increase the early resorption. The frequency of abortions was correlated with the presence of DDT or PCBs in the tissues of women and cows even though blood plasma concentrations of ovarian steroids were not changed. The early exposure to pesticide residues may contribute to a spectrum of diseases throughout life involving intra-uterine growth retardation, disorders of ovulation, metabolic syndrome and sensitivity to cancer.

In a recent field study, about 15.1 to 17.5% dairy animals of low pesticide usage and high-pesticide usage area were detected positive for pesticide residue(s), and the blood serum

concentrations of pesticide residues were high in dairy animals of the high-pesticide usage area compared to low pesticide usage area (27.5±21.0 vs 65.6±68.5 ng/ml). Actually, in dairy animals of a slaughterhouse, as well as low-pesticide usage and high-pesticide usage areas of Punjab state, the proportion of blood samples positive for >1 pesticide residue was 66.7%, 25.0% and 16.7%, respectively. In same study, the presence of pesticide residues in high concentrations in buffaloes subjected to slaughtering was suggested as the causative factors underlying their infertility scenario. About 36.2% blood samples, 58.6% ovarian tissue samples and 21.4% ovarian follicular fluid samples of the buffaloes subjected to slaughtering were detected positive for pesticide residues. Moreover, blood, ovarian and follicular fluid concentrations of pesticide residues were alarmingly high (211.0±284.0, 245.10±330.3 and 526.1±617.4 ng/ml, respectively) in slaughtered buffaloes compared to dairy animals of low-pesticide usage and low-pesticide usage areas (27.5±21.0 to 65.6±68.5 ng/ml) of Punjab state.

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Challenges in Bovine reproduction

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Poor fertility in buffalo is mainly attributed to late attainment of puberty, seasonal reproductive patterns, poor estrus expression and prolonged postpartum anestrus. Till date, there is meager genetic selection of buffalo for fertility. Nevertheless, to understand the reasons underlying poor fertility of buffaloes, numerous studies on buffalo endocrinology and exogenous hormone therapies are being carried out.

Delayed puberty

The age at onset of puberty (age at first estrus) is difficult to establish in buffalo heifers due to poor expression of estrus and is generally extrapolated from the age at first calving. Literature suggests that puberty is usually obtained when the heifers are 18-46 months old (most common: 26-30 months) with a body weight of 250-400 kg. The time elapsed between the first estrus and first conception varies between 50-440 days. Various factors viz., nutrition, management and season could influence the age of puberty. Nutritional management of buffalo calves should start from birth to ensure the attainment of appropriate body weight at an early age. Calves with body weight gain of 630 g / day may attain puberty at 600 days, whereas a gain of only 440 g / day may delay the puberty to 660 days. Under the routine management of Egyptian buffaloes, the age and body weight at puberty was 25 months and 310 kg, respectively, whereas under improved managemental practices, puberty was attained at 270 kg body weight. Moreover, Indian Murrah buffaloes kept under routine management practices, attained puberty at 36 months with 360 kg body weight, whereas improved management and use of water sprinklers during the summer season decreased the pubertal age by 3 months at the body weight of 322 kg. Even in buffaloes with appropriate body weight, seasonal depression of estrus activity can delay the age at puberty. Early induction of puberty ($\leq$ 20 months) was obtained with the utilization of a progesterone releasing intravaginal device (PRID) for 12 days along with equine chorionic gonadotrophin (eCG) at the time of PRID removal. This protocol was better for estrus induction in non-cycling buffalo heifers that exhibited 60% conception rate (CR) in comparison to 22% in controls.

Seasonal pattern of reproduction

Maximum percentage of Indian buffaloes exhibit estrus during short day-length (74%) and the minimum during long day-length (26%). Moreover, Murrah breed displays an

abbreviated duration of estrus during summer and highest cases of unobserved (silent) estrus were observed in April (70%) and lowest in December (10%). Calving season in Indian buffaloes is between July-December and the calving interval is longer for those calving between February-June, indicating that: a) resumption of post-partum ovarian activity is delayed in summer calvers compared to those calving during the breeding season (from 38-64 to 116-148 days), and b) there is a decrease in the conception rate during the spring and summer. In fact, during winter, early embryonic mortality in buffaloes was 20% which increased to 45% in summer. To reduce the impact of low breeding season on fertility, calving of buffaloes should be avoided during the summer season. This can be achieved by synchronization of estrus along with breeding during the winter season.

During off-breeding season, true anestrous buffaloes exhibit a clear follicular wave pattern with the dominant follicle attaining ovulatory size (>12mm) but failing to ovulate. Endocrinological studies of buffalo during summer season reveal suboptimal functioning of the hypothalamus-pituitary gonadal axis, low FSH and LH release, variable plasma progesterone and elevated plasma prolactin. This off-breeding season coincides with the period of highest temperatures, relative humidity and day lengths. Thus, seasonal suppression of reproductive activity in buffaloes is suggested to be due to high environmental stress together with under-nutrition due to lack of green fodder. However, a distinct seasonal reproductive pattern is also found in buffaloes of temperate regions (Italy) where buffaloes are fed on a constant balanced diet throughout the year. Moreover, reproductive period of buffalo is longer near the Equator where the light and dark ratio is steady throughout the year and reproductive period is mostly dictated by the nutritional status. Thus, reproductive seasonality in buffaloes does not appear to depend on nutritional status, whereas climate and especially photoperiod may play a key role.

Endocrine signal of the light-dark rhythm is a pineal gland hormone (Melatonin) that is secreted during the night and can modify hormones involved in the regulation of reproductive function. The best indication of genetic predisposition of buffaloes to daylight variation is inferred from the fact that over the years the Italian buffalo breeders have selected animals less sensitive to daylight variation. Buffaloes with a clear seasonal reproductive trend had highest melatonin during the night compared to the day. Moreover, night-time melatonin was highest in December and decreased progressively towards June. On the contrary, buffaloes which had persistently high plasma melatonin during the day with a lack of evident increase during the night exhibited a uniform parturition frequency around the year. Regarding the impact of melatonin on reproductive axis, an increase in plasma melatonin may lead to at least 10-fold increase in plasma GnRH and gonadotrophins, thus leading to follicular growth and ovulation. Using melatonin implants, ovulatory size non-ovulatory follicles present in summer anestrous buffalo heifers can be

successfully ovulated to initiate ovarian cyclicity. This suggests the effectiveness of melatonin implants for breaking summer anestrous of buffalo heifers.

Prolonged postpartum anestrus

Prolonged postpartum anestrus is a main contributor to the long calving interval in buffaloes. Complete involution of the uterus in dairy buffaloes takes a minimum of 15 days and a maximum of 74 days, with 66% of the females requiring 35-45 days. Under most favorable conditions buffaloes restart cyclicity by 30-90 days, however only 34-49% buffaloes exhibit estrus within 90 days, while 31-42% remain anestrus for more than 150 days. The sensitivity of buffaloes to photoperiod, together with factors viz., poor body condition, poor nutrition, suckling and season of calving can play an important role in duration of uterine involution, the resumption of post-partum ovarian activity, and subsequent conception. Buffaloes with appropriate body condition score at calving had an earlier first postpartum estrus than buffaloes with poor body condition score. Moreover, well-fed buffaloes that are allowed once per day suckling resume cyclicity within 30-60 days after calving, whereas under-fed buffaloes with free suckling remain anestrus for 150-200 days. Out of the buffaloes that suckled their calves, about 32% display first ovulation within 96 days after calving, whereas 68% remain anestrus for 150 days post-partum. Data suggests that the season of calving is more important than suckling. A shorter postpartum anestrus period is displayed by the buffaloes that calve during peak (August-January, 56 days) compared to low (February-July, 91 days) breeding season. Schemes suggested for alleviating prolonged postpartum anestrus include adequate nutrition during peripartal period, restricted suckling and the use of wallowing and water sprinklers during summer months.

Low popularity of artificial breeding

Accurate estrus detection is essential when AI is to be practiced in place of natural breeding. However, buffalo is a shy-breeder. Estrus behavior in buffaloes is much more subtle and estrus is difficult to detect because of weakness of estrus symptoms and the variability of estrus length. Homosexual behavior is rare (only in 20%) in female buffaloes. Secondary signs such as restlessness, bellowing and frequent urination are not the reliable indicators of estrus as their expression is extremely weak and is inconsistent. Acceptance of the male is the most reliable estrus indicator. Routine observation of buffaloes may help to detect external physical signs such as vestibular reddening, vulval swelling and effacement of wrinkles present on the vulva's external surface. Mucus discharge during estrus is less copious and usually does not hang as strands from the vulva. On average, estrus behavior lasts for 33h (range: 4-64h), although 59% estruses were observed between 10.00PM and 6.00AM in Murrah buffaloes. For optimal estrus detection, buffaloes should be observed vigilantly in the early hours of the morning, the late hours of the evening and at 4-5h

intervals during the day. In a study, only 37% buffaloes exhibited any type of estrus sign. False estrus was displayed by 17% buffaloes as the estrus symptoms were exhibited during luteal phase. About 62% buffaloes had silent estrus in which endocrine activity was normal and the only estrus sign in these animals was mucus discharge. Silent estrus is another largest factor responsible for poor estrus detection in buffalo. To compensate for the lack of overt estrous behavior among buffaloes, estrus can be detected with the aid of teaser bulls. In this case, standing estrus is the most reliable sign of ovulation, although a large variability exists between the interval from the start of standing estrus and LH surge (124 h before to 6 h after the LH surge). The end of bull acceptance is a reliable sign that may indicate the end of estrus. However, keeping a teaser bull is not practically possible for the marginal farmers. Difficulty in predicting the correct time of AI relative to ovulation limits the correct use of AI in buffaloes. Ovulation cannot be predicted from secondary estrus signs as they are very variable in relation to ovulation time. In general, ovulation occurs in Murrah buffaloes about 24-60h (mean: 42h) after the onset of estrus, or 6-21h (mean :14h) after the end of estrus. This wide range for ovulations was due to preovulatory LH surges occurring at 0-34h after the onset of estrus. In fact, for the buffaloes that conceived, the average interval between LH surge and ovulation was 25h and this interval was 46h for those that failed to conceive. Thus, delayed LH surge is a contributing factor for the unsuccessful inseminations in buffaloes. Moreover, in Italian buffaloes, the lengths of estrus period are categorized as short (<12h), medium (13-24h), long (24-48h) and very long (>48h). In the short and medium estrus period, ovulation occurs after the end of estrus, around 6-72h and 24-60h after the onset of estrus, respectively. Depending on the ovulation time, the short estrus often continues as silent estrus. In some of the long and very long estrus periods, ovulations occurs before the end of estrus period.

Estrus synchronization during low-breeding season

During low-breeding season, $PGF_{2\alpha}$ administered 12 days apart in cycling buffaloes was highly efficacious for synchronizing ovulation and thus, permitting fixed-time AI at 72 h after second-$PGF_{2\alpha}$ which lead to good enough fertility. Moreover, during the breeding and low-breeding season, $PGF_{2\alpha}$ administered at 11 days interval induces estrus and ovulation in 60-80% buffaloes, in which, the average duration from $PGF_{2\alpha}$ treatment to estrus was 88h (48-144h; 78% from 72-96h), and from treatment to ovulation was 100h (60-156h; 81% from 84-108h). Thus, following this protocol, recommendation of fixed-time AI appears difficult. Moreover, estrus signs after $PGF_{2\alpha}$ display wide variation and are less apparent. Nevertheless, following AI after this protocol, the CR was 53 and 25% in peak-breeding and low-breeding season, respectively.

Progesterone implants are effective in improving fertility during the low-breeding season and in females with ovarian cysts. Between seasons, the interval from PRID removal to LH

surge and from PRID removal to ovulation was 47h and 72h in November and 61h and 96h in March, respectively. Therefore, in synchronized buffalo cows most appropriate times for insemination in the autumn are 48h and 72h after PRID removal, and at 72h and 96h in the low-breeding season. Prepubertal anestrous buffaloes during low-breeding season have shown that long-term (15 days) PRID placement is highly successful for inducing estrus response and 60% CR was obtained when these animals were inseminated at 48h and 72h after PRID removal. Synchronization of buffaloes during summer with PRID and eCG (500 IU) produces higher pregnancy rate (50%) compared to synchronization with PRID alone (8-28%). Another $PGF_{2\alpha}$, eCG and PRID based schedule ensures a good ovulatory response during low-breeding season and the fertility rate is comparable to that observed during peak-breeding season (44-46%). Furthermore, a protocol was used to decrease the variation in ovulation time. In this protocol, a CIDR (with estradiol benzoate, im; controlled internal drug release) was kept in place for 9 days. At the removal of CIDR, $PGF_{2\alpha}$ and eCG (500 IU) were administered. On day 11, buffaloes received 1500 IU hCG, with AI 14h later. Thus, compared to 28% CR using ovsynch, higher CR (53%) was obtained using a CIDR with eCG and hCG in low-breeding season.

Ovsynch protocol (GnRH-day 0, $PGF_{2\alpha}$-day 7, GnRH-day 9) improves the efficiency of fixed-time AI by synchronizing ovulation in a short period of time (24-30h after 2^{nd} GnRH). In fact, AI was required only at 24h after 2^{nd} GnRH in 82% ovsynch-treated prepubertal anestrous buffalo heifers. Pluriparous buffaloes respond better in comparison to primiparous buffaloes (51 vs 35%). With this protocol, the CR during peak-breeding season, transition to seasonal anestrus and low-breeding season was 49, 36 and 7%, respectively. Moreover, improvement in CR following application of ovsynch in prepubertal anestrous buffalo heifers during low-breeding season needs further attention as their CR is low (18%).

To improve the success rate with respect to degree of estrus synchronization and associated CR in heifers or anestrous buffaloes especially during low-breeding season, the most effective schemes appear to be: a) initiating treatment in the presence of a dominant follicle, b) supplementation of ovsynch protocol by PRID for 7 days between the 1^{st} GnRH and $PGF_{2\alpha}$, and c) substitution of 2^{nd} GnRH with hCG. The use of hCG improves quality of the CL, progesterone levels are increased and embryo losses may be reduced, especially during the summer season. No difference in the interval from treatment to ovulation (26.5 vs 24.4h) was observed following GnRH or hCG based ovsynch protocols.

However, the use of PRID in a ovsynch protocol is instrumental for the improvement of CR in non-cyclic buffaloes, although no significant improvements was observed in cyclic buffaloes. With this modification, the CR increases from 19 to 30%, but remains still lower

compared to the CR observed during breeding season (45%). To further modify the ovsynch protocol, a CIDR was kept between day 0 to day 8 along with $PGF_{2\alpha}$ plus eCG (500 IU) administration at day 7, followed by single AI at 14h after 2nd GnRH. A substantial improvement in the ovulation rate was observed in non-cyclic buffaloes and the CR achieved in anestrous lactating buffaloes, whether cyclic and non-cyclic, approached the rates observed in buffaloes inseminated at natural estrus.

Embryo production and transfer

Despite the attention focused on the improvement of superovulation protocols in buffaloes, an optimal protocol remains obscure. Although there is an appreciable ovarian response, fewer transferable embryos are retrieved from a responsive animal. The five possible reasons for the low retrieval could be: a) limited follicle population in a buffalo, b) inability of the superovulated follicles to ovulate which continue to produce estradiol, c) failure of oocyte entry into the oviduct that could be due to higher plasma estradiol persisting for longer duration following $PGF_{2\alpha}$ administration in superovulated buffaloes. Estradiol may cause loss of ovulated oocytes into the peritoneal cavity due to reverse peristalsis in the oviduct, d) low survival of young embryos under high estradiol environment, and e) embryo development is rapid in a buffalo and hatching usually occurs 6.5-7 days after the onset of estrus compared to 8.5-10 days in cattle. After hatching, the characteristic landmark of embryo (zona pellucida) is lost and the identification of embryos in the recovery medium is difficult. Thus, embryo recovery needs to be done between days 5 and 6 after the onset of estrus. However, on day 5 the CL is small, soft and difficult to palpate rectally. The proper evaluation of synchronized recipients on day 5 is must for the transfer of embryos to the horn ipsilateral to the CL.

Efficiency of *in vitro* fertilization in buffalo is much lower and several bottlenecks need to be removed. Oocytes collected per ovary from abattoir-ovaries was 0.43-0.70 in India, and 2.4-3.3 in Italy. This remains much lower compared with cattle in which 8-12 good quality oocytes are usually obtained from abattoir-ovaries. However, by transvaginal ultrasound guided puncture (ovum pick up), about 2.25 oocytes can be collected and this technique appears to be superior to superovulation keeping in view the number of transferable embryos per donor. Although, a high oocyte maturation, fertilization, and cleavage rate is obtained, there is low blastocyst yield and calving following the transfer of *in vitro* produced buffalo embryos. Embryo cryopreservation is important for buffaloes because: a) synchronization of recipients may not be efficient and a less number of buffaloes may be available for the transfer of fresh embryos, and b) embryos can be transferred in a period of the year favorable for buffaloes. However, buffalo embryos have reduced capacity to withstand freezing temperature due to a high lipid content. Recently, solid surface vitrification procedures have been successfully employed to cryopreserve buffalo

blastocyst. Although buffalo calves have born after the transfer of *in vivo* derived cryopreserved embryos, factors restricting the success rate of embryo transfer programmes are lack of breeding stock, inherently lower level of fertility, delayed puberty and seasonality of the reproductive pattern.

To counteract the increase in milk prices and to decrease the reduction of fertility during the summer season, various measures can be adopted such as: a) control of environmental temperature and humidity, b) providing quality nutrition, and c) stimulating the appetite by increasing the number of feedings per day. Due to inherent infertility of buffaloes, a slow progress has been made in the application of assisted reproductive technologies. Embryo transfer and *in vitro* embryo production remain in the area of testing as the embryo yield is limited due to a low superovulatory response, low efficacy of oocyte aspiration and low blastocyst yield.

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An extension team approach for linkages between reproduction research, education and field application of technologies

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Livestock is the rising star and a ray of hope for the poor farmers which has provided the much needed diversification in agriculture through dairying resulting in significant reduction of income disparities between rural and urban population. Livestock plays an important role in the state economy of Punjab contributing 11.77 percent to the gross domestic product of state. The per capita availability of milk in Punjab is 962g per day which is at par with the best in the world. Today, farmers are rearing the milch animals not only for their household purposes but for commercial purposes also. Now, dairy farming is adopted by many farmers as a commercial enterprise, so more production ought to be derived from these animals which is possible through ideal reproduction of dairy animals and ideal nutrition along with proper management. As the majority of the problems/diseases faced by the dairy farmers are of reproduction viz repeat breeding, anestrous, prolapse, retention of placenta etc. Production in a simple way can be enhanced only when the reproduction is smooth in that farm. Field Veterinarians are mainly concerned with the treatment of the diseases and same is the farmers view. But the policy of the Govt., Universities, State Animal Husbandry department or any other animal welfare agency, is the first prevention of the diseases along with the treatment aspect. For the prevention of diseases, farmers should be updated about the various management practices/ technologies / innovations that can be helpful to reduce the incidence of diseases. To make the farmers aware about the technologies/innovations, the role of extension is most important; however sadly the extension aspect is mostly ignored. Scientists/Vets are doing research in the labs at molecular level / biotechnological level, doing the treatment with newer antibiotics or hormones, but at the field level, the farmers are not even aware about the possible advantages of AI and the importance of feeding mineral mixture. So, the extension can help a lot in getting the farmers aware and then adoption of these technologies. If someone wants to do some serious efforts for the adoption of correct reproductive technologies one should know the answers of the following questions.

- What is known & what is to be done & why? e.g. How many % age of farmers go for A.I. – or know about it , what % age of adoption and what is our aim? what is to be done?
- Some farmers accept new idea quickly why?

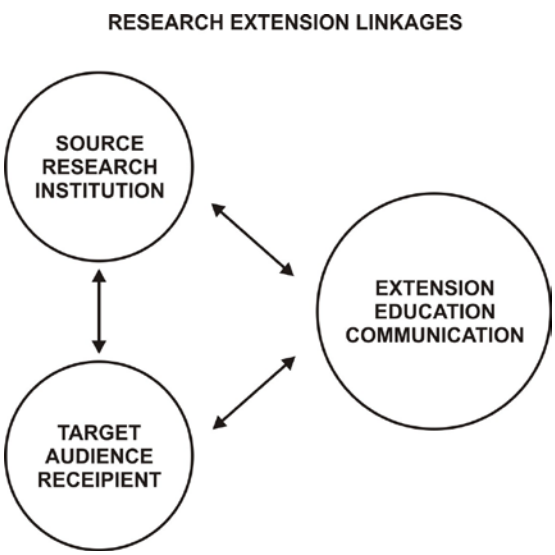
- May be education level high, More cosmopolite sources of information, more exposure to mass media

- From where farmers get new idea?

- May be fellow farmers, May be relatives, May be V.O., May be University scientists/ extension persons

- Why some of these ideas are put into practice by some farmers quickly?

Reproduction scientists bring out new technologies or innovations. The extension worker carries this new scientific information in the field and passes it on to farmers. The farmers may have some problems in day to day farming while applying new practices and techniques. The extension worker may or may not have answer to all the problems of farmers and he seeks answers. The research scientist conduct experiments and passes on his results as answers. So, extension worker has dual responsibility of bringing the results of research to the farmers and at the same time has also the responsibility to transmit the problems faced by the farmer to the research scientist for solution. Thus, extension is a two way channel. Extension service has the role and responsibility of bridging the gap between research and users of technology through transplanting, transmitting and translating research results/technology in to practice by way of establishing coordination and linkage with institutions of higher learning i.e. research institute on the one hand and the farmer's institutes and organizations on the other hands.



Diffusion of innovations is a theory that seeks to explain how, why, and at what rate new ideas and technology spread through cultures (Everett Rogers, 1983) . Diffusion is the process by which an innovations communicated through certain channels over time among the participants in a social system.

- Innovation means "an idea, practice, or object that is perceived as new by an individual or other unit of adoption".
- Communication channels- "the means by which messages get from one individual to another".
- Time- "The innovation-decision period is the length of time required to pass through the innovation-decision process". "Rate of adoption is the relative speed with which an innovation is adopted by members of a social system".
- Social system- "a set of interrelated units that are engaged in joint problem solving to accomplish a common goal"

Farm people learn best in different ways: some by listening, some by seeing, and still others through discussion. All people do not learn at the same speed, some may be at the stage of trying a new practice and want to know the details of how to do it, while others are barely aware of the practice or are just interested. But, the essential thing for every technology is that whether it will be adopted by end users or not . So, knowledge of different stages in adoption of a new technology is must , which are as under

Five stages of the adoption process

Stage	**Definition**
Knowledge	The individual is first exposed to an innovation, but lacks information about the innovation. During this stage the individual has not yet been inspired to find out more information about the innovation.
Persuasion	The individual is interested in the innovation and actively seeks related information/details.
Decision	The individual takes the concept of the change and weighs the advantages/disadvantages of using the innovation and decides whether to adopt or reject the innovation. Due to the individualistic nature of this stage, Rogers notes that it is the most difficult stage on which to acquire empirical evidence.
Implementation	The individual employs the innovation to a varying degree depending on the situation. During this stage the individual also determines the usefulness of the innovation and may search for further information about it.
Confirmation	The individual finalizes his/her decision to continue using the innovation. This stage is both intrapersonal (may cause cognitive dissonance) and interpersonal, confirmation the group has made the right decision.

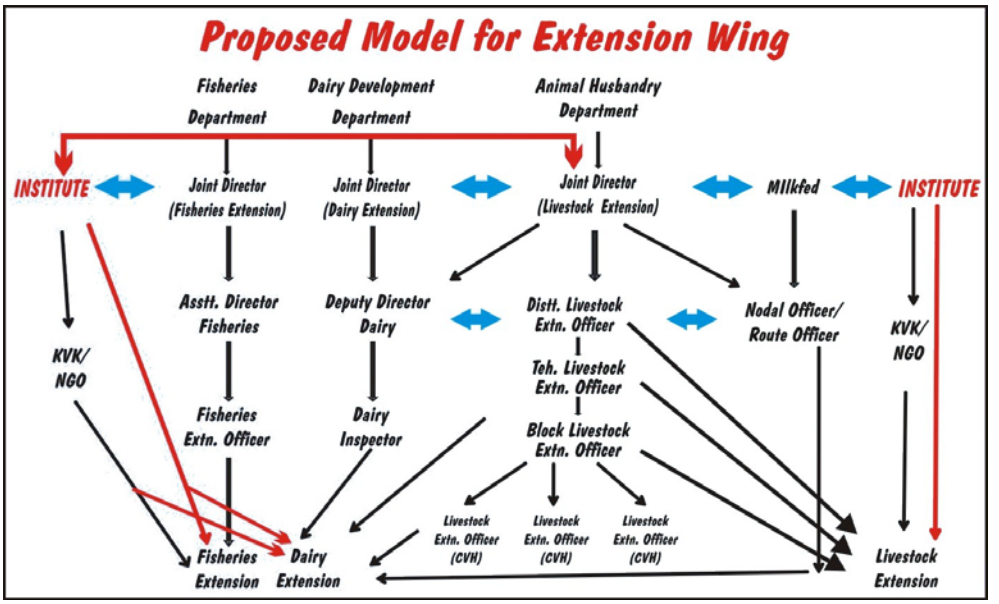
Different extension methods can be used for different stages of adoption process

- For Knowledge or awareness of technology:- All printed material, film-show, Radio, TV, Posters, Newspaper, Leaflets, Banners, Posters etc.
- For Persuasion or interest stage: Personal contact, Meetings, Radio talk, Farm magazines, Recorded Cassettes/DVD/CD, TV, Leaflet, Folder
- For Decision or evaluation of technology:- Demonstration followed by discussion, Cassettes, Field trips, Field day, Farmer's experience in printed form
- For implementation or trial of technology: Personal contact, Method demonstration, Result demonstration, Farm magazine, Field trips, Field days, Leaflet, Folders.
- For conformation or adoption of technology: Group discussion, Method demonstration, Result demonstration, Field trips, Trainings, Campaign, Farm magazines.

The different rate of adoption of new technology and its field application can be attributed to the intrinsic characteristics of an innovation, which are as under:

- Relative advantage: How improved an innovation is over the previous generation. The degree of relative advantage is mostly measured in economic terms, but satisfaction and convenience are also important. The greater the perceived relative advantage of a technology, the more rapid its rate of adoption.
- Compatibility: It is the degree to which a technology is perceived as being consistent with the existing values, past experience and needs of potential adopter. Only compatible technology is adopted.
- Complexity: It is the degree to which an innovation is perceived as difficult to understand and use. New ideas that are simpler to understand will be adopted more rapidly than technologies that require the adopter to develop new skill and understanding.
- Trialability or Divisibility: It is the degree to which a technology may be experimented with on a limited basis. New ideas that can be tried on the installment plan will generally be adopted more quickly than technologies that are not divisible. A technology that is trial able represents less uncertainty to the individuals who is considering it for adoption, as it is possible to learn by doing.
- Observability: A technology, which has observable result, is easily adopted.

The research worker should be acquainted with the present farming conditions so as to develop the technology according to needs of farmer and the developed technology will have higher rate of adoption.



Present Day Dairy Farms: Present day dairy farms show a trend towards -Increased size of farms, Increase milk production per cow, High level of mechanization ,difficult and complicated farm organization , detection of health disorders, Control of production costs, More attention for sub clinical diseases, Health problems are considered to be the major constraint for profitability, Formulation of least cost ration as feed costs are the highest variable costs in production. Development of farming skills, Management of animals, equipment and farming conditions and regular economical estimation.

Progressive Dairy Farmer: These have an advantage over the common farmer by these features as :Invest in knowledge through practitioners, Mind set up with self assessment and positive evaluation considered as risks takers, Commercially market oriented, decide based on broad information from various sources, Innovative, planners and turn threats into opportunities, Highly skilled in communication so far newer ways of making profit, Group parturitions through technological interventions.

Dairy farming in the state is run by two groups of farmers one who follow traditional dairy farming practice and can be categorized as common farmers, others those who have adopted and are in the scientific raising of dairy cattle and belong to the category of progressive dairy farmers. The number of progressive dairy farmers in the state has increased significantly from around 100 in the initial stages to around 1200 at present. The enormous increase in the number can be credited to the extension services provided by the institute and the state department along with mass media approaches. Some salient features from the evaluation of 180 dairy farms (30 from each agro-climatic zone) during a study

conducted in the department about the farmer's knowledge level for appraisal indicated that:

Overall incidence of reproductive disorders in different agro climatic regions of Punjab was 19.11% in buffaloes and 15.05% in cows. Major reproductive disorder in buffaloes was anoestrus (31.98%) whereas in cows it was repeat breeding (35.06%). Anoestrus and repeat breeding were related with deworming, mineral mixture supplementation and concentrate feeding in animals.

Maximum adoption of A.I. in cows was 41.67% in Flood plain region, however, in buffaloes the adoption was 17.78%. Adoption of A.I. was related with education, herd size, extension contacts, mass media exposure, and distance from civil veterinary hospital and total dairying experience. Miscellaneous perceptions still exist in the dairy farmers regarding adoption of A.I. and dairy farmers prefer natural service in buffaloes in specific areas of Punjab.

Linkage between Reproduction research education and adoption of technology: Innovation and research outcome of the university over the years have led to the propagation of various livestock development strategies to the farmer, through various extension methodologies. Various reproductive technologies specifically artificial insemination and to some extent initiation of multiple ovulation and Embryo Transfer (MOET) programmes have made remarkable impact in milk production and development of elite crossbred germplasm in the state. The embryo transfer programmes run by the State Animal Husbandry Department along with Veterinary varsity is quite a success and now more and more farmers are interested in going for it. However the dissemination of the following procedures /methods is urgently required in the field for enhancing productivity in the dairy animals:

- Ideal body weight of the animals at maturity
- Methods for heat detection
- Optimum time of insemination
- Proper insemination/mating
- Record keeping
- Timely pregnancy diagnosis
- Preventing exposure to abortifacient agents
- Drying off
- Balanced feeding
- Intercalving interval
- Parturition hygiene
- Post-parturient care of the animal for next breeding

For effective linkage between research scientist and farmer adopting technology, following measures can be taken in to account:

- Research and extension interface should be organized regularly, in which research scientists and farmers should participate and there is open discussion between them.
- Adoption studies of every newly invented technology should be carried out.
- The developed technology should be farmer friendly i.e. always keep farmer aspect in mind
- Feedback of farmers i.e. solutions to problems in adopting technology should be incorporated in standard procedure of developing technology.
- Reproductive disorders and developed technologies should be addressed immediately through modern extension approaches like kiosk, e- extension, cyber extension, sms alert services coupled with more extension contacts and network.

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Ultrasound imaging: A tool to transform reproduction research capabilities

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Ultrasonography has been accessible to the medical community since early 1970's and considered to be a technological breakthrough in the diagnostics. The technology is well adaptable for the study of internal reproductive events in animals. With the use of ultrasound scanner an operator can visualize organs which were previously reachable by tactile sense. In recent time, ultrasonography is employed in an extremely diverse range of circumstances, not only as a diagnostic tool in the routine clinical workup of a range of species, but also for disease diagnosis, herd management and clinical research. The clinial uses of ultrasonography in female involve assessment of pubertal status, seasonal status of ovaries, stage of cycle, prediction of ovulation, pregnancy diagnosis, fetal viability, fetal age and sex, amnio-allantocentesis, ovulation failure, ovarian and uterine tumors, follicular/luteal cyst, pyometra, mucometra, hydrometra, embryonic loss, postpartum involution, ovarian response to hormonal treatment. The clinical uses in males involve evaluation of external and internal genitalia, inflammatory conditions and tumors of testes and accessary sex glands and routine method for breeding soundness evaluation. The technique has various research appliocations viz. Sereal examination (follicular and luteal dynamics), qualitative assessment (visual scoring), quantitative assessment (image analysis) and vascular flow dynamics (color doppler).

Ovarian follicular dynamics in cattle and buffalo involves the development of follicles and the interactions of various cellular and hormonal components. The successful development of an ovulatory follicle involves integrated sequence of biological events involving ovary, corpus luteum, uterus and hypothalamus-pituitary- gonadal-axis. There are several factors including season, nutrition, post-partum period etc which can alter the process of folliculogenesis and can be serially detected by ultrasonography. Hormonal and non-hormonal regimes to manipulate follicular dynamics in favourable and unfavourable conditions needs to be integrated with current methods to obtain synchrony of estrus (necessary for fixed time AI), optimum fertility (healthy dominant follicle with competent oocyte) and effective follicular development (necessary for follicular aspiration and superovulation).

In cattle numerous studies over the last decades have shown that during the ovulatory cycles, follicular development occurs in wave like pattern. These waves consist of

synchronous growth of a large number of small antral follicles (4-5 mm in diameter) i.e. recruitment, followed by selection and growth of one dominant follicle that achieved the greatest diameter, suppressing the growth of the other subordinate follicles. In the absence of luteal regression, the dominant follicle becomes atretic and new follicular wave resumes. The dominant follicle regulates the growth of subordinate follicle, since the emergence of next wave is accelerated if the dominant follicle is destroyed and delayed if the lifespan of dominant follicle is prolonged experimentally. During bovine estrous cycles, 2or 3 successive wave emerge and wave emergence was detected on average, on the day of ovulation (day 0) and day 10 for 2-wave cycle and on days 0, 9 and 16 for 3-waves cycles. Apparently, the basal levels of gonadotrophins during estrous cycle are sufficient to allow emergence of waves about every 7-9 days and that the time of luteolysis relative to time of appearance of the last follicular wave, determines whether an individual cycle will have two or three waves of follicular development Incidence of 2 or 3-wave cycles depends mainly on follicular size (>10 mm) and estradiol concentration (>5.0 pg/ml), When both are attained after emergence of the 2nd wave, the cycle will be 2-wave cycle, and when not attained, the cycle continues to be 3-wave cycle (Noseir, 2003).

Ovarian follicular dynamics in buffalo are similar to those in cattle. There is a predominance of two waves with the first wave beginning around Day 0 (day of ovulation) and the second wave around Day 9 or 10 (Ali et al 2003). Both 3-waves and 1-wave cycles are also observed in buffalo. The hormonal profiles during estrous cycle in buffaloes are closely resembled to cattle (Singh et al 2000).

The initiation of each follicular wave is preceded by a transient increase in the concentration of FSH and the peak of the FSH surge occurs at or near the time when the future dominant follicle of the resulting wave is emerging. This increase in FSH could be the stimulus for the recruitment of the cohort of ovarian follicles and for wave emergence i.e. when the first follicle of the follicular wave reached $\geq$ 4mm diameter. Following emergence, follicles continue to growth but FSH begins to decline until the time of follicular deviation. Follicular deviation is defined as the beginning of the greatest difference in growth rates between the largest follicle and largest subordinate follicle. At the time of follicular deviation the diameter of largest dominant follicle averages 8.5 mm and the largest subordinate follicle averages 7.2 mm (Wiltbank et al 2002). This diminished FSH continues until a few days before emergence of subsequent follicular wave. The two primary inhibitors of FSH that are secreted by the follicle are inhibin and estradiol. Inhibin appears to be secreted by follicles of all size however; estradiol only appears to increase after dominant follicle has been selected following deviation (Kulick et al 1999). At the time of emergence the maximum FSH levels are because of low circulating inhibin and estradiol. As follicular wave progresses, inhibin produced by follicles is responsible for the

reduction of circulating FSH and the increase of circulating estradiol (~0.2 to ~1 pg/ml) near the time of follicular deviation causes final depression in FSH. The inhibition of FSH by estradiol is synergised with inhibin.

The selected non-ovulatory dominant follicle of first wave continues to grow, gains LH receptors on granulosa cells and secretes greater amount of estradiol and follicular regulatory products to establish dominance and thus decreasing FSH levels by negative feedback mechanism and depriving subordinate follicle at an early stage of development (Ginther, 2000). It now becomes dependent on LH and its final fate, regression or ovulation is determined by LH secretion (Ginther et al 2001). Because of the presence of functional corpus luteum and high progesterone concentration, this dominant follicle does not cause and LH surge or behavioural estrus and does not continue to ovulation. The end of each dominance is preceded by the incapability of suppressing FSH, by the loss of estrogen producing capacity and by the loss of FSH and LH receptors (Burke et al 2007). At the same time new wave emerges, which is again preceded by the transitory increase in FSH (Roche et al 1997). At this time dominant follicle continues to growth until sufficient circulating estradiol is achieved to induce LH surge and ovulation of the dominant follicle. Dairy heifers were found to ovulate follicles of 14.8±0.2 mm, but lactating cows ovulated follicles of 17.4± 0.5 mm (Sartori et al 2000). All these aforesaid events are integrated part of research on follicular dynamics and necessarily involve use of ultrasonography. These cascades of events suggest that functional dominance is not only dependent on gonadotrophins and estradiol but some intraovarian factors are also involved to alter the secretion of gonadotrophins at the pituitary level or to modulate their effect at the ovarian level (Guilbalt et al 1993).

The revolutionery changes in research approaches related to ovarian follicles have been realized through advancements in imaging technologies over the last two decades. New special technologies, such as computer assisted image analysis, ultrasound-based biomicroscopy and transvaginal ultrasound guided ovum-pick up indicate a new era in our understanding of the basic processes of reproduction and their clinical importance.

Computer asssisted image analysis

This is considered a new method of determining follicle status at a single examination. An ultrasonographic image is collection of thousands of picture elements, or pixels. Each pixel represents a discreet tissue reflector and can assume one of 256 shades of grey (ranging from black to white). We can perceive “smoothness” of an image with increasing shades of grey, but can only distinguish between 18 and 20 shades of grey. Computer assisted pixel analysis will allow researchers and clinicians to assess the physiology underlying ovarian follicular growth and development.

The ultrasonographic appearance or image pattern of a tissue is termed echotexture and is determined by the histologic structure of the tissue (Singh and Adams 2000). Computer algorithms have been designed specifically for analysis of ultrasound images to overcome the inconsistencies of subjective visual evaluation, and to provide a quantitative approach to grey-scale pixel-value analysis (Synergyne 2©, Version 1.1, WHIRL, Saskatoon, SK, Canada). These algorithms have been used extensively in studies characterizing the echotexture dynamics of ovarian structures at different phases of the follicular wave (Singh et al 2003).

The image analyses for the assessment of ovarian follicular function require original, standardized data point to be easily incorporable into computer analysis software. The image analysis can be done through various digital processing steps to extract numerical data (spot and line analyses), enhance visual interpretation (region analysis and time-series analysis), and automate analysis of visual data (wavelet analysis and mathematical modeling).

Ultrasound biomicroscopy

Commercial ultrasound machines provides a lateral resolution of 0.7–1.0 mm. This level of resolution is sufficient for many clinical uses, but the full potential of image analysis can be exploited with an instrument that provides microscopic resolution (i.e. <0.2 mm). A new ultrasound instrument produced by a Canadian company capable of microscopic resolution (http://www.visualsonics.com) is available.

This ultrasound biomicroscope (UBM) uses a single crystal to release a sound wave with a frequency of 30–70MHz and will produce an image with a resolution of 30–50μm. In addition, the instrument has spectral Doppler capability to measure blood flow through vessels as small as 50 μm in diameter. Major disadvantages of the biomicroscope are depth of penetration (approximately 10mm at 30 MHz, 5mm at 50 MHz), field of view (imaging width, 1 cm), and frame rate (8 frames/s).

Ultrasonic biomicroscopy is feasible for identifying small antral follicles (0.4–3 mm in diameter) and COCs within the ovary of cattle in vivo (Pfeifer et al 2009). The transvaginal method is a more effective approach for imaging small follicles compared to the transrectal method. Moreover, the ovaries of heifers were more successfully imaged than cows. The ability to sequentially monitor the dynamics of small follicle development and COC in vivo could lead to breakthroughs in the study of ovarian physiology.

Transvaginal-ultrasound-guided follicular aspiration in bovines

Assisted reproductive technologies like *in vivo* embryo production, production of clones or transgenic animals and establishment of oocyte banks have made a significant step in recent years. It started with conventional collection following superovulation, laparoscopic collection from live animals and from slaughterhouse ovaries, which have several limitations. The technique of ultrasound guided transvaginal oocyte retrieval (TVOR) in domestic animals has several advantages. It allows repeated collection of oocytes from the same donor. It is safe and does not interfere with normal cycle of the animals. The requirement of follicular aspiration/ovum-pick up comprised of an ultrasound scanner with a transvaginal transducer, needle guidance system and a suction pump. The transducer is having a casing to guide the long needle precisely into the follicle to be aspirated. The entire operation of aspiration is displayed on monitor. Suction apparatus provides a constant vacuum pressure to retrieve oocytes from follicles. Apart from oocyte collection the method can be used as a non-hormonal approach for induction of wave emergence (Honparkhe et al 2014).

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Cystic endometrial hyperplasia and pyometra in dogs: implications for fertility

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Pyometra is defined as accumulation of pus within the uterus. The condition is commonly accompanied by histological lesions of cystic glandular hyperplasia of the endometrium that is otherwise found in older bitches without the signs or evidence of pyometra. Because of the association between cystic endometrial glandular hyperplasia and pyometra, the condition is referred as cystic endometrial hyperplasia (CEH) - pyometra complex.

Pathophysiology

Progesterone induced endometrial hyperplasia usually precedes the development of pyometra (Dow, 1958). The disease results from bacterial interaction with an endometrium that has undergone pathological changes from prolonged or repeated progesterone stimulation (Hardy and Osborne, 1974; Smith, 2006). Progesterone during diestrus stimulates the endometrial growth. There is increase in the size and number of endometrial glands. Progesterone also stimulates the myometrial activity and results in accumulation of glandular secretion in the uterus. These secretions provide an excellent medium for the growth of bacteria that gain entry into uterus from vaginal vault (ascending infection) through the relatively dilated cervix during proestrus and estrus. Most likely these organisms are resident bacteria of vaginal vault (Baba *et al*., 1983). The other bacterial sources for the uterine infection are urinary tract infections or transient bacteremias. Predominantly, *Escherichia coli* has been recognized, as cause of uterine infection in bitches and it may be the secondary to the ability of this organism to adhere via specific antigenic sites to receptors in progesterone stimulated endometrium and myometrium (Sandholm *et al.*, 1975). In addition, *Staphylococci*, *Streptococci*, *Pseudomonas*, *Proteus* and many other bacteria have been isolated in bitches suffering from pyometra (Dow, 1958; Nelson and Feldman, 1986; Meyers-Wallen *et al.*, 1986; Memon and Mickelsen, 1993; Gandotra *et al*., 1994; Dhaliwal *et al.*, 1998, Pradhan *et al*., 1999). Bacterial growth is further enhanced by inhibition of the leukocyte response to infection in the progesterone-primed uterus (Hawk *et al.*, 1960).

Estrogen, by itself, is not usually associated with the development of CEH-pyometra. However, estrogen enhances the stimulatory effect of progesterone on the uterus. Higher than the physiological concentration of estrogen administered exogenously during estrus or

diestrus, to check conception in mismated bitches, increases the risk of developing pyometra (Nelson and Feldman, 1986).

History and Symptoms

Pyometra is primarily a disease of middle-aged cycling bitches, with a mean age of six to eight years. The condition also attracts younger animals and in these cases CEH may be absent. There may be correlation between the disease in young animals and the administration of estrogen to prevent pregnancy. Out of 8 bitches reported to suffer from pyometra under the age of three years, five had received estradiol cypionate within six months before diagnosis (Wheaton *et al.,* 1989). Nulliparous bitches have a moderately higher risk of developing pyometra than primiparous and multiparous animals. The reason for the apparently protective effect of pregnancy is unclear. The original endometrium is lost after pregnancy, and the new endometrial lining might differ in the susceptibility of its receptors to estrogen or progesterone (Niskanen and Thrusfield, 1998).

The clinical disease varies greatly in severity. The symptoms are influenced by the patency of cervix and the condition is referred as either open-cervix pyometra or closed cervix pyometra. A sanguineous to mucopurulent vulval discharge is the main or sometimes only symptom observed in open cervix-pyometra. The discharge is usually first noticed 4 to 8 weeks after standing heat or as late as 12 to 14 weeks after the end of standing heat. The discharge is lacking in bitches suffering from closed-cervix pyometra. The other observations associated with pyometra include lethargy, anorexia, depression, abdominal distension, polyuria, polydipsia and vomiting. Fever is a variable sign and, if recorded, is associated with uterine inflammation and secondary bacterial infection as well as septicemia or bacteremia. These signs are more severe in closed-cervix pyometra and in conjunction with anorexia, polyuria, and vomiting leads to dehydration, shock, coma and death.

Clinical Pathology

The white blood cell counts of bitches with pyometra are extremely variable (Hardy and Osborne, 1974 and Gandotra *et al.,* 1994). The total WBC is increased in the bitch with closed-cervix pyometra usually exceeding 30,000 cells per mm^3. Increased WBC may be found in the bitch with open-cervix pyometra, normal WBC is also encountered. An absolute neutrophilia with variable degrees of cellular immaturity develops secondary to the significant infection and septicemia, which may ultimately result in a degenerative left shift with toxic neutrophils (Nelson and Feldman, 1986). A mild normocytic, normochromic, non-regenerative anemia (PCV 28 to 35 ml/dl) is often associated with pyometra. The anemia resolves ones the pyometra is corrected.

Serum biochemical evaluation reveals concomitant hyperproteinemia and hyperglobulinemia that may result from dehydration and/or chronic antigenic stimulation of immune system. Serum urea nitrogen concentration is increased if dehydration is present. Occasionally, alanine aminotransferase (ALT; SGPT) and alakanine phosphatase concentration are mild to moderately increased due to hepatocellular damage caused by septicemia and/or diminished hepatic circulation and cellular hypoxia in the dehydrated bitch.

Urine specific gravity is variable (<1.008 to >1.030; normal 1.008 to 1.015). Toxemia, following secondary bacterial infection particularly due to *E. coli* interferes with resorption of sodium and chloride in the loop of Henle (Asheim, 1965; Hardy and Osborne 1974); reducing renal medullar hyper tonicity and impairing the ability of collecting tubules to resorb free water. This leads to polyuria and compensating polydipsia. Glomerular and tubular lesions have been associated with *E. coli* pyometra (Stone *et al*., 1988). The damaged renal tubules as a result of E. coli endotoxins are less responsive to anti diuretic hormone (ADH) resulting in polyuria and compensatory polydipsia. Prolonged polyuria polydipsia cause renal medullar solute washout, further impairing the kidney's ability to conserve water and urine becomes progressively more dilute (Sp. Gravity <1.008).

Diagnosis

Pyometra can be easily diagnosed and should be suspected in any bitch with consistent clinical signs that appear during or immediately following diestrus. The diagnosis of pyometra is confirmed by:

Clinical signs: Sanguineous to mucopurulent discharge, depression, anorexia, polyuria, polydipsia, vomiting etc.

Physical examination: Abdominal palpation of enlarged uterine horns.

Clinical pathology: Increased WBC along with absolute neutrophilia hyperproteinemia and hyperglobulinemia, increased BUN, SGPT and alkaline phosphatase concentration, change in specific gravity of urine etc.

Vaginal Cytology and Culture: Vaginal smear from a bitch with an open-cervix pyometra contains severely degenerated neutrophils. Vaginal culture results in heavy bacterial growth, which sometimes can be due to vaginitis. Results of vaginal culture and antibiotic sensitivity can be used to make a rational choice regarding antibiotic therapy.

Radiology: The uterus can be visualized in bitches suffering from pyometra. Radiographic signs of pyometra include the presence of large fluid filled dense luminal structures in the caudo-ventral abdomen, displacing loops if intestine dorsally and cranially (Nelson and Feldman, 1986; Root and Spaulding, 1994 and Ayyappan *et al.,* 1997).

Ultrasonography: This has been helpful in diagnosis of early pyometra, even before development of significant clinical signs. Ultrasonography allows determination of uterine size, the thickness of uterine wall and the presence of fluid accumulation within the lumen (Nelson and Feldman 1986; Root and Spaulding, 1994; Younis *et al.,* 2014). The fluid filled uterine loops can be seen to be continuous and coalescent. The uterine horns can usually be traced to the body of the uterus, between the urinary bladder and the descending colon. Luminal contents range from hypoechoic (relatively acellular) to homogeneously isoechoic with small mobile echogenic foci suggesting purulent material (cellular debris).

The success rate of different diagnostic tools varied between 35 to 89 percent and hence a combination of these techniques be used for accurate diagnosis of pyometra (Pande *et al.,* 2006a).

Treatment

Pyometra in the bitches can be managed surgically or with medicines. Irrespective of the management practice chosen, treatment with antibiotics is indicated. Ideally the antibiotic should be chosen on the basis of bacterial culture and sensitivity test (CST). Pending results of CST, a broad spectrum, bacteriological antibiotic likely to be effective against *E. coli* should be chosen (Memon and Mickelson, 1993).

Surgical Treatment

Ovariohysterectomy: This is preferred treatment for pyometra unless the reproductive potential of the bitch must be saved. Injectable broad-spectrum antibiotics and intravenous fluid therapy with appropriate electrolyte supplementation should be started immediately to improve the chances of survival in severely affected bitches. Supportive therapy should be continued during and after surgery and antibiotics should be continued 7-10 days after removal of the infected uterus.

Surgical Drainage: Surgical drainage of the uterus has been attempted to treat the pyometra surgically (Nelson and Feldman, 1986). The technique helps in preserving the uterus and ovaries. Purulent material is aspirated and each uterine horn is flushed with an antiseptic solution for several days after surgery through indwelling tubes/catheters. The tubes are removed after 4 to 7 days.

Medical Treatment

The aim of medical treatment is to evacuate the uterus of the pus exudates. Treatment with estrogens, androgens, ergot alkaloids, quinine, oxytocin or antibiotics is usually not successful (Nelson and Feldman, 1986). The use of repeated low doses of prostaglandins alone or in association with either dopamine agonists or progesterone-receptor antagonists has been demonstrated to be a viable alternative for valuable breeding dogs (Verstegen *et al.*, 2008).

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$): Sokolowaski (1980) reported successful use of $PGF_{2\alpha}$ in the treatment of pyometra. $PGF_{2\alpha}$ acts upon uterine myometrium, cervix and corpora lutea. $PGF_{2\alpha}$ stimulates myometrial contractions and relaxation of cervix. Smooth muscle contractions induced by $PGF_{2\alpha}$ stimulates the movement of purulent contents of the uterus towards the cervix and outside as there is a progressive decrease in the concentration of endometrial PG receptor sites and myometrial smooth muscles closer to the cervix (Davidson, 1995).

$PGF_{2\alpha}$ is administered @ 0.25 mg/kg body weight sc every 24 h in divided doses and @ 0.1 mg/kg body weight sc upto three times daily for 3-5 days (Nelson and Feldman, 1986; Memon and Mickelsen, 1993 and Davidson, 1995) or intravaginal @ 0.15 mg/kg body weight (Gabor *et al.*, 1999). In addition, concurrent administration of broad-spectrum bactericidal antibiotics and supportive therapy, as needed is advised.

Several side effects are observed in bitches after administration of $PGF_{2\alpha}$ and include restlessness, panting, salivation, emesis, tenesmus, diarrhea, urination and mydriasis etc. These reactions disappear with in one hour after $PGF_{2\alpha}$ injection. After each subsequent $PGF_{2\alpha}$ administration reactions diminish in severity and duration.

Indication of a successful response to $PGF_{2\alpha}$ therapy includes loss of clinical signs, development of serous vulvar discharge, which then stops completely, a decrease in the palpable uterine diameter and a return to normal leukogram. The bitch should be re-evaluated two weeks after the completion of PG treatment and if the symptoms of pyometra (vulvar discharge, neutrophilia, uterine enlargement) are still present, treatment with $PGF_{2\alpha}$ may be useful for treatment of pyometra with lesser side effects (Gabor *et al.*, 1999).

Prostaglandin $F_{2\alpha}$ should be used with caution in bitches with closed cervix pyometra, under normal conditions the cervix dilates in response to hormonal stimuli and internal pressure against it. In closed cervix pyometra there is risk that uterine contractions may cause rupture of the abnormal uterine wall or will force infected material out the uterine tubes, causing peritonitis.

Precautions and contradictions for $PGF_2\alpha$ treatment: The bitch under treatment with $PGF_{2\alpha}$ should be young (Preferable 8 yrs. of age) and other wise healthy. The bitch should not be pregnant. Only naturally occurring $PGF_2\alpha$ should be used at the recommended dosage. Synthetic $PGF_{2\alpha}$ analogues are much more potent in its action than in natural $PGF_{2\alpha}$ and their use at the recommended dosage could result in shock and death. Because prostaglandins are not approved for use in dogs, informal consent of the owner must be obtained prior to their use.

Breeding of the bitch is recommended at the next estrus following treatment to avoid the risk of developing pyometra again. Successful conceptions have been reported in bitches after $PGF_{2\alpha}$ treatment of pyometra.

Antiprogestins: More recently, antiprogestins have been evaluated in the treatment of pyometra (Wiebe and Howard 2009). The availability of antiprogestin based drugs has completely changed the clinical approach to pyometras in which the only solution has been ovariohysterectomy. Aglepristone, an antiprogestin, has been examined as a treatment for open pyometra in dogs and cats. A dose of 10 mg/kg on days 1, 2, and 7 in combination with antibiotics was successful in treating 21 bitches and 4 cats with only 1 recurrence over a 14-month period of observation. No side effects were observed, and 2 of the bitches went on to be successfully bred. Another retrospective study of bitches with pyometra, subjected to medical treatment with aglepristone on a median of four occasions in combination with antimicrobial therapy was successful in 75% of the bitches studied and the recurrence rate was 48% (Ros *et al.*, 2014).

Prognosis

Pyometra affected azotemic dogs having BUN >30mg/dl and creatinine >2mg/dl are at higher risk of mortality (Pande et al., 2006b and Kuplulu *et al*., 2009). Substantial improvements in the treatment of pyometra have been made over the last decade. Results are good and continuously improving with the availability of better medications to achieve luteolysis and prevention of progesterone effects, uterine contraction and evacuation, uterine regeneration, and inhibition of bacterial development (Verstegen *et al.*, 2008). Successful conceptions have been reported in bitches after medical treatment. Bitches that have been treated should be bred to a fertile stud dog at her next estrous cycle to maximize her chances for pregnancy, with expected conception rates of 50–65%. Recovered bitches that fail to conceive or complete a subsequent cycle without being bred have a high incidence of recurrence of pyometra (Smith, 2006).

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Sperm uterine interactions in mares

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Fertility varies markedly amongst the mares. Apart from poor management and incorrect timing of mating, probably the most important reason for low pregnancy rates is persistent endometritis and is ranked as 3rd most frequently occurring medical problems in adult horse (Traub-Dargatz *et al.,* 1991). For more than 30 years since the study of Bryans (1962) it was believed that massive numbers of bacteria are introduced into uterus of the mare during breeding which elicit an acute inflammatory reaction in the uterus. This inflammation is transient one as the number of bacteria introduced during mating is low and that too are eliminated by uterine defense mechanisms (Katila, 1995). Later on it was observed that aseptic inoculation of semen into uterus gave inflammatory response similar to bacterial inoculation and the term sperm induced leukocytoisis was given (Kotilaninen *et al.,* 1994 and Troedsson *et al.,* 1995). This indicated that both bacteria and spermatozoa are introduced at artificial insemination (AI)/ natural service is capable of triggering transient endometritis (Troedsson *et al.,* 2001).

Sperm uterine interaction

The uterus of domestic animals, including the horse, has a dual role in the interaction of the uterus and sperm. On one hand, uterine contractions carry sperms toward the oviduct and on the other hand the uterus eliminates excessive spermatozoa.

i) Transport of spermatozoa towards oviduct

Regardless of mares being bred by NS or AI, semen is deposited directly into the uterus. From here, it is transported to sperm storage site of the oviduct i.e. caudal isthmus. Spermatozoa have been found to reach the tip of uterine horns and oviducts within 8-20 minutes and 13-29 minutes of semen deposition, respectively (Katila *et al.,* 2000). But considering the fact that individual spermatozoa move too slowly (60 μm/sec) to account for such rapid transport, there must be other mechanism responsible for movement of spermatozoa towards the storage site rather than active spermatozoa movement. Investigations on this aspect had shown that insemination, visual contact with stallion, and teasing were associated with a rapid onset of myometrial contractions simultaneous with oxytocin secretion (Madill, 2000). Campbell and England (2004) showed that it is not the teasing but vaginocervical manipulation that may be important in promoting contraction of mare's uterus with increased plasma concentration of oxytocin. Moreover, potent ecbolic oxytocin was detected in semen, extracts from the testis and epididymis of stallions

(Watson *et al.,* 1999). Besides this, other ecbolic prostaglandins have been isolated from seminal plasma of humans. But contrary to this, oxytocin administered post insemination reduces pregnancy rates (Rigby *et al.,* 1999). This may be because of exogenous oxytocin resulting in unphysiological high endogenous oxytocin levels that evacuate the uterus prior to colonization of spermatozoa. Thus like other mammalian species, myometrial contractions in mares have been shown to play a physiological role in helping the spermatozoa to progress towards the site of fertilization rather than active sperm movement (Katila, 2001 and Campbell and England, 2004).

ii) Elimination of excessive spermatozoa

Of the billions of spermatozoa ejaculated by a stallion into the uterus of a mare, only small fractions are routinely recovered from the oviduct following breeding. The majority of spermatozoa are eliminated within four hours after insemination and hardly any spermatozoa is left after 48 hrs (Katila, 1995). The two methods by which elimination of excessive sperm takes place are following.

a) Mechanical clearance: Campbell and England (2004) described the phenomenon of mechanical clearance. They found that uterus distended due to large volume of semen which resulted in activation of stretch receptors and strong myometrial contractions. These contractions are aimed at clearance of uterus from endometrial debris, sperms, seminal plasma and inflammatory by-products via cervix/lymphatics.

b) Phagocytosis/inflammation: Uterine inflammatory response after natural mating does not differ from that of AI with fresh semen. Spermatozoa act as an antigen and activate complement system resulting in cleavage of factors C5 and C3. C5a, leukotriene B_4 (LTB_4), PGE, PGF_2 act as chemotactic mediators for polymorphonuclear cells (PMNs) and C3b opsonises spermatozoa thus facilitating their phagocytosis by PMNs. (Troedsson *et al.,* 2001)

The acute inflammatory response due to spermatozoa causes release of cytokines and PG from the inflammatory cells. Cytokines especially the IL-6 has both pro and counter inflammatory role. It would initially promote acute inflammation and PMN recruitment; later on it would induce PMN apoptosis and phagocytosis by monocytes, which would become inflammatory macrophages, leading to the termination of the inflammation (down regulation of inflammation) (Kaplanson *et al.,* 2003). Both the cytokine and PG induces uterine contraction and reduces further inflammation.

PMNs, the first line of defense is found 30 minutes after insemination and their population peaks in about 6-12 hr. It subsequently subsides after 24 hr and very few numbers of PMNs

are found at 48 hr post insemination. It is considered that the transient inflammation induced by contaminants is of 24-36 hrs and the majority of inflammatory products cleared from the uterus (Katila, 1995) within this time.

Spermatozoa binding and role of seminal plasma

Spermatozoa were observed to bind to neutrophils by the acrosomal region of the sperm head and due to this aggregation only few spermatozoa reach the oviduct (Alghamadi, 2004). Interestingly there are some other observations like; live and dead sperms induced a similar PMN response (Katila, 1997), some sperms seemed to be resistant to PMN binding and reach to oviduct even in highly inflamed uterus (Alghamadi, 2001). Thus till date, the mechanism of a possible specific binding between spermatozoa and PMNs selection criteria and its importance to fertility is unknown.

Seminal plasma has been shown to have a dose dependent suppressive effect on PMN chemotaxis, phagocytosis of spermatozoa and complement induced cytotosis *in vitro* (Troedsson *et al.*, 2000). Troedsson *et al*, (2001) found that presence of seminal plasma in an insemination dose shortened the duration of breeding induced inflammation. A recent *in vitro* study by Alghamadi (2004) proved that equine seminal plasma reduces sperm binding to PMNs and improves fertility of fresh semen inseminated into inflamed uteri. Fractionation of equine seminal plasma according to molecular weight, revealed a 50-100 kDa protein fraction to be most suppressive on PMN chemotaxis (Troedsson *et al.*, 1999). Further studies showed that 6 mg/ml of this precipitated protein fraction had similar effect like whole seminal plasma (Alghamadi, 2004).

Intra uterine fluid (IUF)

The inflammation is often, but not always, accompanied by accumulation of intra uterine fluid. Secretions from the uterine glands and transudate from the blood are generally believed to be source of uterine fluid in mammalian uterus (Tunon *et al.*, 2000) and considered as physiological at estrus and after service (Watson, 2000). The incidence of IUF at the time of estrus in mares is as high as 39 per cent (Reilas *et al.*, 1997). Myometrial contractions and lymphatics eliminate the intra uterine fluid. .

In mares fertilized zygote descends into uterus by 5 days post-ovulation which means that the uterus in normal mares must be capable of spontaneously clearing the inflammation debris and fluid by this time. A normal uterus clears the inflammatory by products with in 24-36 hrs and IUF in 12 hrs postmating by down regulation mechanism and myometrial contractions, respectively. The incidence of IUF in diestrus is up to 43 per cent in mixed population of mare as studied by Newcombe (1997) and considered as pathological because it reduces the pregnancy rates (Watson, 2000).

Persistent inflammation

In some mares the physiological transient endometritis persists and changes this inflammation to pathological and resulting environment is not compatible for establishment of pregnancy. Studies have shown that the persistent inflammation is detrimental for sperm motion characteristics (Alghamadi, 2001) and the resultant IUF accumulation is also detrimental for both embryo and sperms (Relias, 1997). If the inflammation becomes chronic, fibrosis will develop and lead to scar tissue formation. The end result is an in hospitable environment for the embryo when it descends into the uterus. This inflammation due to breeding is called Persistent mating induced endometritis (PMIET).

Pathophysiology of persistent mating induced endometritis

Any defect in the uterine defense mechanisms lead to PMIET. The most likely causes are impaired down regulation mechanisms, decreased myometrial contractions and some miscellaneous factors.

a) Myometrial contraction

i) Hormonal defect: The main hormones, which are responsible for myometrial contraction, are oxytocin and prostaglandin. Researchers have found no difference in receptor concentration and plasma concentration of ecbolic hormones in normal and susceptible mares.

ii) Neuronal defects: There is very little information on neurological control of myometrial contractility in mare, but there are a few reports, which suggested that the differences lie at autonomic level.

iii) Myogenic defect: The muscular defect appears to be an intrinsic contractile dysfunction of the myometrium and it is independent of the age, parity, receptors and intracellular calcium concentration (Rigby, 2001).

b) Cytokine production: Fumuso *et al.*, (2003) showed that artificial insemination upregulated in mRNA expression for all three cytokines (IL-1β, IL-6, TNF-α) in both resistant and susceptible mares, with persistence in diestrus in the later, thus probably indicating a point of down regulation failure in these mares.

c) Miscellaneous defects

These defects are mainly acquired and anatomical defects and are as follows:

i) It is thought that mares which accumulate fluid in the uterus during estrus have more glands with large diameter and wider lumen than mares without IUF and due to hypersecretion of mucus, more accumulation of IUF takes place (Relias, 1997).

ii) Mares that accumulate IUF may have cervical fibrosis or anatomical changes (cranial tilting of vulva) often associated with old age and multiparity resulting in a pendulous or entirely tilted uterus. The more ventral position of uterus likely contributes to IUF as less fluid can be cleared by gravity.

iii) Uterine lymphatic drainage may be impaired in susceptible mares. Lymphatics clear particulate matter from the uterine lumen and drain edema from the uterine wall. Endometrial vascular degeneration including elastosis, fibroelastosis and calcification of vessels also contributes to delay in uterine clearance and more accumulation of uterine fluid.

Treatment

Treatment is directed at the rapid removal of intrauterine fluids and inflammatory products after breeding. The line of treatment recommended as follows:

a) Because of sperm uterine interactions it is generally accepted that mares should be mated once during oestrus in the 48 hours preceding ovulation so that there could be more time for further treatment also UDM is more competent in estrus. Equine spermatozoa has a long life and can live more than 48 hours in the uterus, thus there are not any reduction in pregnancy rates if mares bred prior to ovulation.

b) It is recommended to perform large volume lavages with 1-2 l of NS/RL. First lavage at 6 to 12 hours and second lavage at 24-36 hrs after mating in susceptible mares is preferred. It is recommended to delay the lavage until 4 hours after breeding so that viable sperm are not prematurely washed out of uterus. It limits contact between inflammatory by products and the endometrium as the peak PMNs are also found in between 6-12 hours (Katila, 1996).

c) Use of ecbolics is an important aspect in the treatment of PMIE. If there is more than 2 cm of IUF before breeding, oxytocin 20 IU IM/IV along with the lavages, is advised to facilitate uterine emptying. $PGF_2\alpha$ may be useful in the treatment of susceptible mare but needs to be administered with caution to avoid undue complications. It would be better to use cloprosternol 250 µg IM at the time of each lavage. Oxytocin induces strong uterine contractions for 30-50 minutes and $PGF_2\alpha$ produces contractions that persist for 4-5 hrs.

d) If bacteria are isolated from the uterus before breeding, the mare should be treated with appropriate I/U antibiotics for a minimum of 4-5 days and is not advisable to be bred mare in that cycle. The indiscriminate use of intrauterine antibiotic administered to all mares can not be justified and should be discouraged.

Conclusions

After mating there is a physiological transient endometritis triggered both by spermatozoa and microorganisms. Sperm motility probably is not essential to sperm transport thought the uterus, but myometrial contractions carry sperm towards the oviduct. Sperms activate complement, which results in chemotaxis, and phagocytosis by PMNs. Selection process is not known but only a minority of sperm escapes phagocytosis. Seminal plasma inherits PMN spermatozoa binding. Chemotactic role of spermatozoa suggest that transient inflammation is necessary to clear uterus from excess spermatozoa and inflammatory by products, well before the embryo enters the uterine lumen. In susceptible mare inflammation persists may be due to defect in myometrial contraction, down regulation or some anatomical causes resulting in insufficient uterine clearance and the pathological conditions develops. PMIE may be more important cause of infertility in susceptible mare than infections endomeritis. Susceptible mares should be treated by restricted breeding, large volume lavages and ectolics.

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Influence of seminal plasma proteins in semen fertility

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The seminal plasma is a highly complex biological fluid comprising of proteins, enzymes, lipids, vitamins, minerals, hormones, fatty acids, amino acids and trace elements. There are variety of proteins in the semen viz. inhibin, plasmin, androgen binding protein, transferrin, immobilin, calsemine, heparin binding protein, heat shock protein, cellular retinol binding protein, acrosome reaction potentiating protein and forward motility protein (Kulkarni 2003). Few of these proteins may be exploited as potential markers of male fertility. Many proteins have been studied to predict the bull fertility (Dejarnette et al. 2004). The presence of 21, 25, 44 and 239 kDa proteins in seminal plasma have been identified as the potential markers for bull fertility (Roncoletta et al. 2006). The current manuscript is an update of characteristics of different seminal plasma proteins (SPP) that play an important role in governing the fertility potential of semen.

Seminal proteins of reproductive organ origin: A major part of the proteins originate from the tests, epididymis, vas deferens, ampullae, vesicular glands, prostrate and Cowper's glands (Gupta et al. 2002). The biosynthesis and secretion of these proteins is regulated by blood testosterone. These proteins have special role in biology of reproduction and are important molecular markers of male fertility.

Structural and soluble proteins: These proteins have been expressed in the accessory sex gland fluid and associated with bull fertility (Karunakaran et al. 2012). They include osteopontins (OPN), prostaglandin D synthase (PGDS), fertility associated antigens (FAA) and acidic seminal fluid proteins (aSFP). They have an important role in sperm metabolism, cause disaggregation of SPP, protective action against stressful environment and influence heparin binding activity (Killian et al. 1993). The semen cryopreservation causes alterations in protein molecules that influence the metabolism and consequently fertilizing ability of the sperm. Therefore, these proteins are used as markers for differentiation of bull fertility and semen freezing qualities. Cancel et al. (1997) noted that the membrane integrity decreases when spermatozoa are cooled below 15^{o}C. The decrease in membrane integrity may result from a decrease in structural proteins owing to leaking that could alter the fertility of the semen from a particular bull.

Proteins involved in capacitation: They are produced by male accessory glands, secreted into seminal fluid and upon ejaculation bind to sperm (Bellin et al. 1996). Heparin, a

commercially available, sulfated glycosaminoglycan, induces capacitation in sperm from bulls (Bellin et al. 1996). Being a highly differentiated cell, sperm has minimal transcriptional and translational activity and cannot synthesize new heparin. The presence of specific HBP on sperm indicated affinity of sperm to heparin, subsequent ability of sperm to capacitation and thus the fertility potential of a bull (Gerena et al. 2000). Addition of heparin binding protein to epididymal sperm induced heparin-stimulated capacitation (Bellin et al. 1998). Mor et al. (2006) isolated and studied the physiological activities of HBP in goats and suggested that HBP act as a regulatory cell protein on motility.

Proteins involved in acrosome reaction: The proteins identified on the acrosome of mammalian sperm include ubiquitin, caveolin-1, t-SNARE syntaxin, the v-SNARE synaptobrevin, the calcium sensor synaptotagmin and the ATPase N-ethylmaleimide-sensitive factor (NSF). These proteins modulate secretion and release lysosomal enzymes during the acrosome reaction (Hutt et al. 2002). Michaut et al. (2001) reported that stallions with infertility problems were deficient for SNARE and caveolin-1. Other acrosomal proteins which help in zona interaction are proacrosin / acrosin, SP-17, MC-41, SAMP-14, SAMP-32, AM-50 and AM-67. The receptors of these proteins are localized to the inner acrosomal membrane during zona binding (Yang et al. 2006). These proteins have been demonstrated as a potential tool for bull fertility.

Heat shock proteins (HSP): HSP are one of the regulators for sperm adhesion and penetration with zona pellucida (Asquith et al. 2005). They act as a component of syneptonemal complex during meiosis and low level of HSP causes faulty meiosis leading to aneuploidy (Dix et al. 1997). These proteins control final maturation of sperm by helping in the extrusion of cytoplasmic droplets, membrane remodeling and reducing creatinine kinase (Huszar et al. 1992). Consequent upon the sperm production, HSP also activates nitric oxide synthase enzyme which is beneficial for post-thaw sperm motility (Lewis et al. 1996). They protect sperm from oxidative stress during cryopreservation, maintain intracellular protein homeostasis and regulate both spermatocytogenesis and spermiogenesis (Shi et al. 1998). HSP are expressed primarily in pachytene stage whereas little synthesis is in leptotene and zygotene stage. This indicates that expression of HSP is in a stage specific manner during spermatogenesis (Randy et al. 1988).

Sperm surface proteins: These proteins are important for the diagnosis of certain cases of infertility. Low expression of 56 kDa and 57 kDa proteins may be one of the reasons for infertility in men (Rajeev and Reddy 2004). Kim et al. (2001) hypothesized that the particular nature of 56 kDa and 57 kDa may allow them to remain temporarily associated with mouse surface after being exposed to the external environment during the course of acrosomal exocytosis. Another sperm surface protein is P34H. It plays an important role in

the binding to the egg's zona pellucida. Infertility in male human has been explained by the absence of P34H for sperm surface and is therefore proposed to be a fertility marker (Boue and Sullivan 1996). Likewise, P25b is also necessary for the binding to the surface of egg acquired through the interaction between epididymosomes and male gamete. The role of these proteins was revealed through the elucidation of the structures of the proteins, characterization of the functions of the individual proteins and the use of specific antibodies.

Proteins involved in gamete interactions: The success of fertilization depends on several events pertaining to gamete interactions. These proteins are associated to the sperm plasma and inner acrosomal membranes by a glycosylphosphatidyl inositol (GPI) anchor and also possess hyaluronidase activity (Cherr et al. 1996). The known molecules include PH-20 and PH-30, tyrosine kinases, protein kinases, galactosyl transferases, adhesins, integrins, extracellular matrix proteins such as vitronectin, fibronectin and laminin, G proteins and proto-oncogenes like c-kit, c-fos, c-myc and c-ros. Spermatozoa must pass through cumulus oophorus followed by the zona pellucida for fertilization. Sperm bind to zona pellucida though protein ZP3. Several sperm proteins have been proposed as being mediators of the interaction with the zona pellucida proteins. The primary receptors bind to ZP3, which is responsible for the binding of the acrosome intact spermatozoa to the eggs matrix and the induction of the acrosomal exocytosis, whereas secondary receptors maintain the sperm binding to the zona pellucida through interactions with ZP2 following the sperm acrosome reaction (Topfer-Petersen et al. 2000).

Granulocyte Elastase: These are related to an increase in the white blood cell count in the semen in case of inflammation of male genital tract. Their death, releases elastase, leading to production of reactive oxygen species, which have detrimental effect on spermatozoa and thus inhibits penetration of zona-free hamster egg by the sperm (Baker et al. 1996).

Antisperm Antibodies: They inhibit fertilization by interfering sperm zona binding after reacting with fertilization antigen found on sperm (Zraly et al. 2002). Furthermore, experimentally induced antisperm antibodies can block in vitro fertilization (Kim et al. 1999).

Reactive Oxygen Species (ROS): Excessive generation of ROS like H_2O_2 and oxygen free radicals causes lipid peroxidation of sperm membrane reducing their ability to respond to calcium ionophore challenge. This responsiveness is directly correlated with fertilizing ability *in vitro* (Storey, 1997). This damage inhibits sperm ability to bind zona pellucida, undergo acrosome reaction and fuse with the egg membrane. This assay may be used to detect sub-fertile semen samples having normal motility and morphology.

Unidentified proteins: Roncoletta et al. (2006) conducted two-dimensional gel electrophoresis of Nellore bull sperm and indicated 27 spots in the higher fertility group as compared to just one spot in the lower fertility group (SM-244). Out of these spots only two proteins (SM-244 and SM-239) showed a great potential for predicting bull's fertility. The amount of SM-239 was 8.5 times greater in the sperm protein profile of high fertility groups of Nellore bulls. For the other spots potentially associated with fertility were not identified. In another study, Zahn et al. (2006) reported 43 protein bands in equine sperm extracts. However, only two of them were present in all animals (B-18 and B-35) and related with fertility. The other bands between good and regular freezers were not recognized.

Seminal plasma proteins mediate and regulate inter- and intra- cellular signaling during capacitation, acrosome reaction, sperm oocyte fusion and fertilization and govern the reproductive potential in bulls. However, these proteins have not been studied in detail. Some proteins may be associated with fertility while others may be indicator of problems arising at the time of sperm formation and maturation. Thus, a combination seminal attributes and proteins may be worth in detecting fertility potential of males / semen samples.

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Anti-sperm antibodies: a cause of subfertility/infertility in bovines

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Anti sperm antibodies (ASA) are antibodies directed against the sperm. Under normal conditions the immune system develops antibodies to help protect our immune system against illnesses. However, in the case of anti sperm antibodies, the body develops and directs specific antibodies against the sperm which is the wrong approach and can cause negative side effects upon the health status of the sperm and can cause infertility in men as well as animals. ASA may also be defined as immunoglobulins of the IgG, IgA and IgM isotypes, which can be developed against the antigenic proteins of head, mid piece and tail or combination of three. First, IgM antibodies are produced, but these are not secreted into the genital tract because their size is too large to pass the epithelial barrier. Shortly afterwards, antibodies of the IgG class appear, and these can enter the genital tract. The ASA of the IgG class come into contact with the spermatozoa and attach to these. ASA may develop in male as well as female. In some cases and more commonly indeed during infection, secretory IgA-ASA are produced locally in the genital tract, probably the epididymis/ cervical mucus/ uterus. The occurrence of ASA is being connected with infertility in humans, laboratory and farm animals, but major work had been done in humans. Sperm-reactive antibodies can also be present in serum yet undetectable in semen or within female reproductive tract secretions. Antibodies directed to sperm antigens can be detected in seminal fluid, where they may be bound to the sperm surface or solubilized in the seminal plasma. Seminal plasma ASA not attached to the sperm surface are probably of little clinical significance. In humans, dogs, bulls and other species, seminal plasma components do not ascend beyond the vagina, but this does not true for all species. Although the probable reason for the presence of unbound antibodies in seminal plasma traditionally is believed to be due to the excess of antibodie relative to the sperm specific antigens, the presence of unbound antibodies due to a reactivity difference that found on the spermatozoa cannot be excluded automatically when using indirect tests. It is considered that the indirect measurement of sperm antibodies might be grossly unreliable as compared with the results obtained with direct method.

Types of Antibodies

The antibodies formed may be of the circulatory type (in the blood serum) or secretory type (in the tissue). Once sperm antibodies have formed, they can affect sperm in several

different ways, on the basis of which these can be classified into five categories.

Agglutinting Antibodies: Some antibodies will cause sperm to stick together or agglutinate. Agglutinated sperms clump together in dense masses and thus are unable to migrate through the cervix into the uterus.

Opsonizing Antibodies: These antibodies mark the sperm for attack by Natural killer (NK) cells of the body's immune system.

Immobilizing Antibodies: These antibodies cause reactions between the sperm membrane and the cervical mucus preventing the sperm from swimming through the cervix.

Blocking antibodies: Such antibodies can also block the sperm's ability to bind to the zona pellucida of the egg, a prerequisite for fertilization.

Phagocytic Antibodies: There is recent evidence that the fertilized egg shares some of the same antigens that are found on the sperm. It is possible that sperm antibodies present in the mother can react with the early embryo, resulting in its destruction by phagocytic cells.

Causes for Development of ASA

In the case of the testis, many of the antigens expressed by developing spermatogenic cells are not recognized by the immune system as self i.e. can induce an immune reaction and tissue damage. Blood testis barrier (BTB) consists of a tight junctions between sertoli cells and the seminiferous tubules. It prevents testicular cells, which express foreign antigens from coming into contact with lymphoid tissue and immunocompetant cells. The primary purpose of the BTB is to prevent autoimmune reactions from destroying the developing germ cells. Yet, this barrier can be broken, through injury to the reproductive tract, thereby allowing the immune cells to come into contact with the sperm and recognize them as foreign bodies. Once the barrier is broken, immune cells are able to detect the presence of sperm due to their unique antigen surface. This triggers a response by the immune system to treat sperm as an "invader" and attack it.

The male may produce antibodies to its own sperm as a result of contact between blood cells and sperm due to undescendant testicles, twisting of testicles, testicular biopsy, injury to testicles, testicular cancer, varicocele, natural service, frequent semen collection, or sometimes for unknown reasons. ASA are more frequent in females with genital diseases such as metritis and vaginitis, or during mating than in females with ruptures in genital organs. The formation of ASA has also been induced by breeding accidents in stallion, testicular biopsies/ sperm granulomas/ epididymal aspirates in dogs, very low temperature

in swine, cryptorchidism in boars, vasectomy in ram, and excessive male exploitation in rabbits.

Effect of ASA on reproduction

Presence of ASA can inhibit passage of spermatozoa through cervical mucus, prevent membrane fluidity changes needed for capacitation, reduce the ability of spermatozoa to undergo the acrosome reaction, and interfere with binding to the zona pellucida and fertilization. It has also been reported that ASAs cause early embryonic death and functional disorders of spermatozoa or leading to the death of spermatozoa.

Mechanism of Development of ASA

Various protective mechanisms besides the blood-testis barrier and the blood-epithelium barrier have also been identified. Immunosuppressive substances have been found in semen and follicular fluid. Studies on the relationship between inhibitors of T-cell function in seminal plasma and sperm autoimmunity have been complicated by the fact that seminal plasma contains very high concentrations of the polyamines spermine and spermidine These molecules are not lymphosuppressive themselves, but are converted to cell growth-inhibiting oxidized forms by polyamine oxidase, an enzyme found in serum and particularly fetal calf serum. These are unstable and may be further metabolized to the cytotoxic molecules acrolein and putrescine. These sequences of reactions are responsible for lymphosuppressive activity of seminal plasma. Moreover, suppressor T lymphocytes in the human immune system, which partially mediate the normal state of immunologic unresponsiveness toward sperm autoantigens, also play an important role in preventing the autoimmune response. Three complement-regulatory proteins, decay-accelerating factor, membrane cofactor protein and CD59, have been found on spermatozoa and in the extrafetal tissues. It is likely that these inhibitors are essential for normal reproductive function. A soluble form of Fc γ RIII (CD16) has been isolated from seminal plasma, which may modulate immunosuppression of antisperm immune responses in both male and female reproductive tracts. Individuals who were ASA positive had lower detectable levels of Fc γ RIII compared with those who were ASA negative, indicating that the inhibition of CD16 plays an important role in preventing ASA production (Lu et al. 2008).

The possible relationship between ASA production and *Ureaplasma urealyticum* infection was also investigated. It was confirmed that *U. urealyticum* and human sperm membrane proteins share cross-reactive antigens (61, 50 and 25 kDa). Among the cross-reactive antigens, the urease complex component UreG of *U. urealyticum* was determined. Furthermore, the cross-reaction between human nuclear autoantigenic sperm protein (NASP) and UreG was verified. Both anti-rUreG antibodies and antiserum against synthetic peptide NASP393-408 inhibited mouse sperm-egg binding and fusion. After immunization

with rUreG or synthetic peptide, 81.2 and 75% of female mice became sterile, respectively. These findings proved that the cross-reactive antigens shared in sperm and microorganisms induce ASA production and infertility (Wang et al, 2009).

Methods to detect ASA in Blood Serum/ Seminal Plasma/Cervical mucus

Numerous tests have been developed for the detection of ASA in several species. An ideal assay would be an objective assay that detects the presence of ASA bound to sperm, their location, antibody load and isotype, with high sensitivity and specificity and that could be performed on live cells. Anti-sperm antibody detection methods are based on the identification of antibodies present in serum, seminal plasma, vaginal or cervical secretions, or follicular fluid (indirect methods) or the identification of antibodies bound to the sperm cell (direct method).

1. Indirect Methods

a. *Agglutination techniques:* Agglutinates of spermatozoa are observed after serum and donor spermatozoa are brought together in gelatin medium or on microslides.
b. *Sperm immobilization tests:* Complement-mediated sperm immobilization or cytotoxicity assays, count immobilized spermatozoa, when a complex of antibodies is linked with antigens on spermatozoa in the presence of complement.
c. *Techniques using labelled antibodies:* Indirect immunofluorescent antibody technique for spermatozoa requires a FITC labeled antibody, a good fluorescent microscope, preferably equipped with interference filters, adjusted for the fluorocromes used. The immunoperoxidase technique uses specific antibodies to localize antigens. Usually the IgG fraction of the immune serum contains most of the active antibody and the use of purified Fab fragments of IgG helps to ensure that antigen is localized specifically rather than non-specific binding of the Fc piece or other serum proteins occurring. A second antiserum against the species of Fab used in the first step has to be raised, the Fab fragments are prepared, and these are covalently labeled with horseradish peroxidase.
d. *Indirect immunobead test/ SpermMar Test:* Immunobeads/ latex particles of IgG/ IgA/ IgM type are used to locate the presence of this on surface of motile spermatozoa. The region of the sperm surface to which ASA are bound can be determined.
e. *Enzyme-linked immunosorbent assays (ELISA):* ELISA use enzyme-linked anti-human antibodies, which bind to the antibodies on the sperm surface. A substrate for the enzyme is then added, and its product is measured colorimetrically.

2. Direct methods for ASA detection: Antibodies, which are directly bound to sperm

a. *Mixed agglutination reaction:* The mixed agglutination reaction (MAR), is a direct test in which a washed suspension of Rhesus (Rh)-positive human red blood cells (RBCs) coated with Rh-directed human IgG or IgA are mixed with drops of semen. IgG- or IgA- bound sperm are then bridged with the IgG- or IgA coated RBCs after the addition of anti-human IgG or IgA antibody. When spermatozoa in the ejaculate are antibody-bound, they may form mixed agglutinates with the RBCs.
b. *Immunofluorescence assays:* Immunofluorescence assays use fluorescein-tagged anti-antibodies which bind to antibodies on the sperm surface.
c. *Flow cytometry:* Fluorescein-tagged antibodies are used in this technique. This method can be used to evaluate ASA directly bound to motile spermatozoa.

3. Characterization of antigenic proteins: Sperm proteins, separated on acrylamide gels, are transferred to nitrocellulose/ PVDF membranes and reacted with blood serum/ bodily fluids to find sperm antigens, responsible for production.

Studies related to Prevelence of ASA in Animals

Male: Naturally occurring ASA have been recognized in many species including dogs, cows, horses. Perez and Carrasco (1964) reported two naturally occurring cases of agglutinating ASA in bulls associated with reduced spermatozoal motility and infertility. The first occurrence of ASA was recorded in bulls aged 5 to 6 months and boars related to changes occurring in the developing reproductive system (Fayemi et al. 1992 and Zraly et al. 1997). Negative effect of ASA on sperm function during *in vitro* fertilization was demonstrated by Kim et al. (1999) and Lombardo et al. (2001). In a study involving 612 bulls of two different breeds and several ages, serum titer were obtained by the use of an ELISA assay (Zraly et al. 2002). It was also confirmed by Lazarevic et al. (2002) that in male calves, ASA naturally occur before puberty and are more probably the result of cross reactivity with microbial antigens. AI-Kheraije (2010) suggested an increase in antisperm antibodies in the serum of old camels as well as in the fluid of the seminiferous tubules of young and old camels during non-breeding season. Level of antisperm antibodies in the blood serum and seminal plasma of 26 breeding cattle bulls was analyzed using IPA, SpermMar test and ELISA and their relationship with sperm parameters was evaluated in our lab (unpublished work). Out of 26 tested bulls, there were 11 bulls with low values of either HOST or *in vitro* acrosome reaction / CMPT and higher significant level of serum/ seminal plasma ASA. Immunoblotting of sperm extracts of 15 bulls with their respective blood serum revealed ASA against 19 antigenic proteins. Proteins with mol wt of 55, 16, 30 and 24 kDa proteins also cross reacted with anti-HBP, anti-TIMP-2 and anti-FA-1. It

revealed the development of ASA in blood serum of bulls against fertility associated proteins (HBP/Timp-2/FA-1).

Female: Antibodies to sperm or egg yolk had been suggested to be possible causes of subfertility in cows (Hunter, 1972; Coulter et al. 1976). The incidence of ASA was reported in 26% Holstein cows by Farahani et al. (1981). Wang and Xie (1990) found that 34.5% of infertile cows had anti-sperm antibodies in blood serum as compared to 6.7% in non-pregnant cows with a history of normal fertility. On the basis of comparing the blot with the positive sperm agglutinating serum to the blots of the positive controls, Kanchev et al. (1993) identified two buffalo sperm isoantigens of 40 kDa and 120 kDa and concluded that circulating agglutinating anti-sperm antibodies were very rarely detected in buffalo cows with unexplained infertility after several artificial inseminations. Milovanovic *et al* 2005 strongly confirmed the hypothesis that immunemechanisms may be involved in reproductive disturbances due to high levels of ASA of IgA class. Fayemi (2005) serologically investigated the sperm antibodies in 11.75% Zebu cattle and found that the mean age at first calving and the mean inter calving interval were significantly higher in the group positive for sperm antibodies compared to the negative animals ($P<0.001$). The cows having more unsuccessful inseminations showed higher antisperm-antibodies with high titre in serum and mucus (Sarma et al 2009). A study done on 42 cows in our lab highlights the detection of ASA in blood serum and cervical mucus of non-inseminated heifers/ variably inseminated cows/heifers. A combination of immunological tests (IPA, SperMar test, ELISA) revealed sub fertility/ infertility in 22.2 % of the tested animals due to the presence of ASA in serum and CM. Immunoblotting of sperm extracts with blood serum of variably inseminated heifers also indicated the occurance of ASA against 23 proteins and cross reaction of similar sperm proteins with blood serum of variably inseminated cows and anti-fertility associated proteins (HBP, TIMP-2 and FA-1) on immunoblots indicated the formation of ASA against sperm specific proteins.

In general, mechanisms by which ASAs inhibit fertility are unclear and may be unique to each individual's antibodies. Antisperm immunization has only a relative effect on impairment of fertilization, rather than an absolute effect. It is suggested that ASA should be considered as one of the important factor amongst other causes subfertility/infertility.

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Biomarkers and other diagnostic protocols for predicting male fertility in crossbred cattle and buffalo

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Success in reproduction of farm animals depends on male factor, female factor, accuracy of estrus detection and efficiency of the artificial insemination. The male factor is important as semen is disseminated very fast due to extensive use of AI under field conditions. Hence, the procreation potential of the male should be accurately assessed to augment the reproductive performance of the animals. Thus, the male reproductive efficiency is a major concern that determines the economic fate of a dairy farming industry.

Fertility traits of semen can be categorized as compensable or uncompensable defects. Defects in compensable traits (motility and morphology) can be overcome by increasing the number of spermatozoa per insemination. Defects in uncompensable traits affect the function of spermatozoa during the later stages of fertilization and in embryonic development and as such cannot be compensated. Uncompensable traits include nuclear vacuoles, morphological deficiencies that do not suppress movement, defective chromatin structure. Low fertility in bulls has an uncompensable component that includes reduced cleavage rate and delayed pronuclear formation following in vitro fertilization. Currently available fertility assays assess the defects that affect functional competence of spermatozoa (i.e. capacitation, acrosome reaction, sperm-oocyte interaction), however these cannot definitively predict fertility. At present, the molecular nature of sperm fertility defects or biomarkers for accurate fertility prediction is not known.

Several studies have been conducted to study protein markers in males. 20.4% sperm proteins are common and rest are unique to high and low fertility groups. 40.6% of proteins are sperm membrane proteins. Biological process annotation of membrane proteins revealed that majority of membrane proteins involved in transport (33%), cell communication (18%) and metabolism (17%).

Molecular Markers: A genetic marker is a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or trait. The molecular genetic approach could enable us to detect the fertility of males. A number of autosomal and sex chromosomal genes have been found to be associated with the reproduction traits of the

males. A few important genes related to male fertility and seminal attributes have been localized in bovine autosomes and Y chromosome (Xiao *et al*., 1998).

Mammalian Y-Chromosome: Genes like, SRY, RBMY, TSPY genes, the Xp22 genes, and other Y-linked genes present on the Y-chromosome play important roles not only in sex determination, but also in spermatogenesis, male fertility, and growth control.

Testis specific protein, Y encoded (TSPY): Testis specific protein, Y encoded (TSPY) is expressed in spermatogonia, primary spermatocytes and in prostate-cancer tissue and cells. The gene is predominantly expressed in adult testes and to some extent in fetal testes due to lower content of germ cell precursor stages. RT-PCR analysis shows that human testes start expressing TSPY gene by the prenatal age of 22 to 26 weeks (Zhang *et al*., 1992).

Sex-determining region, Y-encoded (SRY): The SRY expression triggers the proliferation of the supporting cells, some of which are the precursors of Sertoli's cells in the XY gonads. The SRY also stimulates migration of cells into the XY gonad from the adjacent mesonephros. The differentiation of gonadal primordial cells into male Sertoli's and Leydig's cells depends on the presence of SRY and Y chromosome.

Ubiquitin Specific Peptidase 9, Y-linked (USPY) gene: Ubiquitin-specific protease 9, Y-chromosome (USPY), was the first gene to be identified in the azoospermia patient. USPY gene is closely associated with sperm production and is absent or partially deleted in the infertile men. The USP9Y gene encodes a protein called ubiquitin-specific protease 9. Ubiquitin is secreted into epididymal fluid and gets covalently linked to the surface of defective mammalian spermatozoa. Since many of such spermatozoa can be found in the semen, ubiquitin is a suitable marker of sperm abnormalities.

RBMY gene: RNA binding motif Y linked (RBMY) gene is associated with the arrest of spermatogenesis at meiosis because azoospermic men have been detected with the deletion of some members of RBMY family. RBMY deleted mice produces high level of abnormal sperm.

Autosomal Genes:
Calicin gene: Calicin (~60 KD), contributes to the formation of the peri-nuclear theca as an architectural element involved in the shape changes in sperms and the intimate association of the sperm chromatin with the acrosome and the plasma membrane. Teratozoospermia results from the alteration of the cytoskeletal element during spermiogenic differentiation.

Aromatase: It is a member of cytochrome P450 protein family and produces mono oxygenase that catalyze many reactions involved in steriodogenesis. Aromatase enzyme is an important factor in sexual development and maintaining normal spermatogenesis. Aromatase deficiency in male mice leads to infertility due to impairment of spermatogenesis associated due to decrease in sperm motility and inability to fertilize oocytes. It catalyzes estrogen biosynthesis from androgens. Overproduction of aromatase leads to estrogen over-production in male.

Fertility associated metabolites in dairy bulls have been identified in seminal plasma as citrate (2.50), tryptamine / taurine (3.34-3.38) and isoleucin (0.74) and leucine (0.78) ppm. Similarly, fertility associated metabolites in serum are isoleucine (1.14), asparagine (2.90-2.94), glycogen (3.98) and citrulline (1.54) ppm. These fertility associated metabolites needs to be validated in an independent larger set of high and low fertility dairy bulls.

Some seminal proteins such as Osteopontin (OPN), Prostaglandin D synthase (PGDS), Bovine seminal plasma proteins (BSPs), Fertility Associates Antigens (FAA) and Acidic seminal fluid protein (aSFP) have been reported (Cancel *et al* 1997, Therien *et al* 1997, Einspanier *et al* 1991, Einspaneir *et al* 1994, Therien *et al* 1998, 1999, Gerena *et al* 2000 and Manjunath *et al* 2002) and the amounts and or presence have been associated with bull fertility (Hynes 1987, Killian *et al* 1993, Cancel *et al* 1997, Bellin *et al* 1998, Parent *et al* 1999, Gerena *et al* 2000, Nauc and Manjunathm 2000, Sprott *et al* 2000 and Manjunath *et al* 2002). Bulls are the half of the herd. Identification of high fertility bulls is very essential. However, none of the methods mentioned above have been found practically usable.

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Dairy herd fertility: role of various factors and associated economics

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Fertility can be defined as the capability of the animal to conceive and produce a viable calf following insemination (Royal *et al*. 2000). It plays an important role in determining the profitability of a dairy farm. Reproduction can have a multitude of impacts on a farm, from altering culling policies, moving primiparous animals into a more productive 2nd lactation, and improving milk production. In other words, reproductive traits are not only a measure of fertility but also of productivity of an animal for life. Because production accounts for more than 88 per cent of the gross income of a dairy farm (Santos *et al*. 2010), it is no surprise that most attention paid to improvements in reproduction evolve around altering milk production during the productive life of animals.

According to LeBlanc (2007) reproductive performance economics are function of two main opportunity costs: 1) The forgone profit as animals remain open beyond approximately 120 days in milk, and therefore spend a more-than-optimal proportion of their lifetime in later lactation; 2) The failure to realize all the profit potential in otherwise profitable cows that are replaced sooner-than-optimally because of the inability to make them pregnant. The magnitude and the source of payback from improved reproductive performance vary among herds, but generally the greatest single component is marginal milk. Marginal milk is additional production without additional capital cost. Even though extra feed is required to make extra milk, there is still net profit, because maintenance costs are diluted into more production. In fact, marginal milk from existing cows is generally the most efficient means to increase profit through management. Additional benefits of improved reproductive performance include more selective and therefore more optimal culling.

Low reproductive efficiency due either to delayed first service, missed estrus, or multiple services per conception continues to be a major problem in dairy herds. Inefficient reproductive performance results in excessively late age at first calving and long lactations. Both of these factors are costly to the dairy producers because of the high replacement costs, breeding expenses and fewer calves being born (Oudah *et al.* 2001). Several reports have showed that poor reproductive performance, manifested as lengthened calving intervals, can result in reduction of milk yield, increased replacement costs and culling rates (Kadarmideen *et al.* 2003 and Sewalem *et al.* 2008). Beever (2006) reported that average

dairy herd fertility is declining, with more services per successful conception, lengthened calving intervals and increased culling due to failure to rebreed, all adding considerable costs to milk production. In other words, the factors like high age at first calving, longer calving interval, late maturity and dry period affect the dairy farm income by decreasing milk and calf production by the animals. It is anticipated that each animal losses 2 to 3 lactations due to poor reproductive efficiency, which largely change the economics of dairy farming. Shortening the calving interval reduces the average days in milk of the herd and, consequently, a greater proportion of animals would be in earlier stages of lactation when peak of milk production and greater income over feed cost occurs, whereas a smaller proportion of animals would be in later stages of lactation producing low amounts of milk with low income over feed cost. The average calving interval can be improved to 12 to 14 months with better management of the animals.

Economic losses due to delayed conception in dairy animals were estimated in different countries. Holmann *et al.* (1983) estimated that the net value per day open was positive ($0.21 to $0.40) for all milking animals when calving interval was extended from 12 to 13 months and on the other side, the value per day open was negative (−$0.04 to −$0.23) when calving interval was extended from 13 to 15 months. So, the 13 month calving interval appears to be most favorable. One day of delay in conception was calculated to cause $2.03 (Lineweaver 1975), $1.24 (DeVries and Conlin 2003) loss in the United States and £2.41 loss in the UK (Esslemont *et al.* 2000) for an average milking cow. Esslemont *et al.* (2000) also reported a loss of £6.52 per day for a high producing cow to become pregnant between 206 and 235 days post-calving (Kafi *et al.* 2007). Shah *et al.* (1991) estimated the economic losses in Nili-Ravi buffaloes due to reproductive failure in Pakistan. The most favorable calving interval for dairy buffaloes was found to be 12 to 13 months. Losses caused by sub-optimal calving intervals were Pakistani Rs. 9–14 per extra day per calving interval. Losses for forced replacement as a result of reproductive failure average Rs. 133 per buffalo present on the farm. Esslemont *et al.* (2000) estimated that per day loss due to per day of delay was £1.73 when the calving interval was extended from 85 days to 100 days post-calving. This loss had risen to £2.86 per day when calving interval was extended from 116 to 145 days post-calving and £3.55 per day when it further extended up to the day when no extra milk came from the current lactation. Khan *et al.* (2008) studied the impact of delayed conception on calving interval of the animal. He stated that as the calving interval increased due to delayed conception, a steady trend was shown, in the low, moderate and high yielding buffaloes. There was a steady decline in milk yield per day of calving interval with delayed conception, associated with lengthened calving interval. An animal that conceives at a later stage of lactation showed a decline in financial returns by 24 to 27 per cent than those that conceived earlier. A study from Pakistan reported that on an average per day loss due to delayed conception in heifer was Pakistani Rs. 212.52, whereas in case of lactating

animals, average per day loss per lactating buffalo was Pakistani Rs. 261.12. Main reason of delayed conception in heifers and lactating animals was only poor feeding (Sadaf 2011).

Other factors beside milk production, which have a tremendous impact on profitability of the dairy farm, are culling and replacement policies. Both of these factors are associated with reproductive efficiency. Improvements in reproduction result in greater flexibility in these policies and allow farmers to take programmed decisions based on economic aspects (Groenendaal *et al*. 2004). Reproductive inefficiency increases cost per pregnancy, increases retention of low-producing animals because of their pregnancy status, and reduces the number of replacements, which diminishes the gain in genetic merit of the herd. Another factor affecting economics is growth rates of heifers. It has been reported that in order to maximize lifetime production, Holstein heifers should calve at approximately 23-24 months of age with ~85% of adult body weight (Gabler *et al*. 2000; Ettema and Santos 2004). Heifers calving at younger age have reduced productive and reproductive performances in the first lactation, whereas those calving at older age have no improvements in productivity at first lactation (Gabler *et al*. 2000; Ettema and Santos 2004), with additional days of unproductive life and feed costs. Under local/Indian conditions, crossbred and buffalo heifers should calve at 28-30 and 34-36 months of age, respectively. In India, the major cause for the late maturity is considered as poor feeding which results in at least loss of one lactation per animal under local environmental conditions.

The profitability of dairy farming also depends upon conception/pregnancy rate at the farm. The direct costs of delayed pregnancy or failure to achieve pregnancy include extra semen, labour, veterinary and drug costs, and the difference between revenue for an animal culled for non-pregnancy and the cost of her replacement. Depending upon milk price and milk yield, each 1 per cent increase (or decrease) in pregnancy rate results in the gain (or loss) of approximately $12 to $25 per cow per year (Overton 2005). Because as pregnancy rate increases, over time, the average days in milk for the milking herd will decrease, leading to higher average milk production per day of lactation, more time per lifetime spent in the most profitable portion of lactation, and less veterinary and breeding costs. As pregnancy rate decreases, average days in milk increases, leading to increased management, feed, and veterinary costs for animals in the least profitable portion of lactation (Joseph and Amin 2009).

Economics of dairy farming

The approximate calculations to work out the economics of dairy farming at current prevailing market prices are presented in table 1.

Assumptions:

- Animals shall be recently calved, lactation no. is less than 3 and raised under stall-fed condition.
- Lactation length shall be 305 days.
- The cost of animals shall be Rs.50,000/buffalo & Rs.60,000/cow.
- The cost of animal shed and feed store= Rs. 2,00,000/- (10 animals).
- The cost of dairy equipments :
  - Buffaloes= Rs. 1,00,000/- (10 animals).
  - Cows= 1,50,000/- (10 animals).
- Interest on capital investment = 10.5 %.
- Depreciation on repair of shed and dairy equipments shall be 5 and 10 % per annum, respectively.
- No depreciation shall be considered on animals during first three lactations.
- No land rent shall be considered.
- Animal insurance= 3.0 % per annum.
- Daily allowance of fodder per animal:
  - Green fodder= 40 kg @ Rs. 80 per quintal
  - Dry fodder= 5 kg @ Rs. 300 per quintal
- Daily requirement of concentrate feed (Rs. 1600/quintal) per animal:
  - 1 kg feed: Buffalo= Upto 5 lt. and Cow= Upto 7 lt. of milk production, respectively.
  - 2 kg feed: Buffalo= >5 lt. (per 2 lt. milk) and Cow= >7 lt. of milk production (per 2.5 lt. milk), respectively.
- Requirement of mineral mixture per animal: 30 g per day @ Rs. 100/kg
- Labour charges: Rs. 300/day (10 animals)
- Miscellaneous charges such as veterinary, electricity, water etc.:
  - Buffalo= Rs. 80/day (10 animals) and cow= Rs. 120/day (10 animals)
- Total milk production Lactation yield (305 days) per animal:
  - Buffalo= 3000 lt. @ Rs. 40/lt. of milk and cow= 4000 lt. @ Rs. 30/lt. of milk
- Returns from farm yard manure : Rs. 30/day (10 animals)

Table 1: Economics of milk production/animal/day

		Buffalo	**Crossbred Cow**
A.	**Capital investment**	**Amount (Rs.)**	**Amount (Rs.)**
i.	Animal	50,000.00	60,000.00
ii.	Animal shed and feed store	20,000.00	20,000.00
iii.	Dairy equipment (cooling unit, Chaff cutter, Chains, Water pump,	10,000.00	15,000.00

	motor, buckets etc.)		
Total		80,000.00	95,000.00
B.	**Fixed cost**		
i.	Interest on investment	23.01	27.33
ii.	Depreciation and Repair on cattle shed and buildings	2.74	2.74
iii.	Depreciation on dairy equipment	2.74	4.11
iv.	Animal insurance	4.11	4.93
Total		32.60	39.11
C.	**Variable Cost**		
i.	Green fodder	32.00	32.00
ii.	Dry fodder	15.00	15.00
iii.	Concentrates	64.00	80.00
iv.	Mineral mixture	3.00	3.00
v.	Labour charges	30.00	30.00
vi.	Miscellaneous charges (Veterinary, electricity, water etc.)	8.00	12.00
Total		152.00	172.00
D.	Total Cost (B+C)	184.60	211.11
E.	Cost per litre of milk	18.84	16.11
F.	**Gross returns**		
i.	From milk	392.00	393.00
ii.	From FYM	5.00	5.00
Total		397.00	398.00
G.	Net income/animal/day	212.4	186.90

Effect of delay in conception on economics

The approximate calculations about the effect of delay in conception on economics are presented in table 2. Based on the previous example (Table 1), per day loss and cost of milk production have been calculated by assuming that:

- The animals shall conceive at a time when peak milk yield (Buffalo: 9.8 and cow: 13.1 lt./day) reduced by 25, 50, 75 and 100 (Dry) %, respectively.
- No concentrate feed and mineral mixture shall be fed to dry animals.

Table 2: Effect of delay in conception on economics

Milk yield reduced by:	Buffalo			Crossbred cow		
	Net income (Rs./day)	Loss (Rs./day)	Cost of milk production (Rs./L)	Net income (Rs./day)	Loss (Rs./day)	Cost of milk production (Rs./L)
0%	212.40	0.00	18.84	186.90	0.00	16.11
25%	128.40	84.00	23.21	120.49	66.41	18.24
50%	68.40	144.00	27.90	54.40	132.50	22.46
75%	-33.60	246.00	55.75	-43.71	230.61	44.85
100% (Dry)	-112.60	325.00	-	-123.10	310.00	-

Economics of reproductive programs

Now days, many synchronization protocols are followed at dairy farms to enhance the profitability. A reasonable alternative to enhance submission rate and the proportion of cows pregnant early after the end of the voluntary waiting period is the incorporation of timed AI programs, either alone or in combination with estrous detection. Timed AI programs are particularly beneficial in farms with low detection of estrous (Tenhagen *et al.* 2004).Low estrous detection rates result in more variable and longer time to first insemination and pregnancy, reduced pregnancy rates, and increased calving interval. The net returns per cow per year are decreased by 4 per cent when estrous detection efficiency is lowered from 65 to 35 per cent (DeVries and Conlin 2003). The timed AI programs such as ovsynch or its modified programs improve reproductive performance of dairy animals and result in economic advantage over only estrous detection when the efficiency of detection of estrous is low. However, fertility and reproductive performance in timed AI programs are not always superior to that of animals inseminated at detected estrous (Chebel and Santos 2010). Therefore, it is important to consider which timed AI program to use and select the one that offers the highest fertility when detection of estrous is completely eliminated (Giordano *et al*. 2011).

To our experience a voluntary waiting period (VWP) of 60±10 days must be given in both cows and buffaloes. If animals come in estrous naturally at the end of this period then insemination should be done. If animals don't show estrous at the end of VWP then timed AI program should be incorporated. Each inseminated animal must be watched carefully for estrous signs around the time of next due estrous cycle and the animals in estrous should be reinseminated after examining the cervico-vaginal discharge. The pregnancy diagnosis can be done at 35-45 days post AI in non-returned animals by transrectal ultrasonography and resynchronization should be done in non pregnant animals. In other words, combination of

timed AI program with estrous detection is more economical than timed AI programs alone (Personal view).

The approximate calculations about the advantage of timed AI program (Double synch) at prevailing market prices are shown in table 3. Based on the previous examples (Table1 and 2), the profit of timed AI program over AI at natural estrous has been calculated by assuming that the animals shall conceive at least one estrous cycle (21 days) earlier by timed AI following synchronization protocol than AI at natural estrous.

Table 3: Profit of timed AI program over AI at natural estrous

Milk yield reduced by:	**Loss/21 days (Rs.)**	**Cost of hormones (1 GnRH analogue and 2 PG injections)**	**Profit of timed AI program (Rs.)**
25%	1764.00	Rs. 888.00 (Institutional price)	876.00
50%	3024.00		2136.00
75%	5166.00		4278.00
100% (Dry)	6825.00		5937.00

Summary

Herd fertility plays a major role in determining profitability of the dairy farm. The losses are mainly caused by decreased milk production and increased non pregnant animals at the farm. The majority of reproductive losses occur due to too long or too short calving interval and dry period, too high services per conception and too old heifers at first freshening. Herd fertility can be improved by adopting latest technologies viz. Transrectal ultrasonography, estrous detection aids etc., improving nutritional as well as health status of animals and sound record keeping system at the farm. In general, incorporating timed AI program for first AI followed by detection of estrous with reinsemination of animals that come in estrous at next due cycle and resynchronization of animals that are non-pregnant at 40-45 days post AI seems to be a profitable strategy.

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Nutritional requirements of dairy animals with reference to season, age, and reproductive status

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The nutrition plays a vital role in animal well being and the economics of animal enterprise. Feeding according to the requirement of the animal, which depends upon the genetic potential , is key to full genetic exploitation of the animal. With crossbreeding programmes and currently with the use of high genetic potential bull germ plasma, the modern dairy cow has been producing large quantities of milk. It is not uncommon for a cow to yield 30-40 kg milk per day. Feeding and management of such high yielders require special skills especially during periods when animal is under production stress. Modern high producing animals are either in lactation or in advanced pregnancy posing a regular metabolic stress to the body. The nutritional requirement depends upon:

- Age of animal
- Physiological state
- Season of the year
- Genetic potential or breed of the animal

The nutrient requirement for different categories of animals is given below:

Calf Nutrition: The new born calf is a non ruminant and its need are entirely different than adult animals. Similarly growing animal need quantity and ratio of nutrients which might differ from other type of animals. Early feeding from 0-4 months of age is very critical for overall development and growth. Feeding of calf starter stimulate the early development of rumen.

The calf is non-ruminant and require high quality diet. The protein quality in calf starter has to be superior in terms of essential amino acids. The feeding schedule of calf is given in table-1.

Table 1: The feeding schedule of calf

Age	Milk Feeding schedule	Calf starter	Green fodder
0-5 d	3.5 kg colostrums in two equal feeding	nil	nil
6-30 d	4 kg/d	Ad lib	nil
31-60 d	3kg/d	Ad lib	Ad lib
61-105 d	2 kg	1 kg/d	Ad lib
Up to120 d	1 kg	2.0 kg	Ad lib

The growth rate of calf during first 4 months of age influences the number of milk secretary cells. The diet deficient in protein reduces the mammary gland parenchyma DNA. The calf starter has to be maize-soybean meal based so that higher energy and high quality protein requirement of calf is met.

Table 2: Nutrient requirement for calf starter (given along with milk).

S. No.	Nutrient	Required in Calf Starter
1	CP, %	18.0
2	ME, Mcal/kg DM	2.46
3	EE, %	3.0
4	ADF, %	8.0
5	NDF, %	18.0
6	Calcium, %	0.6
7	Phosphorus, %	0.4
8	Vit A, IU/kg DM	4000

(NRC, 2001)

Heifers: The heifer growth is paramount to the dairy farm economics. Lower growth rate of heifers in Indian conditions is not entirely due to genetic make-up but poor nutrition is also an important cause. Under feeding during growing phase slows down the growth rate and maturity is delayed. Feeding high energy rations to prepubertal heifers reduces the age of sexual maturity. A heifer can be bred when it attains the 60% of its mature body weight. If adult weight is taken as 550 kg than a heifer can be bred at the body weight of 330 kg. If the growth rate from birth to puberty averages 500 gm/d than a buffalo heifer can be bred at the age of 20 months. This growth rate is quite achievable.

Research on growing Holstein heifers has shown that an optimum growth rate is 800-900 gm/d. Higher growth rates than this impairs the mammary development and ultimately the milk production. Dietary protein content plays very crucial role in the mammary development. The period between 4 months of age to puberty is the critical time in mammary development. During this period the mammary tissues grow at a faster rate than that of other body tissues. The number of parenchyma cells present at the puberty partly dictates the number of milk secretary cells that will be present during the lactation. If heifers are grown on the high energy ration but protein is limited than number of milk secretary cells decreases. The heifers fed high energy (weight gain of 1260 gm/d) had 32 % lower mammary parenchyma DNA than those grown slowly (600gm/d).

In a research done in the university on crossbred heifers it was concluded that TMR containing 15.5%CP and CP:ME of 62.0 g of CP per Mcal of ME should be optimum for rapidly growing heifers than for heifers growing at a standard rate without effecting their

normal blood biochemical constituents, liver and kidney function tests. Besides, the CP:ME ratio can be a reliable indicator of energy protein needs of growing heifers. Paul and Patil (2007) has given requirements of protein and energy for growing nili-ravi buffalo heifers.

Table 3: energy and protein requirement for buffalo heifers.

Body weight	ME , KJ/g gain	CP, g/g gain
125-150	32.8	0.21
151-200	30.2	0.18
201-250	26.0	0.20
251-300	33.7	0.31
301-350	40.6	0.24
351-400	54.0	0.24

There are reports that indicate that buffalo heifers tend to deposit less fat in tissues compared to cattle of similar age. On the basis of this observation it could be theorized that buffalo heifers can support higher growth rate than cattle without impairment of milk production capacity of udder, but to support this hypothesis there are not many studies on buffalo heifer growth. Most of studies on buffalo are on male animals which could not be taken as representative of female growth pattern as the energy content of growth are different between males and females.

Lactating Animal: Requirement of energy, protein, calcium and phosphorus depend upon body weight and milk production. For dairy cows the use of grains in ration is quite common. Increasing the amount of starch in ration increases the energy density in the diet but if total DMI is depressed than the benefit of high energy density ration will be negated. When concentrates are offered separately and fodder *ad lib* then it has been observed that the fodder DMI is reduced.

Table 4: Nutrient requirements for milk production.

Milk production kg/d (3.5% fat)	DMI (kg/d)	NE_L (Mcal/d)	CP %	Calcium %	Phosphorus %
20	17	23.0	16	0.62	0.35
30	21	31.5	18	0.70	0.38

The degree of forage DMI depression depends upon starch digestion in rumen and its effect on rumen pH. In addition to the frequency of feeding, the type of cereal grain and its processing determines the negative effect of grains on fiber digestion and DMI. Rapidly digestible starch of wheat and largely reduces DMI more as compared to maize or bajra grain. The animal producing > 20 kg milk can easily consume and tolerate 3 kg grain per

day. The high feeding of grains should be accompanied by buffer feeding. The feeding of buffer ($NaHCO_3$ + MgO in 3:1 ratio) helps in maintaining the proper rumen pH and prevents the depression in DMI and milk fat which are invariably accompanied by high grain feeding. The dose of buffer should be 1-1.5% in the concentrate mixture.

The total fat in the ration should be maximum 7% but should preferably be 6%. Out of this 1-2% should be from protected or saturated fats. Protected fats should be included in ration slowly to overcome the palatability problems and should be fed only upto 120 days of lactation.

Meeting the protein needs of a dairy cow is a very complicated business. A part of the dietary protein is degraded in rumen (RDP) to ammonia and VFA's and a part of it passes as such to abomasums (UDP). The microbes use this ammonia for the synthesis of their own protein which intern is used by the host animal. The rumen system has some maximum capacity for synthesis of microbial protein. The capacity of synthesis microbial protein depends upon the digestible organic matter. Around 21 gm of microbial protein is synthesized for each 100 gm of organic matter degraded in rumen. If the requirement of animal is more than this capacity then animal require extra protein in form of UDP or rumen-protected protein which escapes digestion in rumen and is digested in abomasums and small intestine. Around 20 kg milk per day can be produced without making allowances for extra UDP. For increasing the UDP of diet, the ingredients rich in UDP are used. These are maize gluten meal, heat-treated soyabean meal, fish meal, cotton seed cake etc.

Late Pregnancy to early Calving (transition period): Grummer(1995) defined the transition period as the last 3 weeks of pregnancy to 3 weeks of lactation . During this phase the animal is shifted from high roughage diet during pregnancy to high concentrate diet immediately after calving. This change in diet coupled with metabolic changes during calving makes it challange to both cow and the farmer/herd manager. Most of the infections like mastitis, metritis etc. and metabolic disorders like milk fever, ketosis, acedosis,displaced abomasums, occur during this period. During early lactation the animal often uses its own body reserves to support milk production. During first two weeks of lactation the loss of body tissue energy ranges from 28 to 95 MJ/d (Vermorel *et al* 1982) and loss in body weight continues upto about 20 weeks of lactation. The extent and duration of negative energy balance affects both milk production and reproduction of the animal. The DMI of animals is lower during early lactation. The DMI in last week of pregnancy is decreased by 30% (Bertics *et al* 1992) and post calving DMI is lower for cows having high body condition score (BSC > 3.0 on scale of 1 to 4.0) at calving than cows in moderate BCS (2-2.5).

Table 5: The nutrient requirement of transition dairy cow.

Nutrient	Before Calving	After Calving
NEL, Mcal/kg DM	1.45-1.55	1.62-1.67
Total Fat, %	3-5	4-6
Added Fat, %	0-1.5	0-3%
NDF, %	32-36	29-33
ADF, %	25-29	20-21
CP, %	14-15	18-19
UDP, % of CP	33-35	35-38
DCAD, meq/100 gm DM	0-(-15)	>+35

(NRC, 2001)

High NDF content of diet also reduces DMI as NDF is degraded slowly in in the rumen and stays there for longer duration. The total NDF in diet during early lactation should be around 35%. Mature fodder, dry fodder like wheat straw, paddy straw are high in NDF so these should be avoided in the ration of high yielding animals. The digestibility of NDF is also lower for poor quality dry roughages. The NDF digestibility is positively correlated to DMI and milk yield (Oba and Allen, 1999). The fodder at the right stage of growth has high digestibility of nutrients.

Post Partum Reproduction and Nutrition

Effect of Energy: As the milk production per animal is increasing, there is tendency for reproductive performance to decrease. In early lactation when animal is producing maximum milk, the reproduction suffers. In New York state When milk production increased by 33% the first service conception rate declined from 66% to 50%. Butler and Smith (1989) reported an inverse relationship between milk yield and conception rate, but at the same time by analyzing the data of different surveys they concluded that the decline in conception rates for lactating cows reflects the effects of greater milk production (or negative balance of nutrients), not selection for lower fertility.

DeVries *et al* (1999) analysed the data from over 100 lactation for modeling energy balance and to see its effect on first detected estrus postpartum. They observed that the interval to nadir of energy balance occurred at 4.8, 5.4 or 2.5 day in milk (DIM) and post partum interval of return to positive energy balance was at 56.2, 85.3 or 85.4 DIM for first lactation cows, second lactation cows, or third and later lactation cows, respectively. They reported that for each additional decreased of nadir of estimated energy balance by 5.20 MJ of NE_2/d, the first estrus post partum was detected a day later.

Negative energy balance result in decrease in size of CL and decrease in progesterone within CL as compared with cows on a high plane of nutrition. Armstrong *et al* (2001) studied the effect of low and high energy diets on follicular growth and early embryo development in heifers. They observed that high energy rations increased insulin-like growth factor (IGF-1) in ovaries, thus increasing the sensitivity of follicles to FSH. These changes, in combination with increased peripheral concentrations of insulin and IGF-1 in heifers offered high energy diet, contribute to the observed increase in growth rate of dominant follicle. But at the same time increased protein in diet resulted in decreased oocyte quality, due to increased plasma urea concentrations. Stapler *et al* (1990) reported that post partum ovarian activity starters earlier in cows with an improved energy status.

Effect of Protein: Canfield *et al* (1990) fed diets containing either 16.5% CP or 19.2% CP to cows and heifers from calving to 20 days after first breeding. The first service conception rate was lower (31% Vs 43%) and plasma urea higher (18.5 mg% Vs 13 mg%) in animals fed high protein diet. Other parameters such as days to first ovulation, days first service, pulsatile LH secretion, days to negative energy balance nadir, and average daily energy balance were not affected by dietary treatments. The timing of energy balance nadir and first ovulation were highly correlated (r=0.75). The authors postulated that most likely mechanisms by which high protein diets affect reproduction and depress fertility involves alterations of uterine environment. The high protein diets elevate urea nitrogen in plasma and the bovine reproductive tract.

Jordon and Swanson (1979) also reported that feeding excess protein (19.3%) of DM) to dairy cows impaired fertility by increasing days open and services preconception as compared to lower percent of dietary protein (12.7% of DM). Patil and Deshpande (1981) reported that gir cows that gained weight in the first three months after parturition comes into estrus cycle while those that lost weight remained anestrus. The cows that lost weight had lower blood glucose and serum proteins than cows that gained weight. Carroll *et al*(1988) fed diet containing 13% and 20% CP to freshly calved cows. They observed no difference in days to first observed estrus, days to first service, days open on services per conception. The plasma urea was high for high CP group (25 mg/100ml Vs 10 mg/100 ml). They concluded that the cows managed with an intensive program for detection of estrus and for reproductive health did not show differences in reproductive efficiency when fed 13 or 20% CP rations. For optimum post partum reproductive performance the animal should be moderately conditioned at calving and the days of negative energy balance should be minimized.

Feeding During Hot Season: The DMI of crossbreds during heat stress is reduced. The effect of heat stress on DMI is more severe for high milk producing animals. The body heat

production of cow producing 18.5 kg milk per day and 31.6 kg milk per day was 27.3 per cent and 48.5 per cent higher as compared to dry cows of similar body weight (Purwanto *et al*, 1990). The DMI is reduced by 0.85 kg per day for every 1^{o}C increase in temperature. Cooling of cows by showers, sprinklers and fans etc. helps in maintaining the DMI and milk production during summer. Increasing the CP % by 2 percentage units in diet is helpful in maintain the milk production during heat stress. Feeding of buffers is very important during summer as cows are more prone to rumen acidosis during heat stress. A modern dairy cow is quite a different animal from the low producing subsisting on crop residues native animals. The nutritional needs of such animals pose a challenge to both the farmer and a nutritionist. A well balanced ration in all the major and minor nutrients is powerful tool for increasing the growth, production and reproduction.

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Role of PUFA to enhance reproductive efficiency of farm animals

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Declining fertility and low reproduction rate in ruminants is a major concern internationally despite variations in breed and management systems. Reproductive performance is negatively correlated with milk production. It is thought that genetic selection for high milk production results in a parallel unintended selection for metabolic-endocrine events that are detrimental to reproduction (21). In addition to genetic variation, nutrition status that varies between herds has been recognised as a major factor affecting fertility (5). One of the major constraints in dairy cow fertility is the problem of NEB brought on by low feed intake along with high lactational nutrient drainage in high milk producing cows. NEB can affect many factors such as follicle growth, oocyte maturation and changes in follicular fluid composition including fatty acid composition (28,29). An increase in dietary fat in high energy diets seems to have abated the severity of NEB (4) and there is recent evidence which supports supplemental fat having a positive effect on cow fertility (57). The main hindrance towards supplementing fat in ruminant diet is process of biohydrogenation in rumen. Protecting fats against rumen biohydrogenation have shown beneficial effects on energy metabolism and ovarian function (2). In addition to increasing energy stores, fatty acids (FA) are known to affect reproduction by moderating prostaglandin production, steroidogenesis, maintenance of cell membrane properties and cholesterol metabolism (52). Many studies to date that have reported effects of polyunsaturated fatty acids (PUFA) on fertility have been inconsistent and have varied between species (57). Due to the vast number of pathways PUFAs are able to affect, studies are only able to focus on a few of these aspects and the larger picture of the interactions of PUFA in the ovary remains unclear.

PUFA Supplementation and Reproductive Performance

Long chain PUFAs play a major role in regulating the reproductive processes in dairy cattle. The potential mechanism by which fat supplements improve reproductive performance may include altered follicular growth and ovulation (40), increased plasma P4 levels during the luteal phase (8), prolonged life span of the CL and suppressed activity of estradiol (E2) and prostaglandin F2α (PGF2α) around maternal recognition of pregnancy (37: Figure 1). Altering the relative concentrations of both n-6 and n-3 PUFAs in feeds can influence PGF2α synthesis and conceptus development.

The conception rate in lactating dairy cows supplemented with PUFAs may be improved by 1) sparing a glucose molecule to stimulate the anterior pituitary to release LH which may in turn stimulate the differentiation of granulosa cells to luteal cells (luteinisation), 2) increased production of progesterone associated with improved fertility by increased uptake and circulatory concentrations of cholesterol, and 3) inhibiting the estradiol and PGF2α production thereby delaying the luteal regression and prolonged life span of CL.

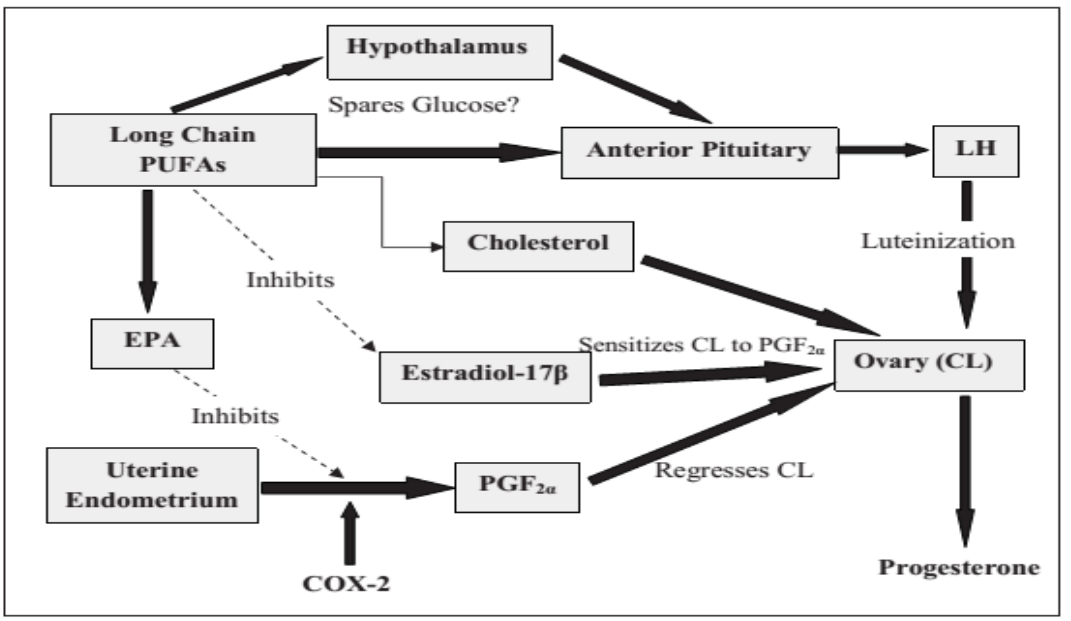

Figure 1: Suggested mechanism of action by which PUFAs may improve reproductive performance in dairy cattle [Source: Staples et al. (50)].

Effect of omega-3 fatty acids on reproductive hormones

Omega-3 (or simply n-3) FAs may affect a number of factors associated with the synthesis and metabolism of important reproductive hormones such as the steroid hormones, progesterone (P4) and estradiol (E2). Diets high in n-3 PUFA are associated with lower plasma cholesterol concentrations (48), while others found increase in cholesterol concentration (15). Antiluteolytic effects of EPA and DHA has been suggested by Petit et al. (44), who reported that cows fed a diet high in omega-3 fatty acids had larger corpora lutea than cows fed linseed oil or no fatty acid supplement. In addition to the antiluteolytic effect of EPA and DHA, Bilby et al. (7) found that cows fed an n-3 FA enriched diet had larger pre-ovulatory follicles as compared to non-supplemented cows; suggesting a potential role of EPA and/or DHA in folliculogenesis. Alpha-linolenic acid present in flaxseed is known to induce granulosa cell proliferation and cause an increase in size of developing follicle, which subsequently generates large sized

corpus luteum with increased plasma progesterone secreting capacity (1,31). Rectal palpation of n-3 supplemented cows revealed a greater number of CL compared to the control (6). Furthemore, cows fed marine algae had a larger CL while the diameter of CL in fish oil fed cows remained intermediate (6).

Different researchers have reported no effect (3,9,35,46); even reduced (26,48) or enhanced (6,18) release of plasma progesterone in lactating dairy cattle supplemented with n-3 FA sources. Although, increased n-3 was associated with lower plasma concentrations of P4 in dairy cows in the mid-luteal phase (48) and lower P4 production in bovine luteal cells (22), inhibition of PGF2α by high n-3 may prevent regression of the corpus luteum (CL) resulting in sustained P4 release (38). Furthermore, certain PUFAs may be associated with 1) increased peroxisome proliferator activated receptors (33), 2) increase serum concentration of insulin and this in turn may enhance synthesis of P4 through steroidogenesis (10,53), 3) reduce hepatic expression of some enzymes of the cytochrome P450 complex which catabolize progesterone (27) resulting in reduced P4 clearance (19). Some *in vitro* studies have demonstrated that P4 and E2 metabolism can be inhibited by high concentrations ALA in the culture media (45). In addition, Hughes et al. (25) suggested that n-3 PUFA increases P4 production solely in theca cells and that this is associated with an increase in STAR transcript expression. Inhibition of PTGS2 activity is known to facilitate cAMP-induced steroidogenesis in mouse Leydig tumour cells via increased STAR protein expression (55), and n-3 PUFAs are potent inhibitors of PTGS2 activity (47).

The effects of n-3 PUFA on P4 secretion seem to be dependent on the species, state of differentiation of the cells and type of FA. The discrepancy between *in vitro* and *in vivo* studies is unknown and warrants further investigation. Although effect on P4 production appears to be multifarious, plasma E2 concentrations increases invariably by n-3 PUFA supplementation (39,48). Other studies reported that no change in plasma E2 levels was observed during the luteal and follicular phase of the estrous cycle of lactating dairy cows fed with Ca-LCFA (30). It may be beneficial to have a prolonged life span of CL as a result of a reduced level of E2 caused by fat supplementation. However, it might negatively affect estrus behaviour and expression (49).

Effect of omega-3 fatty acids on prostaglandin synthesis

Supplementation of dairy cows with fish meal containing EPA and DHA affects uterine PGF2α secretion of lactating dairy cows (51). Dietary supplementation with PUFA alters the membrane phospholipid composition of cells throughout the body. Altered phospholipid bilayers within the cell may lead to competitive inhibition of the enzymes, COX-1 and COX-2, by changing the availability of substrates. In the presence of increased

quantities of EPA and DHA, AA metabolism can decrease (57). Supplementation with n-3 FAs could lead to displacement of arachidonic acid (AA) with EPA or DHA which could decrease production of series-2 prostaglandins and shift to series-3 prostaglandin production. Mattos et al. (36) concluded that EPA and DHA inhibit secretion of $PGF_{2\alpha}$ through a mechanism different from that of bIFN-τ. The effect of EPA on $PGF_{2\alpha}$ secretion may be caused by competition with AA for COX-2 activity or reduction of COX-2 activity. In men, it has been shown that dietary supplementation with n-3 FAs enhances the synthesis of 3-series prostaglandins (23).

Results of experiments examining the effects of direct n-3 feeding on circulating PG concentrations have been variable due to the type of lipid and dietary supplements fed, parity and days post-partum. Different researchers have reported an increase (15,44), decrease (18,34,35,41) or no effect (48) of oxytocin treatment on plasma PGFM of cattle where flaxseed or fish meal was supplemented in diets. Moreover, in vitro cell cultures of bovine endometrial cells incubated with DHA and EPA had inhibited $PGF_{2\alpha}$ release (36). The addition of fish meal to the diet suppressed oxytocin-induced uterine $PGF_{2\alpha}$ secretion in heifers that had low, but not in heifers that had normal, luteal-phase progesterone suggesting fish meal supplementation may improve fertility in cows with low luteal-phase progesterone following mating by suppressing uterine prostaglandin $F_{2\alpha}$ release during the period of maternal recognition of pregnancy (54). Similarly, bovine endometrial cells cultured for 24 hours with EPA decreased concentrations of $PGF_{2\alpha}$ by 75% as compared to controls (11). Furthermore, the inhibition of $PGF_{2\alpha}$ secretion was decreased when the ratio of n-6 to n-3 fatty acids increased (11). Rats fed diets rich in DHA had lower levels of placental $PGF_{2\alpha}$ compared to rats fed a linolenic supplemented diet (43). DHA was more effective in lowering $PGF_{2\alpha}$ and PGE_2 than EPA in equine endometrial explants cultures, whereas, enzymes related to PG synthesis were not affected by 24-h culture with EPA or DHA (42).

Eicosapentaenoic acid has been shown to inhibit the activity of COX-1 (57), although the conversion of EPA into 3-series prostaglandins via COX-1 is poor. Mattos and colleagues (36) found $PGF_{2\alpha}$ to be suppressed *in vitro* in bovine cell cultures supplemented with PUFAs, possibly due to reductions in PGHS-2 and PLA_2 enzyme activity and PGHS-2 mRNA concentrations and protein expression. The myometrial concentrations of PGHS-2 mRNA (the rate-limiting enzyme in the synthesis of 2-series PG,) were lower in ewes intravenously infused with a lipid high in long-chain n-3 compared with long-chain n-6 (32). In contrast, EPA did not influence the detectable mRNA of COX-2 in cultured bovine endometrial cells (11). Similarly, lactating dairy cows supplemented with fish meal had increased uterine n-3 FA concentrations, but there was no affect on endometrial COX-2 protein or $PGF_{2\alpha}$ production in response to an oxytocin challenge (40).

Enzyme expression related to PG synthesis varies with tissue type and concentration, source and duration of fatty acid supplementation. Although DHA and EPA decreases prostaglandin production, it has been shown altering of enzyme expression is not the only mechanism inhibiting PG secretion; therefore other mechanisms must be involved in the n-3 mediated inhibition of $PGF_{2\alpha}$ release. Possible mechanisms for this inhibition include 1) shifting production from 2-series prostaglandins to 1 or 3-series prostaglandins, through competition of EPA and DHA with AA for lipid metabolism and incorporation in the phospholipid membrane (12,36) or 2) DHA is possibly affecting the lipid composition of the membrane influencing signaling pathways within the cell (13).

Effect of omega-3 fatty acids on gene expression

It was previously thought that PUFAs regulated gene expression by altering the make-up of phospholipid membranes or through eicosanoid production. However the existence of nuclear receptors capable of binding FAs which therefore affected gene transcription was discovered by Gottlicher and colleagues (20). Since then, other transcription factors that utilise FAs to control gene expression have been discovered, and it seems that these factors are involved in a complex interplay to maintain tissue-specific biological functions throughout the body. Within the context of reproductive function very few studies have investigated the role of dietary PUFAs on gene expression (15,16), but PUFAs are known to regulate a wide range of genes which are expressed within the ovary, oocyte and embryo.

Waters et al. (56) studied the effects of dietary n-3 PUFA supplementation on global uterine endometrial gene expression in cattle. Beef heifers were supplemented with a rumen protected source of either a saturated fatty acid (Control; palmitic acid) or high n-3 PUFA (n-3 PUFA; 275 g) diet per animal per day for 45 days and global gene expression was determined in uterine endometrial tissue using an Affymetrix oligonucleotide bovine array. A total of 1,807 (946 up- and 861 downregulated) genes were differentially expressed following n-3 PUFA supplementation. These included prostaglandin biosynthesis, steroidogenesis and transcriptional regulation, while effects on genes involved in maternal immune response and tissue remodeling were also observed. Most notable among them were: 1) Phospholipase A2 (26.9-fold downregulated); 2) Prostaglandin reductase 2 gene (downregulated); 3) Endothelin 1 and endothelin receptor type A (upregulated); 4) Estrogen receptor 1 (downregulated). The effect on all the above gene has a potential of altering the paracrine milieu resulting in a net luteotrophic effect observed in n-3 PUFA fed animals. Moreover *OXTR* gene expression was found to remain unchanged due to n-3 PUFA supplementation and the same result was also observed previously (17). In an similar microarray experiment involving effect of n-3 PUFA supplementation on ovine granulose

cell gene expression, two genes were significantly altered in granulosa cells from n-3 vs n-6 PUFA fed ewes viz., IL18 and STAR. In both cases transcript expression was greater in n-3 group than n-6 fed ewes; with (24).

Conclusion

Dietary fat may enhance follicular development via metabolic hormones that act on the central nervous system to stimulate GnRH secretion and through metabolic hormones acting at ovarian level. Increased folliculogenesis, total number of follicle and increased ovulatory follicle size have been reported following n-3 and n-6 PUFA supplementation. Moreover, n-3 PUFA is beneficial for enhancing the conception rate by increasing the chances of embryo survival and therefore may be included in the diet. However, further studies are warranted to investigate the more appropriate and optimum dose and schedule of PUFA in the diet to enhance reproductive efficiency of farm animals.

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Application of diagnostics and research for maximizing bull fertility

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A. Breeding Soundness Evaluation: Breeding bull soundness evaluation implies a complete evaluation of all factors contributing to normal reproductive potential. It is a relatively quick and economically prudent procedure for screening potential bulls. It is done before the purchase of bull and before the start of breeding season. It is essential as purchase of lower performance bull will lead to decreased fertility and thereby hampering dairy economics. In range breeding, introduction of low fertility bulls will lead to lower conception.

Procedure

Breeding soundness evaluation involves a thorough and systematic format for identifying problems affecting male fertility. It involves following steps:

Identification:

- Date of birth
- Dam
- Sire
- Colour
- Special identification mark

History: Thorough breeding history of a bull can assist in determining diseases and presence of lethal/ economically undesirable recessive genes in bulls.

- Health record
- Vaccination
- Breeding performance
- Genital infections

Structural soundness: It includes general health and normal body configuration, which affects fertility. Poor health status affects libido, mating ability, semen production and its quality. It includes functional feet, associated joints and overall body confirmation. Any disease conditions, which impair mobility, will hinder the performance of the bull.

Thorough examination of systems/organs is recommended for breeding soundness evaluation of bulls.

Reproductive soundness: Reproductive organs should be examined for fitness:
External: Scrotum, Prepuce, Penis, Testes and Epididymis.
Internal: Pelvic urethra, Prostate gland, Seminal vesicle, Ampulla & Inguinal ring.
External and internal reproductive organs are examined for any diseases/abnormalities.

Techniques of scrotal circumference measurement:

- Testicles are palpated and held firmly in to the lower part of the scrotum so that they are side by side to minimize the scrotal wrinkles that may inflate the measurements.
- A looped measuring tape is kept around the greatest diameter of the scrotum and tape is pulled in such a way that in may be in close contact with the entire circumference.

Libido: Libido is defined as the willingness and eagerness to mount and attempt service, with mating ability described as the ability to complete service. Deficiencies in either can affect the herd production seriously. High libido bulls are advantageous to herd fertility and have beneficial effects on the fertility of subsequent female progeny. Breeding soundness evaluation for bull is widely accepted but little emphasis is put on libido testing.

Semen analysis: The most important aspect of breeding soundness evaluation is the semen analysis. Semen analysis is done to assess the availability of potential sperm which take part in fertilization process.

Sperm viability and morphology

- A small drop of semen is placed on a prewarmed glass slide and mixed with a relatively larger drop of the nigrosin eosin stain by an applicator stick, and a thin and uniform smear is made. After air-drying the smear, 100 sperms are observed using microscope at 100x (oil emersion lens) for unstained heads of sperm (live) and stained/partial stained heads of sperm (dead). Dead sperms are stained pink.
- Rose Bengal staining is performed for total sperm abnormalities. One hundred sperms are observed using a microscope at 100x (oil emersion lens) for heads, midpiece, and tail abnormalities.
- Live sperm percentage = Total live sperms counted / Total number of sperms counted *100

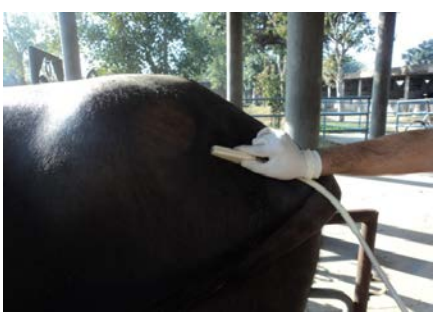
- Abnormal sperm percentage = Total abnormal sperms counted / Total number of sperms counted*100

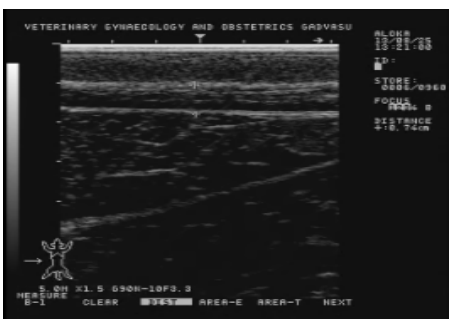
Semen freezability

- Semen is diluted and straws are filled, sealed and kept at 4ºC for 4 hours for equilibration. Then the straws are frozen at ultra low temperature using biofreeze. Retrospective analysis of data is done to calculate the freezability as the proportion of ejaculates with acceptable post thaw motility (>50%) of the total ejaculates that are subjected to cryopreservation.

B. Evaluation of fertility associated antigen using Lateral Flow Cassette: FAA could be detected in high and low fertility bulls by Reprotests (Lateral flow cassettes) Repro Tec Inc, Midland Bio Products, Boone, USA. Freshly ejaculated semen (1 ml) is diluted with 1 ml of buffer in a sterile buffer dilution vial supplied with the kit. The semen is gently mixed with buffer and gently poured 4-5 drops in the sample well of the Reprotec which kept on a level floor and allowed the cassettes to stand for 20-30 mts. A positive test for FAA is indicated by 2 lines at 'C' and the 'T' positions. The negative test for FAA is indicated by only one line at 'C' position only.

Reprotest (Lateral Flow Cassette) showing two lines at 'C' and 'T' positions indicating positive for Fertility Associated Antigens (FAA)

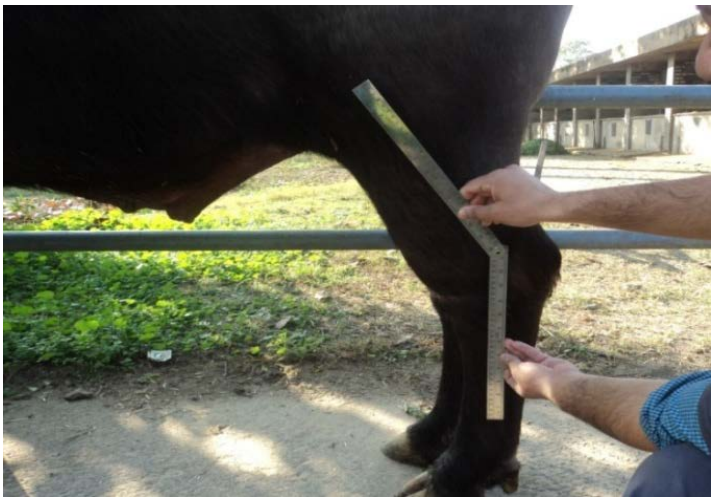




C. Rump Fat thickness using Ultrasonography: Linear ultrasonographic probe of 5 MHz is successful in demarcation of fat from the skin layers above and muscle layers below fat. Ultrasonographic probe of 5 MHz is placed at different points between hook bone and pin bone. Homogeneity of ultrasonographic measurement of rump fat is obtained in the area lying in the halfway between the hook bone and the pin bone. Centre point between hook and pin bone is marked for placing of ultrasonographic probe. The probe is placed longitudinally and slightly dorsal to the halfway between pin bone and hook bone. The rump fat layer obtained on ultrasonographic image is usually parallel in bulls. Two distinct muscles, the gluteus medius and the biceps femoris are clearly visible at that location. The subcutaneous fasia is hyperechoic and appeared as thin white layer surrounding the fat from upper side as well as from the lower side. Fat is hypoechoic with low range of contrast. The diameter of rump fat is measured including the thick fat layer and the two thin subcutaneous fascia layers.

D. Endocrine status: Endocrinology of dairy bulls might indicate the hormonal milieu. Testosterone concentration is similar ($p>0.05$) in good and poor libido bulls (4.07 ± 0.48 ng/ml versus 3.57 ± 0.42 ng/ml). Estradiol concentration in good libido bulls is significantly ($p<0.05$) lower (46.29 ± 6.79 pg/ml) as compared to poor libido bulls (86.44 ± 16.04 pg/ml). Serum prolactin levels are found similar ($p>0.05$) in both the groups (87.53 ± 2.92 ng/ml in good libido and 86.69 ± 2.73 ng/ml in poor libido). Serum TSH is found to be similar ($p>0.05$) in both the groups (26.89 ± 3.05 uIU/ml in good versus 26.03 ± 3.20 uIU/ml in poor). Serum concentrations of T3 and T4 are similar ($p>0.05$) in good and poor libido bulls (1.40 ± 0.16 ng/ml and 4.62 ± 0.42 µg/dl versus 1.93 ± 0.31 ng/ml and 4.93 ± 0.55 µg/dl, respectively).

E. Standardization of hock joint angulation: A new instrument has been devised which consisted of two measuring scales, connected with each other by a screw. Screw resulted in opening of the scales to a desired angle and the screw was tightened to such an extent that once opened the angle does not change by itself. The buffalo bull is made to stand in normal position with the planter surface of hind feet in total contact with the ground surface. One scale is positioned in front of the metacarpals and the other scale is positioned in front of tibia. The screw is positioned in front of the centre of the hock joint. After placing the instrument and opening the instrument to the level of hock joint angulation, the instrument is removed and the angle is measured.



Bull fertility is dependent on several above-mentioned factors. Evaluation of all above-mentioned parameters will help in identifying potential bulls.

A student-centered approach to teaching animal reproduction

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The teaching-learning process should be based on the understanding that education is constituted by a dynamic combination of knowledge and experience that had little to do with previous static ideas of intellectualism and memorization. Students and professors have their own experiences that can be used during the learning process. However, most education institutions continue to follow the original model of classroom-based learning where professors deliver their knowledge to the students, who in turn take notes and memorize what was said in order to get good grades in exams. Technology has changed this style of teaching slightly, allowing professors to prepare more sophisticated presentations, especially with the use of Microsoft PowerPoint. However, this is only a nicer wrapping for the same old concept in which professor talk and students take notes. In higher education sector, there is need to leave the obsolete way of teaching and adopt new concept of education where students can be active, participative and collaborative.

The general impression of veterinary students is that there is little mentoring during their graduation and very few stimulating educators. University administration does not seem to award teachers who understand that entertainment captures the audiences. Keeping in view the importance given to research in teaching hospitals, it appears that teachers think that they are required to spend a percentage of their time teaching, and often give the impression that they would rather stay in their laboratories and be a researcher.

In cattle and buffaloes, theriogenology is the farmer's livelihood. Therefore, teaching methodology should be aimed at thoroughly understanding animal reproduction concepts, acquiring knowledge, integration of knowledge, development of interest, motivation to read and ultimately development of sensitivity towards animal needs. Animal reproduction division at veterinary schools must concern themselves with implementing appropriate educational strategies to graduate competent theriogenologists. Thus, the purpose of the present lead paper is to introduce some teaching methodologies that can be used in the teaching of animal reproduction according to the new instructive concept of interactivity and constructivism.

Teaching Transrectal Palpation of Genital Tract

Achieving combination of high production and acceptable fertility is a greatest challenge in modern dairy farming. Many animal-specific and management-specific factors may perturb fertility. Delayed resumption of ovarian activity and failure to conceive are the two major causes of suboptimal fertility, the early diagnosis of reproductive disorders and early detection of pregnant and open animals are crucial tools for improving a herd's fertility status. Thus, rectal examination of the genital tract is an indispensable skill for veterinarians. Despite widespread access to ultrasonography, teaching of rectal palpation is still vital in reproduction education. However, the technique requires considerable training before adequate dexterity is obtained. To set concrete goals for education, it is important to know how many live cattle need to be palpated to obtain acceptable dexterity. A recent study suggested that although students can successfully localize the cervix, uterus, and ovaries, they had difficulties in interpreting these structures, suggesting that palpation of 200 cattle is insufficient to reach a consistent level of expertise (Bossaert *et al* 2009).

Rectal palpation in cattle and buffaloes is a cost-effective procedure that requires considerable practice to develop the necessary skills to diagnose pregnancy in the animals, assess reproductive physiological status, and diagnose oviductal, uterine and ovarian pathologies. Student access to bovines at instructional herd/university dairy farms is limited because of student numbers, reluctance from practitioners and farmers in having students palpate valuable dairy animals, animal welfare considerations and concerns over induction of embryo mortality because of inexperience of the student (Masterson *et al* 2004).

Simulation Model for Rectal Palpation Teaching in Bovines: Teaching in live bovine is difficult, since the teacher is not able to see or feel what students are palpating and hence cannot comment on their actions. This is unfortunate, during the early stages of education, since novice students often have problems locating structures through feces and the rectal wall or in an air-filled rectum.

Recently, sophisticated models have been developed to teach rectal palpation (Ballie *et al* 2003). The benefits of a virtual-reality simulation teaching model (Breed'n Betsy) have been described (Ballie *et al* 2003, 2005). All participants agreed that this teaching method increased their palpation skills. More specifically, the opportunity to palpate the virtual cow without any risks or animal welfare implications and the ability to provide feedback during palpation was greatly appreciated by students and teachers. In another study, a group of students receiving training in live cows combined with the simulation model. These students performed significantly better at *in vivo* palpation than students trained only in live cows. However, others suggest that Breed'n Betsy cannot fully replace training in live cows, but may be a valuable addition to the classical teaching method (Bossaert *et al* 2009).

The use of models often do not impart the real feeling of palpating a live cow, can be expensive and may limit the variety of physiological and pathological conditions that can be found in real-life situations.

Practicing Transrectal Palpation in Live Buffalo at the Abattoir: To overcome these limitations, practical classes of bovine rectal palpation can be taken at a slaughterhouse, with small groups of students (Lopes and Rocha 2006). The goals established for palpation at slaughterhouse should be include: to locate cervix and horns and assess their relative size and consistency in non-pregnant animals; diagnose pregnancies equal or superior to 60 days; identify cows in peri-estrus based on turgidity of the tract and diagnose ovarian cysts. Following this way of teaching in a Portugese Institute of Biomedical Science, 67-84% students correctly identified pregnancies during the exam and the general opinion on the usefulness of the classes by former students was by and large positive (96%). While other techniques such as virtual reality-based teaching tools are not widely available (Ballie *et al* 2003, 2005), teaching rectal palpation at the slaughterhouse seems a realistic and worthwhile proposition (Lopes and Rocha 2006).

Development of 3D Technology for Teaching Veterinary Obstetrics

In a recent study, a group of students were taught veterinary obstetrics in a classroom with traditional modules like photographs, text, and two-dimensional graphical presentations. However, other group of students was taught by three-dimensional (3D) media like linear animations and interactive QuickTime Virtual Reality models. The students taught in the latter group had significantly high scores in the assessment of the subject matter. Thus, this approach of education may help to better prepare students for efficiently dealing with obstetrical cases (Scherzer *et al* 2010). However, only limitation at the present time is the availability of 3D material required for teaching obstetrics.

Traditional Problem-Based Learning

Problem-based learning (PBL), an instructive approach that challenges students to “learn to learn” working cooperatively in groups to seek solutions to real world problems is one of the concepts that can be introduced in the entire Veterinary education curriculum with or without some technological tools (Garcia 2006). Problem-based learning has received increased attention in recent years. Several studies have documented veterinary staff and students’ generally have positive feedback on group learning activities (Thurman *et al* 2009). There is need for veterinary teachers to better prepare students for group learning activities.

In PBL, clinical cases are carefully selected to provoke deep student learning by the acquisition of both basic scientific and clinical knowledge critical to the case; cultivate

problem-solving abilities; and encourage the development of team-building, self-directed learning, communication, and self- and peer-assessment skills. Problem-solving skills, understanding of the basic sciences and clinical performance are all improved by the PBL process (Lane 2008).

Traditional PBL system involves facilitated small group discussions conducted over several sessions, where the students form their own learning objectives. In this approach, every session of two hours duration can be repeated at weekly interval for 8-10 weeks. On the first day of PBL, the students began to analyze the clinical case and identify basic science learning objectives for each member in the group to present in the next session a week later. During presentation and discussion of the learning objectives, students use reading material from books, online library and notes etc. Moderator can be provided to students during their discussion sessions whose role is only to monitor the performance of the group members. Moderator can encourage the group members who provide less input towards group discussion. At the end of 8-10 weeks, students and their moderator evaluate the performance of each participant in the group.

This method allows students to analyze, read, listen, discuss and solve problems in a large group format for every clinical condition. The active participation of students increases their satisfaction and enjoyment and hence their retention of factual knowledge Moreover, PBL enable students to develop the lifelong learning skills required of a veterinary professional, such as critical scientific thinking, problem solving, group interaction, and positive interdependence. Nevertheless, students should be more positive toward several aspects of the activity as well as toward group work in general.

An Australian study recommended that there was a significant increase in the percentage of students who strongly agreed that they had better understanding of the subject using PBL, and had learned to apply principles from this class in new situations. The only consistent criticism by students was directed at the extra time required compared to traditional lecture-based subjects (Rand and Baglioni 1997).

Computer Assisted Problem-Based Learning

Asynchronous way: Clinical case can be presented online and, after that, students can have some days to discuss about the case in a web forum and to propose diagnosis and treatment. This kind of interaction is called asynchronous because students and teachers don't need to be online at the same time. They can watch the case report and post messages in their own time.

Synchronous way: This can be carried out via a web-based conference system. This can be in the form of; a) distance learning lecture with large groups of students present in the

classroom, b) distance learning lecture with students outside the classroom, and c) virtual clinical rounds. However, this system warrants a certified and secure client-server system. In spite of difficulties related to the stability and security of the internet connection, the synchronous use of information and communication technologies is a viable technical and instructive solution, with good prospects of improving the educational process in veterinary medicine (Garcia 2006)

Video Teaching Resources

Video technology is a source of teaching and learning due to sound instructive reasons (Ghuman *et al* 2011). The students had a positive perception of video usage in higher education especially prior to practical examinations (Roshier *et al* 2011). Video usage can provide a specific learning resource (a practical technique) by enhancing student engagement and promoting deeper learning (Andrews 1996). Videos may also be used for problem based learning, reviewing practical laboratory techniques, observing live surgical procedures, for teaching undergraduates how to take history and how to perform clinical examinations (Gul *et al* 1999, Parkin and Dogra 2000). Nevertheless, the important concerns for students are good sound quality, accessibility including location of videos within electronic libraries, inability to download, and video content. Nevertheless, videos should have reference to other teaching material, e.g. relevant lectures and practicals.

Early Clinical Exposure (ECE) Program

Veterinary students typically begin their exposure to clinical cases of reproduction in the last professional year of college and often struggle to apply their basic sciences knowledge in unfamiliar clinical scenarios (Elsheikha and Kendall 2009). Diverting away from the practice of having only traditional instructive lectures, ECE program can be included in teaching animal reproduction to the veterinary students (Sathishkumar *et al* 2007). This program involves an active, experiential learning from animal patients with a clinician. This method provides a link between basic physiological concepts and clinical presentations. In this approach, each lecture can began with the projection of one to three clinical problems followed by several questions from student. This can follow traditional lecture, after which the clinical problems can be projected again with the questions. Using the student responses, the professor can emphasize the content of the lecture. Moreover, professor can use the answers of the students to further reinforce the lecture concepts. In a study, students were more receptive to ECE approach (Sathishkumar *et al* 2007). Feedback from clinical students revealed that they were better equipped to analyze clinical problems as well as find and apply appropriate basic science knowledge compared to traditional curriculum. The challenge of ECE methodology is increased faculty time for teaching (Richard *et al* 2001).

e-learning in clinical skills

The use of e-learning has not been yet established in many veterinary universities in India, therefore, the effectiveness of e-learning is difficult to quantify. However, there are concerns that such educational activities may be driven more by novelty, than instructive evidence especially for clinical skills. A study assessed undergraduate students for their ability to perceive IT ability and accessibility, and attitudes towards e-learning in basic clinical skills education, compared to other teaching methods (Gormley *et al* 2009). It was observed that students value the use of e-learning in clinical skills education. It was revealed that e-learning had a positive impact on their learning of clinical skills and was comparable to other traditional forms of clinical skills teaching and acknowledges its integration in a blended approach. Students who displayed deep learning traits when using e-learning, performed better in clinical skills. Developers of clinical skills curricula need to ensure e-learning environments utilize media that encourage deeper approaches to learning.

Concluding remarks

Internet provides new opportunities to deliver distance and e-learning to the veterinary profession both at undergraduate and postgraduate levels. Development of numerous computer-based educational projects in animal reproduction education is in progress which may provide useful models for veterinary teaching. These will challenge academicians to adapt their teaching methodologies and students need to develop new ways of learning (Short 2002).

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Contemporary practices for improving uterine health of dairy animals

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One of the major key for excellent fertility in dairy herds is a healthy uterine environment that facilitates implantation and development of the embryo (Kaufmann *et al* 2009). The conception rates are about 20% lower for cows with endometritis that result prolonged calving to conception interval and there are 3% more animal culled because of failure to conceive (Sheldon *et al* 2009). The endometritis and pyometra are the most commonly encountered anomalies causing infertility in cattle under field or farm conditions (Singh *et al.* 2009). The incidence of subclinical endometritis varied between 20-90% during first 2-3 months post-partum (Gilbert *et al.* 2005, Lincke *et al.* 2007, Cheong *et al.* 2011). However, clinical endometritis was encountered in 21-35 % cows those revealed mucopurulent discharges (Potter *et al.* 2010, Plontzke *et al.* 2011).

Diagnosing endometritis at the earliest often reduces the chances of rising complications due to delayed interventions in affected animals. Routine methods for diagnosing endometritis involve uterine biopsies, lavage and swabs but these may cause irritation and distortion of cells. An inconsistent success following conventional therapies is achieved due to lack of diagnostic standards (Kasimanickam *et al* 2005). Therefore, most of the recent studies have been focused on sophisticated diagnosis of endometrial alterations beyond clinical signs of endometritis. Recently, it has been proposed that uterine cytology in post-partum dairy cows is useful and accurate procedure for detecting existence and severity of endometritis (Dolatkhah *et al* 2013). For uterine cytology, two methods are described viz. by lavage technique or cytobrush method (Barlund *et al* 2008, Gilbert *et al* 2005, Kasimanickam *et al* 2005). Both the methods are based on number of neutrophils (%) in uterine samples. Cows with subclinical endometritis do not have uterine discharge but fail to conceive. Oral *et al* (2009) compared the cytobrush technique, vaginoscopy and transrectal ultrasonography for the diagnosis of endometritis in postpartum cows and concluded that endometrial cytology through cytobrush technique could be used safely and effectively for the diagnosis of subclinical endometritis. In could also be used for the follow up of recovery.

The threshold values of PMN varied from 4% to 18% and suggested to define subclinical endometritis by uterine cytology (Gilbert *et al.* 2005, Barlund *et al.* 2008,Santos *et al.* 2009). Dubuc *et al.* (2010) reported that >6% PMNs or mucopurulent vaginal discharge

was the most appropriate indicator of endometritis in cows 35±3 days in milk whereas, >4% PMNs was the most appropriate in cows 56±3 days in milk.

Following diagnostic accuracy, one has to use either appropriate antibiotherapy or any other alternative therapy. The systemic and local (intrauterine) antibiotic therapies have been tried to combat with uterine infections in dairy animals but that often requires compulsory milk disposal and frequent administration. Apart from high cost of the antibiotic therapy, it also results into development of microbial resistance, and decrease in phagocytic activity of polymorphonuclear cells (Chastant-maillard 2006). The intrauterine lugol's iodine (0.25 - 0.5%) had been reported as an effective treatment of endometritis (Singh *et al* 2010b) but its inadvertant admnistration may causes intense irritation to endometrium and ovaro-bursal adhesion. Modulation of uterine immunity has been proposed as an alternate therapy which involves single intrauterine infusion of *E. coli* lipopolysaccharide (LPS; 100 µg in 20-30 ml normal saline) or of oyster glycogen (500 mg in 50 ml normal saline) in endometritic cows. This cleared the uterine infections in about 75 - 85% cows probably through enhanced phagocytosis and yielded good (45-83%) conception (Singh *et al* 2003, Prasad *et al* 2009).

The drug like levamisol is known for its general immunomodulation but the literature on its effect on uterine immunity in endometritic cows is scanty. Pancarci *et al* (2009) administered levamisol intramuscularly and observed that treatment accelerated the involution of the cervix uteri in cows with normal vaginal discharges when compared with pathological discharges. There was an earlier recruitment of the follicular wave in cows with normal vaginal discharge compared to those with pathological vaginal discharge.

The use of proteolytic enzymes for the intramammary treatment of mastitis has been described as non-antibiotic therapy. It has been observed that certain enzymes viz chymotrypsin, trypsin and papain have fibrinolytic and proteolytic activity and are supposed to support cellular defense mechanism and inhibit growth of microorganisms (Drillich *et al* 2005). Trypsin, Chymotrypsin and Papain are hydrolytic enzymes (Hydrolases) that have the capacity to split proteins and fat bonds. The immunomodulatory effect of the proteolytic enzymes occurs both directly and indirectly. Papain works directly as a cystein-protease, similar to the bacterial cystein-proteases from gram-negative anaerobes, on the CD14 molecule of macrophages and monocytes and raises up their level of efficacy as the instigator of the acute-phase-reaction. Chymotrypsin, trypsin and papain have fibrinolytic activity in inflammed tissue and supposed to support the uterine cellular defence mechanism and also inhibit the growth and survival of micro organisms. The proteolytic enzymes in combination have more comprehensive effect against microorganisms. Gram positive and gram-negative bacteria, yeasts, protothecals, surface structures and toxins contain proteins, lipids or combinations of both and are degraded

through these enzymes. In this way these enzymes has direct effect on microorganisms. The changes in the membrane protein cause stasis in growth or the death of the bacterium. This is responsible for significant reduction in bacterial load post treatment and can yield even up to 70 % pregnancy rates in cattle (Singh 2014). There is paucity of information regarding use of proteolytic enzymes for the treatment of uterine infection and requires comprehensive studies on this aspect.

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Superovulation and embryo transfer technology: research update in cattle and buffalo

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The objective of superovulation is to obtain the maximum number of fertilized and transferable embryos with a high probability of producing pregnancies. The ultrasonographic studies of ovarian follicular dynamic has lead to designing of various interventions and advances which have made superovulation treatments more "user friendly" and have helped in the widespread application of the embryo transfer technology around the world. However, there is still a high degree of unpredictability in superovulatory response in cattle & buffalo that creates problems affecting the efficiency and profitability of embryo transfer programs.

The conventional protocol of initiating ovarian superstimulation during mid-cycle was originally based on experimental information in which a greater superovulatory response was reported when superstimulatory treatments were initiated 8 - 12 days after estrus (Mapletoft et al. 2002). However, none of these early studies evaluated follicular status at the time when gonadotrophin treatments were initiated. The ultrasonographic evidence has shown that the second follicle wave begins 8.5 days post-ovulation (Day 9.5 of the cycle) in 3-wave cows and 9.5 days post-ovulation (Day 10.5 of the cycle) in 2-wave cows (Adams 1994). Therefore, the initiation of conventional superstimulation protocol between day 8 – 12 after esturs would be the approximate time of emergence of the second follicular wave in 2- or 3-wave cycles and a cohort of growing follicles would be present for undergoing superstimulation. Higher superovulatory response was observed when superstimulatory treatments were initiated at the time of wave emergence; as little as 1 day asynchrony significantly reduced the superovulatory response compared to initiating treatments on the day of wave emergence (Nasser et al. 1993). Therefore, the superovulatory protocols are developed to initiate superstimulatory treatment at the beginning of wave emergence through manipulation of follicular dynamics.

Manipulation of follicle wave emergence

There are three methods of synchronizing follicle wave emergence for superstimulation.

Follicle Ablation: One approach to synchronize follicle wave emergence involves transvaginal ultrasound-guided follicle ablation of all follicles ≥5 mm, regardless of stage of the estrous cycle (Garcia & Salaheddine 1998). This removes the suppressive effects of

follicle products (estradiol and inhibin) on FSH release; as a results FSH surges and new follicular wave emerges 1 day later. Superstimulatory treatments were then administered, beginning 1 day after ablation. The timing of estrus was more synchronous when a progestin device was inserted for the period of superstimulation and 2 injections of PGF2alpha were administered (12 hour interval) on the day of progestin removal. Baracaldo et al. (2000) reported that the ablation of the 2 largest follicles at random stages of the cycle was as efficacious as ablating all follicles ≥5 mm in synchronizing follicular wave emergence for superstimulation. However, it was always advised that a progestin device be used during superstimulation.

Estradiol and Progesterone: Estradiol causes regression of FSH and LH dependent follicles through suppression of FSH and possibly LH. Once follicle regression begins and the exogenously administered estradiol was metabolized, FSH surges and a new follicle wave emerge 1 day later. The use of a short acting estradiol-17β in progestin implanted cows was followed by the emergence of a new wave, approximately 3 to 5 days later regardless of the stage of follicular growth at the time of treatment (Bo et al. 1995). Estradiol-17β was normally injected with 50 to 100 mg of progesterone at the same time as placement of a progestin device (Bo et al. 2005). The progesterone prevents an estrogen-induced preovulatory-like LH surge in those animals that do not have a functional CL, and appears to cause regression of LH dependent follicles. The use of other estrogens like estradiol benzoate and valerate for induction of follicular wave has also been reported and could be used in those countries where estradiol-17β is not readily available for commercial use.

Use of GnRH: Another method of synchronizing follicular wave emergence for superstimulation involves the use of GnRH or porcine LH (pLH) to induce ovulation of a dominant follicle followed by emergence of a new follicle wave 2 days later (Martinez et al. 1999). However, the administration of GnRH or pLH does not always induce ovulation, and if ovulation does not occur, follicle wave emergence will not be synchronized. Therefore, the reported asynchrony in follicular wave emergence suggested that GnRH-based approaches may not be feasible for superstimulation (Martinez et al. 1999). However, the results from commercial embryo transfer practitioners (Wock et al. 2008) and a research report involving 411 dairy donors (Steel & Hasler 2009) using GnRH have revealed more promising results. Basically, a progestin device was inserted at random stages of the estrous cycle and GnRH was administered 2 or 3 days later with superstimulation treatments beginning 1.5 to 2.5 days later.

Lengthening the superstimulatory treatment

García et al. (2012) investigated the effect of lengthening the superstimulatory treatment protocol from the traditional 4-day protocol to 7 days to recruit more follicles into the wave

and provide the time required for them to acquire the capacity to ovulate. Lengthening the FSH treatment protocol to 7 days, without increasing the total amount of FSH administered, increased the number of ovulations and the synchrony of ovulations, and tended to increase the mean numbers of total ova/embryos, fertilized ova, and transferable embryos. In other words, the lengthened superstimulatory treatment protocol resulted in more follicles reaching an ovulatory size and acquiring the capacity to ovulate with an increased number of ovulations, and with no decrease in oocyte/embryo quality. In a recent study, the 7-day FSH treatment protocol resulted in 2.5 times more transferable embryos per animal after ultrasound-guided oocyte collection and in vitro embryo production than the regular 4-day FSH treatment protocol (5.6 and 2.5 embryos per oocyte donor, respectively (Dias et al. 2013). Further studies are required to validate these results.

Follicle numbers, Level of AMH and superovulatory response

The number of follicles in ovarian follicular waves in cattle was reported to be highly variable among animals, but highly repeatable within individuals (Ireland et al. 2007). Singh et al. (2004) have reported differences in superstimulatory response between cows that had more than 30 follicles of 3 to 5-mm diameter compared with those with fewer than 30 follicles. Similarly, Ireland et al. (2007) also reported that beef heifers with a low number of follicles (<15 follicles, >3 mm in diameter) at the time of wave emergence also had lower superovulatory response than those with high number of follicles (i.e., >25 follicles) at wave emergence. Therefore, it might be possible to select donor cows based on the number of follicles present at the time of follicle wave emergence detected using ultrasonography.

Ireland et al. 2011 has reported that follicle numbers were associated with circulating concentrations of anti- Mullerian hormone (AMH). Anti-Müllerian hormone is a glycoprotein belonging to the transforming growth factor-b family that is produced by the granulose cells of all growing follicles and is greatest in healthy small antral follicles, which contribute most significantly to AMH endocrine levels. In adult cows and goats, AMH levels were highly variable between individuals, but characteristic of each animal over a period of several months (Monniaux et al. 2013).

Therefore, it might be possible to evaluate the ovarian reserve of gonadotropin-responsive follicles through detection of AMH concentrations in the circulation. Rico et al. (2012) have shown a correlation between AMH production and superovulatory response in Holstein donors and have suggested values that could be used to differentiate between cows producing more or less than 10 transferable embryos.

Additional treatments and superovulatory response

Basic studies on follicular development have shown that FSH is required for follicle recruitment and growth, until the dominant follicle reaches 8.5 mm in diameter in Bos taurus breeds of cattle (Ginther et al. 1996) and 6.2 mm in Bos indicus cattle (Sartorelli et al. 2005). After selection, dominant follicles acquire LH receptors and become LH-dependent. Therefore, follicles of superstimulated cattle might benefit from the inclusion of LH near the end of the treatment protocol. Equine chorionic gonadotropin is a gonadotropin with FSH and LH activity and could provide a constant stimulus to the LH receptors of the growing follicles near the end of a conventional FSH superstimulation treatment protocol.

Barros et al. (2008) conducted an experiment in which Nelore cows were superstimulated with FSH over 3 days; the last two FSH injections (on the fourth day) were replaced with two injections of 200 IU eCG. Treatment with eCG significantly increased the number of ova/embryos and increased the number of transferable embryos over that in control animals. Although, Davis et al. (2012) found no beneficial effect, eCG treatment resulted in more transferable embryos in Red Sindhi, Brangus, Brahman, Senepol, and Angus donors. In most of the studies showing a positive effect of eCG treatment, donors in the control group produced average to below average numbers of transferable embryos, whereas in the study by Davis et al. (2012), donors in the control group produced above average numbers of transferable embryos, suggesting that this treatment might be especially useful when embryo production is depressed. More recently, Barros et al. (2013) reported a similar improvement in embryo production when 1 mg pLH was added to each of the last two Folltropin-V administrations in Angus donors.

Repeated superstimulation

Traditionally, donor cows have been subjected to embryo collection at approximately 60-day. However, more recent information suggests that cows can be superstimulated as often as every 30 days (Bó et al. 2007). Once multiple CL has been induced to regress by the administration of PGF2alpha, and the cow ovulates, normal follicular wave patterns are reestablished and the cow can be superstimulated again. Furthermore, the estradiol + progesterone plus progestin approach to superstimulation makes it possible to superstimulate cows that are not cycling or have abnormal ovarian function (Steel & Hasler 2009).

Fixed-time AI of donors

Barros and Nogueira (2005) have developed a superstimulatory protocol for Bos indicus cattle that they refer to as the P-36 protocol. In this protocol, the progestin device that is inserted before the initiation of superstimulatory treatments is left in place for 36 hours after PGF2a administration and ovulation is induced by the administration of pLH 12 hours

after withdrawal of the progestin device (i.e., 48 hours after PGF2a administration). Because ovulation occurs between 24 and 36 hours after pLH administration, FTAI is scheduled 12 and 24 hours later, eliminating the need for estrus detection.

In a series of experiments in which the timings of ovulations were monitored ultrasonically, Bó et al. (2006) developed a protocol for FTAI in Bos taurus donors without the need for estrus detection and without compromising results. Although donors are typically inseminated twice, 12 and 24 hours after administration of pLH or GnRH, Baruselli et al. (2006) were successful using a single insemination of high quality semen 16 hours after pLH.

Reducing the numbers of gonadotropin treatments

Because the half-life of pituitary FSH is short and traditional superstimulatory treatment protocols consist of twice daily intramuscular injections over 4 or 5 days. This requires frequent attention and might cause undue stress in donors with a subsequent decreased superovulatory response and/or altered preovulatory LH surge (Stoebel & Moberg 1982). However, superovulatory responses were improved in Holstein cows when the single injection was split into two; 75% of the FSH dose was administered subcutaneously on the first day of treatment and the remaining 25% was administered 48 hours later, when PGF2a is normally administered (Lovie et al. 1994).

In a series of experiments in which FSH diluted in a 2% hyaluronan solution was administered as a single intramuscular injection (to avoid the effects of body condition), a similar number of ova/ embryos was produced as in the traditional, twice-daily FSH protocol (Tríbulo et al. 2011). However, 2% hyluronan was viscous and difficult to mix with FSH, especially in the field. The use of hyluronan was improved by splitting total dose of FSH into two injections 48 hours apart as was done with single subcutaneous injection of FSH in Holstein cows (Tríbulo et al. 2011).

Although the number of transferable embryos per donor cow superstimulated has not increased, the protocols that are used today have increased the numbers of transferable embryos recovered per unit time and have facilitated the application of on farm embryo transfer programs in cattle. They are practical, easy to administer by farm personnel, and more importantly, they eliminate the need for detecting estrus.

Superovulation and Embryo recovery in Buffalo

The bovine embryo transfer technology has been successfully applied in the water buffalo, however, the progress has been slow and results have been modest to poor. The first successful transfer resulting in the birth of buffalo calf through non surgical transfer was

performed by Drost et al 1983 in the United States of America. This work aroused considerable interest in buffalo-rearing countries leading to subsequent reports and birth of buffalo calves in other countries. In India the birth of the first calf through surgical (Misra et al, 1988b) and non-surgical embryo transfer (Misra et al., 1988a) was reported at the National Dairy Development Board, Anand. The procedure commonly used in buffalo for ovarian superstimulation has been adopted and is similar to that employed in cattle. Superovulation is even less predictable in buffalo than in cattle; an optimal treatment regimen with FSH remains elusive.

For many years the superovulatory effect of PMSG and FSH have been used to increase ovulation rates in buffaloes and have been applied in conjunction with progestagen and/or prostaglandin F2α treatments to regulate the oestrus cycle. Although the number of corpora lutea was often similar in FSH- and PMSG treated buffaloes, the recovery of embryos after flushing often favoured FSH. Ovarian follicular growth in buffaloes was similar to that observed in cattle and was characterized by waves of follicular recruitment, growth and regression Baruselli et al. 1997. Several authors have attributed the poor superovulatory response of buffaloes to inherent endocrine patterns as well as to the characteristics of the follicular population and ovarian folliculogenesis. The buffalo ovary has a smaller population of recruitable follicles at any given time than the ovary of the cow, at birth an average of 12,000 primary follicles in the buffalo (Presicce 2003) versus an average of 133,000 in the cow (Baruselli et al. 1997).

It was initially assumed that the low embryo recovery rate in buffalo was related to a poor follicular response to exogenous gonadotrophins. However, in studies undertaken by ultrasound evaluation, 9 to 14 ovulatory size follicles were consistently observed in buffaloes stimulated with FSH (Baruselli et al., 1999). This was associated, on average, with ovulation rates of 62.8 percent, a value similar to that found in cattle. In the same study, the number of ovulations presented a high correlation with the number of corpora lutea found on the day of embryo collection, but only one to three ova/embryos were recovered (average recovery rate/CL = 30 percent). In a subsequent study, evidence was obtained for a relatively low rate of transfer of oocytes to the oviduct in buffaloes (Baruselli et al., 2000). It was concluded that the recovery of a low number of embryos in MOET programmes was not necessarily a result of poor superstimulatory responses; rather, it would appear that the failure of oocytes to enter the fallopian tubes and/or impaired transport of ova/embryos in the reproductive tract are major contributing factors to low embryo recovery. A negative correlation between the number of large (>0.8 mm) follicles present on the day of embryo collection and the number of embryos recovered was observed: follicles not ovulating in response to the endogenous LH surge continued to secrete large amounts of estradiol, adversely affecting the functionality of the infundibulum

and passage of ova into the oviducts. A GnRH agonist-LH protocol, developed in cattle (D'Occhio et al. 1999) was used in buffaloes to verify whether it consistently induced ovulations and increased embryo recovery. In females treated with a GnRH agonist the endogenous pre-ovulatory surge release of LH is blocked and ovulation is induced by injection of exogenous LH. Zicarelli et al., (2000) failed to find significant differences in ovarian follicular response in buffaloes treated with GnRH agonist LH protocol and in those treated with a conventional MOET protocol. Gianluca Neglia et al. 2010 reported that progesterone supplementation during the first 2 days of the superovulation treatment seems to enhance the superovulatory response & recovery rate in buffalo species Mediterranean buffaloes.

It is clear nowadays that the application of cattle ET technology to buffalo has met with limited success and much remains to be done in developing procedures specifically for this species.

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Role of oxidative stress and antioxidants in different sperm functions

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Gametes are susceptible to reactive oxygen species (ROS) attack. When manipulated *in vitro* during assisted reproductive techniques, these cells run the risk of generating and being exposed to supra-physiological level of ROS. Defective sperm functions are the most prevalent causes of male infertility and a difficult condition to treat.Many environmental, physiological and genetic factors have been implicated in the poor sperm functions and infertility. Thus, it is very important to identify the factors/ conditions which affect normal sperm functions. Among various causes, oxidative stress (OS) has been attributed to affect the fertility status and physiology of spermatozoa. The term oxidative stress is generally applied when oxidants outnumber antioxidants. The imbalance between the production of reactive oxygen species (ROS) and a biological systems ability to readily detoxify the reactive intermediates or easily repair the resulting damage is known as oxidative stress. The main destructive aspects of oxidative stress are the production of ROS, which include free radicals and peroxides.

Mammalian spermatozoa membranes are rich in high unsaturated fatty acids and are sensitive to oxygen induced damage mediated by lipid peroxidation. Limited endogenous mechanisms exist to reverse these damages. Assessment of such oxidative stress status (OSS) may help in the medical treatment of this male factor infertility by suitable antioxidants

Sperm functional tests

The ultimate goal of a spermatozoan is the successful fertilization of ovum resulting in normal conception. In order to achieve this, the spermatozoa after spermiation must mature within the male genital tract, travel through the female reproductive system, undergo capacitation and acrosome reaction, bind to and penetrate the zona pellucidae of the ova as well as the oolemma, and finally fuse with the female pronucleus. Normal spermatozoa should properly undergo through all of these steps in order to fertilize the ova. This suggests that the routine semen analysis (measurement of seminal volume, spermatozoal motility, density, viability and morphology) does not necessarily provide complete diagnostic information.

As a result of active research in the area of evaluation of semen, a series of sperm function assays have been developed (Table 1). However, no single test is capable of evaluating all

of the steps involved in fertilization. At present only a combination of assays complementing each other can provide a comprehensive evaluation of sperm functions.

IVF: *in vitro* fertilization; CASA: computer-aided sperm analysis; SPA: sperm penetration assay; HOST: hypo-osmotic swelling test; IPA : Immunoperoxidase assay; IBD: Immunobead assay.

Table 1: Different sperm functional assays/parameters

Routine Evaluation	Seminal fluid volume, Sperm count, motility, Morphology, Viability, Leukocytes in semen, Sperm antibodies
Specialized Sperm Function	Membrane integrity, Sperm-cervical mucus interaction, CASA, Capacitation, Acrosome reaction, Zona pellucida binding, Zona pellucida penetration, Oocyte-sperm fusion
Sperm Function Assays	HOST, IBD Assay, IPA assay, SPA, Acrosome reaction tests, Immunoflorescence assays, IVF

Oxidative stress

"Oxidative stress" (OS) is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants commonly known as reactive oxygen species (Figure 1).

Seminal plasma oxidative stress levels can also be quantified either by direct methods, such as chemiluminescence assays, cytochrome-c and nitroblue tetrazolium reduction, flow cytometry, electron spin resonance spectroscopy, xylenol orange-based assay, and by indirect methods which measure the levels of biomarkers of OS, such as thiobarbituric acid reactive substances, level of antioxidant enzymes [(superoxide dismutase –SOD, glutathione peroxidase –GPX, glutathione reductase (GR)], glutathione (GSH), isoprostane, DNA damage and total antioxidant capacity. Under normal circumstances, there is an appropriate balance between pro-oxidants and antioxidants. A shift in the levels of ROS towards prooxidants in semen can induce an oxidative stress on spermatozoa. Concomitantly, a decrease in antioxidant activities (e.g., SOD, catalase, GSH peroxidase and reductase, GSH) in semen will correlate with idiopathic infertility. It is possible that an increased rate of ROS production (suggesting high oxidative stress) may inhibit the action of these antioxidant enzymes, or alternatively the inherent decreased expression of these antioxidant enzymes may cause increased oxidative stress. This will result in increased LPO, decreased sperm motility, viability, and function, and, ultimately, infertility.

Reactive oxygen species (ROS) and oxidative stress (OS)

Reactive oxygen species (ROS) are highly reactive oxidizing agents belonging to the class of free-radicals. A free radical is any compound (not necessarily derived from oxygen) which contains one or more unpaired electrons. The most common ROS that have potential implications in reproductive biology include superoxide (O_2^-) anion, hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals and the very reactive hydroxyl (OH^-) radicals. The nitrogen-derived free radical nitric oxide (NO^-) and peroxynitrite anion ($ONOO^-$) also appear to play a significant role in the reproduction and fertilization.

The assumption that free radicals can influence male fertility has received substantial scientific support. The proposed mechanism for loss of sperm function upon oxidative stress has been shown to involve excessive generation of ROS. The H_2O_2 has both beneficial and damaging effects on sperm and thus can influence the fertilization process. Hence, free radicals and ROS are associated with oxidative stress and are likely to play a number of significant and diverse roles in reproduction.

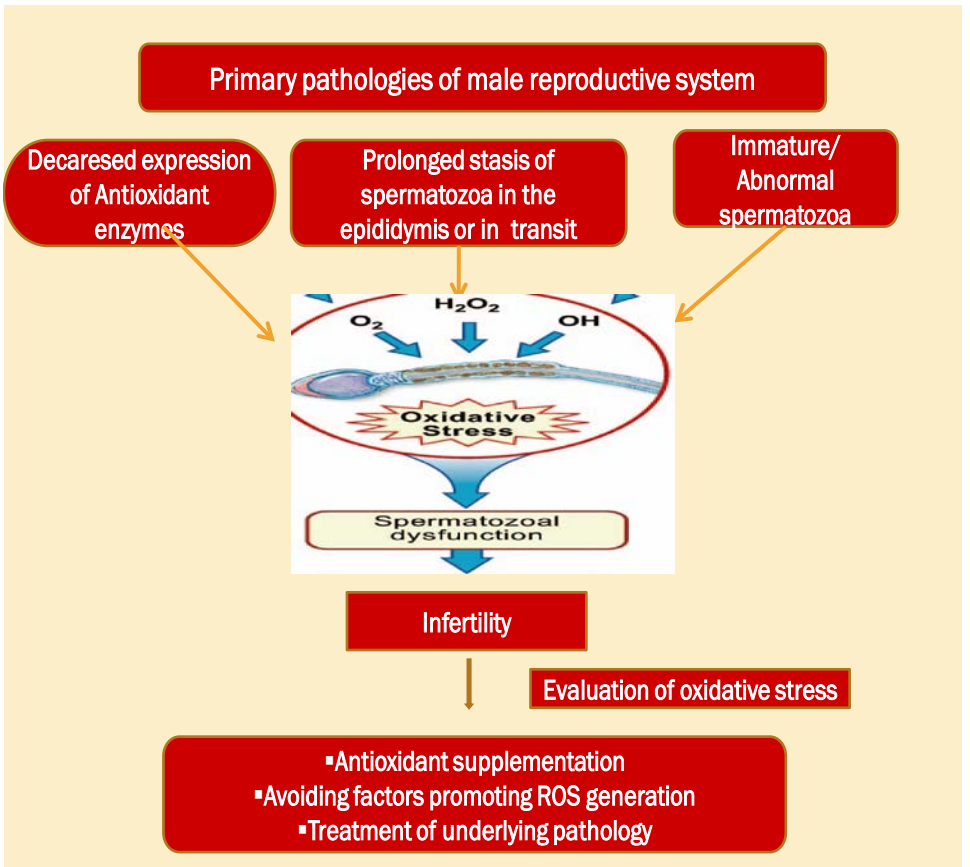

Figure 1: Factors contributing to oxidative stress-induced male infertility

Causes of ROS generation in semen

There are many causes of ROS production in semen, but important ones are following:

- In male, a hypothetical NADH oxidase at the level of sperm membrane and low sperm diphorase (mitochondrial NADH-dependent oxidoreductase) are the two main ROS producing systems(Figure 2).
- In bovine semen, ROS are generated primararily by dead spermatozoa via an aromatic amino acid oxidase catalyzed reaction(Figure 2) .
- Under normal physiological conditions, seminal plasma and normal spermatozoa do not produce ROS, but morphologically abnormal spermatozoa can produce.
- Leukocytes and immature spermatozoa are also the main sources of ROS. Leukocytes particularly neutrophils and macrophages have been associated with excessive ROS production, and, they ultimately cause sperm dysfunction.
- Absence of endogenous defense mechanisms and exposure of gametes & embryos to various manipulation techniques are also the causes of ROS generation under *in vitro* conditions.

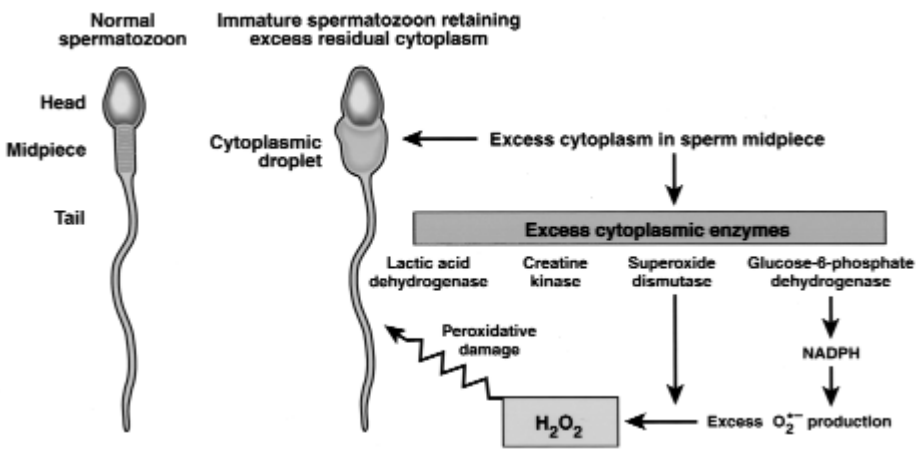

Figure 2: Mechanism of increased production of reactive oxygen species (ROS) by abnormal spermatozoa

Mode of action of ROS

Mammalian spermatozoa are rich in polyunsaturated fatty acids and, thus, are very susceptible to ROS attack which results in a decreased sperm motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability, and increased midpiece morphological defects with deleterious effects on sperm capacitation and acrosome reaction. Lipid peroxidation of sperm membrane is considered to be the key mechanism of this ROS-induced sperm damage leading to infertility.

Positive and negative effects of ROS: The production of ROS is a normal physiological process but an imbalance between ROS generation and scavenging activity is detrimental to the sperm and associated with male infertility.ROS generated by spermatozoa play an important role in normal physiological processes such as sperm capacitation, acrosome reaction, maintenance of fertilizing ability and stabilization of the mitochondrial capsule in the mid-piece in bovine .Controlled generation of ROS may function as signaling molecules (second messengers) in many different cell types , they are important mediators of sperm functions. Evidences have been reported that superoxide anion ($.O_2$) are required for the late stage of embryo development such as two germ cell layers and egg cylinder .Although a significant negative correlation between ROS and IVF fertilization rate has been found. Yet, controlled generation of ROS has shown to be essential for the development of capacitation and hyperactivation ; the two processes of sperm that are necessary to ensure fertilization.In vivo physiological concentrations of ROS are involved in providing membrane fluidity , maintaining the fertilizing ability and acrosome reaction of sperm. The maintenance of a suitable ROS level is, therefore, essential for adequate sperm functionality. ROS cause adverse effects on the sperm plasma membrane, DNA and physiological processes, thereby, affecting the quality of spermatozoa.The axosome and associated dense fibers of the mid-piece in sperm are covered by mitochondria that generate energy from intracellular stores of ATP depletion. Excessive ROS impairs motility and capacity of fertilization .Mammalian spermatozoal membranes are rich in polyunsaturated fatty acids (PUFAs) and are sensitive to oxygen induced damage mediated by lipid peroxidation, and, thus are sensitive to ROS attack which results in decreased sperm motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage, decrease sperm viability and increased mid-piece sperm morphological defects with deleterious effects on sperm capacitation and acrosome reaction.Thus ROS are independent markers of male factor infertility .

Lipid peroxidation: Lipids are considered to be the most susceptible macromolecules and are present in sperm plasma membrane in the form of polyunsaturated fatty acids (PUFA); fatty acids that contain more than two carbon–carbon double bonds. ROS attacks PUFA in the cell membrane leading to a cascade of chemical reactions called lipid peroxidation. One of the by-products of lipid peroxidation is malondialdehyde (MDA), which has been used in various biochemical assays to monitor the degree of peroxidative damage sustained by spermatozoa. Results of such assays exhibit an excellent correlation when examining the relationship between impaired sperm function, discussed in terms of motility, and the capacity for sperm-oocyte fusion. Recently peroxidative damage to the sperm has been measured using a probe.

Role of oxidative stress in sperm capacitation and acrosome reaction

For successful fertilization, mammalian spermatozoa must undergo a preparation period known as capacitation .In physiological terms, capacitation can be considered as the sum of biochemical and biophysical changes that take place in sperm cell during its transport through the female genital tract. Some studies show that sperm capacitation and acrosome reaction are oxidative processes; low concentration of ROS (H_2O_2,$^{\bullet}O_2^-$), exogenously added or minute amounts generated by spermatozoa are needed to trigger this phenomenon *in vitro*. Extracellular but not intracellular production of $^{\bullet}O_2^-$ by spermatozoa is important for sperm capacitation and acrosome reaction, indicating that sperm membrane is the first target of ROS . The involvement of specific ROS may depend upon incubation conditions and on the species of spermatozoa. Substantial evidences exist to suggest that small amounts of ROS are necessary for spermatozoa to acquire fertilizing capabilities. Low levels of ROS have been shown to be essential for fertilization, acrosome reaction, hyperactivation, motility, and capacitation. Co-incubation of spermatozoa with low concentrations of hydrogen peroxide has been shown to stimulate sperm capacitation, hyperactivation, acrosome reaction, and oocyte fusion.ROS such as nitric oxide (NO) and the superoxide anion have also shown to promote capacitation and the acrosome reaction.Furthermore, ROS have also been implicated in sperm oocyte interaction. Controlled generation of ROS may function as signaling molecules (second messengers) in many different cell types, they are important mediators of sperm functions. Evidences have been reported to especially superoxide anion ($^{\cdot}O_2$) are required for the late stage of embryo development such as two germ cell layers and egg cylinder. Although a significant negative correlation between ROS and IVF fertilization rate has been found . Yet, controlled generation of ROS has shown to be essential for the development of capacitation and hyperactivation; the two processes of sperm that are necessary to ensure fertilization. In vivo physiological concentrations of ROS are involved in providing membrane fluidity, maintaining the fertilizing ability and acrosome reaction of sperm. The maintenance of a suitable ROS level is, therefore, essential for adequate sperm functionality.

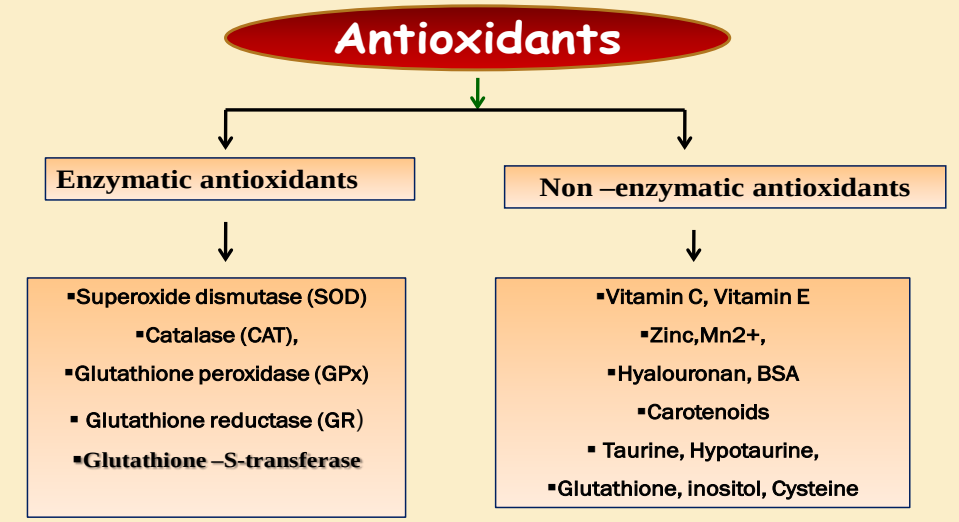

Figure 3: Schematic representation of enzymatic and non-enzymatic antioxidants

Antioxidants: ROS/oxidative stress scavenging strategies

Studies have shown that antioxidants protect spermatozoa from ROS producing abnormal spermatozoa, scavenge ROS produced by leukocytes, prevent DNA fragmentation, reduce cryodamage to spermatozoa, block premature sperm maturation and stimulate spermatozoa and improve ART outcome. Seminal plasma contains superoxide dismutase, catalase, and glutathione peroxidase / glutathione reductase in addition to non-enzymatic antioxidants such as ascorbate, urate, vitamin E, pyruvate, glutathione, albumin, vitamin A, ubiquitol, taurine, and hypotaurine Antioxidants, in general, are compounds and reactions that dispose, scavenge, suppress the formation of ROS, or oppose their actions. A variety of enzymatic and non-enzymatic (chemical) antioxidants that attack ROS and LPO are presently under investigation (Figure 3). Within the category of chemical antioxidants, both natural and synthetic products have gained attention by the cosmetic, nutrition, and pharmaceutical industries. Their usefulness in reproduction and management of infertility has not yet been developed. Treatment strategies to reduce seminal oxidative stress levels may enhance natural conception and the outcome of assisted reproductive technologies. Antioxidants are the most important defense against free radical induced infertility. Standard semen analysis and use of the sperm deformity index have been used to identify infertile males with high levels of ROS .

Non-enzymatic Antioxidants

Carnitines: Carnitine is a water-soluble antioxidant that may play a role in sperm energy metabolism and provide the primary fuel for sperm motility. Spermatozoa exhibit increased L-carnitine and L-acetyl carnitine content during epididymal passage and acquisition of motility. Carnitines enhance the cellular energetics in mitochondria by facilitating the entry and utilisation of free fatty acids within the mitochondria and also restore the phospholipid composition of mitochondrial membranes by decreasing fatty acid oxidation. In addition, carnitines protect sperm DNA and cell membranes from ROS-induced damage and apoptosis.

Vitamin E (α tocopherol): Vitamin E appears to be the first line of defence against the peroxidation of polyunsaturated fatty acids (PUFAs) contained in the cellular and sub-cellular membrane phospholipids, because of its lipid solubility. The phospholipids of mitochondria, endoplasmic reticulum and plasma membrane possess high affinity for α-tocopherol, and the vitamin E appears to be concentrated at these sites. It protects sperm membrane against oxidative damage. It is well documented antioxidant and has been shown to inhibit free radical induced damage to sensitive cell membrane. The antioxidant action of α-tocopherol is effective at higher oxygen concentration, and, thus it is not surprising that it tends to be concentrated in those lipid structures that are exposed to the higher O_2 partial pressure. Vitamin E is the major chain breaking antioxidant in membrane by directly neutralizing superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). α-tocopherol is classified as a chain breaking antioxidant because of its ability to break the lipoperoxidative chain reaction through its interaction with lipid peroxyl and alkoxyl radicals. It is a powerful antioxidant and has been shown to afford some protection to mammalian cells from oxidative attack generated both under *in vivo* and *in vitro* conditions. Damage caused by iron catalysed peroxidation can be prevented by including α-tocopherol (Vitamin E) to the medium. It breaks the free radical chain reaction by forming a relatively stable radical tocopheroxy at a concentration of 10 mM.

Vitamin C: Vitamin C (ascorbic acid) is a water-soluble ROS scavenger with high potency. It is found in concentrations 10-fold higher in seminal plasma than serum. It is found that it protects human spermatozoa against endogenous oxidative damage by neutralizing hydroxyl, superoxide and hydrogen peroxide radicals and preventing sperm agglutination. Significantly reduced concentrations are seen in semen samples with excess ROS. Seminal plasma concentrations have been positively correlated with percentage of morphologically normal spermatozoa. Vitamin C may have a dose-dependent effect on sperm quality. At a dose of 1000 mg/l, vitamin C positively influenced the motility of the spermatozoa. However, higher dosages may have damaging pro-oxidant effects which actually reduce sperm motility. Vitamin C supplementation may minimize endogenous oxidative DNA

damages, thereby decreasing the risk of genetic defects, particularly in populations with low vitamin C levels. The hydrophilicity and lipophilicity of vitamins C and E may act synergistically to protect against peroxidative attack on spermatozoa. It is demonstrated that combined supplementation significantly reduced the percentage of DNA-fragmented sperm. At follow-up, there were significantly increased rates of clinical pregnancy and implantation following intracytoplasmic sperm injection (ICSI).

Selenium: Selenium (Se) may protect against oxidative sperm DNA damage and is required for normal testicular development, spermatogenesis, motility and function The precise mechanism by which Se reduces oxidative stress is not well-established. Selenoenzymes, such as phospholipid hydroperoxide glutathione peroxidase (PHGPX) and the sperm capsular selenoprotein glutathione peroxidase may mediate its effects. Se deficiency leads to impaired motility, breakage of the spermatozoa mid-piece and increased morphological abnormalities, mostly affecting the sperm head. The effectiveness of combined treatment with Se and vitamin E has been studied since Vitamin E works synergistically with Se as an antiperoxidant. A prospective, uncontrolled study reported that combined treatment significantly increased motility and mediate seminal plasma glutathione peroxidase (GSHPx) activity.

Carotenoids: Carotenoids such as beta-carotene and lycopene are also important components of antioxidant defense. Beta carotene protects the plasma membrane against LPO in rat. Carotenoids work synergistically with Se and vitamin E. Another carotenoid of interest is lycopene – naturally derived from fruits and vegetables. It has been found to have the highest ROS-quenching rate, with plasma levels higher than beta carotene. Lycopene is found in high concentrations in the testes and seminal plasma, with lower levels in infertile men.

N-acetyl cysteine: N-acetyl cysteine (NAC) replenishes GSH while scavenging free radicals and reducing ROS production in human ejaculate. NAC plays an important role in germ cell survival in human seminiferous tubules in vitro. It was found that incubating semen samples with NAC for 20 min significantly decreased ROS levels and lead to improved sperm motility. It is found that NAC improved sperm concentration and acrosome reaction.

Pentoxifylline: Pentoxifylline has been shown to decrease ROS production, preserve sperm motility *in vitro* and improve semen parameters *in vivo*.It is demonstrated that pentoxifylline improved sperm motion characteristics, such as curvilinear velocity, path velocity and beat cross frequency but did not increase the percentage of motile spermatozoa in asthenospermic males. It is studied the effects of *in vitro* and *in vivo* pentoxifylline treatment on sperm motion parameters in male subjects whose spermatozoa produced

detectable steady state levels of ROS. Treatment decreased ROS formation and preserved sperm motion parameters in vitro. Orally administered pentoxifylline had no effect at a low dosage, whereas a higher dosage increased sperm motility and some sperm motion parameters without altering sperm fertilizing ability.

Trace metal ions: The antioxidant and/or pro-oxidant potential of three trace metal ions namely aluminium (Al), manganese (Mn) and selenium (Se) has been studied. The effects of Al and Mn have been found to be anion dependent and manganese proved to be the trace metal ion of choice. It is well known that Mn^{2+} is a potent inhibitor of *in vitro* LPO in variety of systems . However, the inhibitory mechanisms of Mn^{2+} on LPO have not been fully elucidated. The action of Mn^{2+} at high concentration is not due to scavenging of lipid radicals; but rather Mn^{2+} inhibited the LPO by counteracting $LOO^{\bullet}$ (lipid peroxyl radicals), which are water soluble, resulting in inhibition of LPO. Probably, Mn^{2+} ions compete with iron (Fe^{2+}) at anionic oxygen of phosphate groups in phopholipids to inhibit LPO. It is reported that 1 μM Mn^{2+} significantly inhibited MDA production in LPO of brain homogenates, which may contain a trace amount of endogenous iron. Adequate zinc and copper intake is needed to maintain the optimal functioning level of antioxidant enzymes, such as superoxide dismutase.

Coenzyme Q-10: Coenzyme Q-10 is a non-enzymatic antioxidant that is related to low density lipoproteins and protects against peroxidative damage. It is an energy-promoting agent and enhances sperm motility. It is present in the sperm mid-piece and recycles vitamin E and prevents its pro-oxidant activity. Oral supplementation of 60 mg⁄ day of coenzyme Q-10 was shown to improve fertilization rate using intracytoplasmic sperm injection (ICSI) in normospermic infertile males.

Inositol: Supplementation of inositol to the extender can improve the motility of frozen thawed bull sperm. Inositol has cryoprotective and antioxidative properties, resulting in higher antioxidant activity, acrosome integrity and intact morphological rates.

Cysteine: It is a low molecular weight amino acid containing thiol (-SH); it is a precursor of intracellular glutathione (GSH). It has been shown to penetrate the cell membrane easily, enhancing the intracellular GSH biosynthesis both *in vivo* and *in vitro* and protecting the membrane lipids and proteins due to indirect radical scavenging properties. It is also thought that GSH synthesis under *in vitro* conditions may be impaired, because of deficiency of cysteine in the media, due to its high instability and auto-oxidation to cysteine. Cysteine has cytoprotective effect on the functional integrity of axosome and mitochondria improving post thawed sperm motility. Cysteine has been shown to prevent the loss in motility of frozen thawed bull, ram and goat semen. It has also improved the

viability, the chromatin structure and membrane integrity of boar sperm during liquid preservation, and has enhanced porcine oocytes maturation and fertilization under in *vitro* conditions.

Trehalose or taurine: It is a sulfonic amino acid acts as non-enzymatic scavenger that plays an important role in the protection of spermatozoa against ROS. Trehalse performs better cryoprotective role by improving post thaw fertilizing ability in ram, bull and mouse sperm. Recent study has demonstrated that, in ram, trehalose shows its antioxidant property when semen is incubated at 37^0C for 3hrs. Tuarine displayed antioxidative properties by elevating catalse level in close association with superoxide dismutase concentration in ram, rabbit and bull spermatozoa.

Hyaluronan: Hyaluronan is an essential component of the extracellular matrix and non – sulfated glycosaminoglycan, is involved in important physiological functions such as motility, capacitation of spermatozoa and preserve post-thaw spermatozoa viability and *in vitro* membrane stability. Hyaluronan improves sperm motility, viability and membrane integrity after freezing and thawing procedures and decrease polyspermy with declining motility in humans and boars.

Bovine serum albumin (BSA): BSA is known to eliminate free radicals generated by oxidative stress, and the protection of membrane integrity of sperm cells from heat shock during freezing- thawing of canine semen. Albumin used in sperm washing procedure is likely to serve as antioxidant by providing thiol groups required for 'chain breaking' antioxidant activity.

Glutathione: Glutathione (GSH) is the most abundant reducing agent found in the body, protecting lipids, proteins and nucleic acids against oxidative damage. GSH combines with vitamin E and Se to form glutathione peroxidase. In a placebo-controlled, double-blind, cross-over trial, administration of 600 mg for 2 months by intramuscular injection in 20 infertile men significantly increased sperm motion characteristics, namely improved forward progression .GSH deficiency may render the midpiece unstable, resulting in defective morphology and motility. Glutathione is the most abundant non-thiol protein in mammalian cells. A glutathione efficiency can lead to instability of the mid-piece, resulting in defective motility. It protects plasma membrane from lipid peroxidation, scavenges superoxide, and prevents O2 formation. In a study of infertile men with unilateral varicocele or genital tract inflammation, glutathione led to significant improvement in sperm quality.

Other Non-enzymatic antioxidant: Molecules such as N-acetyl L-cysteine, carotenoids, coenzyme Q10 and carnitines provide excellent antioxidant support. N-acetyl L-cysteine is a precursor of glutathione that improves sperm motility and reduces ROS-induced DNA damage. Carotenoids play an important role in protecting the cells and organisms by scavenging the superoxide radicals. Co-enzyme Q10 protects lipids against peroxidative damage. It scavenges superoxide anion as well as peroxides. Carnitine promotes membrane stability and plays an important role in sperm maturation and development.

Enzymatic antioxidant

Superoxide Dismutase: Superoxide dismutase (SOD) scavenges both extracellular and intracellular superoxide anion and prevents lipid peroxidation of the plasma membrane. In order to act against H2O2, it must be conjugated with catalase or glutathione peroxidase. SOD also prevents premature hyperactivation and capacitation induced by superoxide radicals before ejaculation.

Glutathione Peroxiadse/ Reducatse system: This system forms an excellent protection against lipid peroxidation of plasma membrane of spermatozoa. It scavenges lipid peroxides thereby arresting the progressive chain reaction of lipid peroxidation. It also scavenges H_2O_2, which is responsible for the initiation of lipid peroxidation. Glutathione reductase (GRD) stimulates the reduction of glutathione disulfide (GSSG) to reduced glutathione (GSH). This ensures a steady supply of the reductive substrate NADPH to GPX. Glucose-6-phosphate dehydrogenase (G6PD) is required for the conversion of NADP+ to its reduced form, NADPH. Glutathione peroxidase (GSHPx), a selenium containing antioxidant enzyme with glutathione as the electron donor removes peroxyl ($ROO^{\cdot}$) radicals from various peroxides including H_2O_2 and results in conversion of glutathione reduced (GSH) to glutathione peroxide(GSSG) in sperm. On the other hand, glutathione reductase (GR) regenerates reduced GSH from GSSG as shown in the following equation:

$$2GSH + H_2O_2 \xrightarrow{GSHPx} GSSG + 2H2O$$

$$GSSG + NADPH + H^{+} \xrightarrow{GR} 2GSH + NADP^{+}$$

GSH peroxidase and GSH reductase may directly act as antioxidant enzymes involved in the inhibition of sperm lipid peroxidation. GSH has a likely role in sperm nucleus decondensation. Thus, in view of the great number of mitochondria in spermatozoa, these antioxidant mechanisms are important in the maintenance of sperm motility, rate of hyperactivation and the ability of sperm to undergo acrosome reaction during sperm preparation techniques especially in the absence of seminal plasma. A high redox ratio (GSH/GSSG) ratio will help spermatozoa to combat oxidative stress. It seems that the role

of these GSH enzymes and their associated mechanisms are related to infertility in men , is an important area for further investigation.

Catalase: Catalase detoxifies both intracellular and extracellular H_2O_2 to water and oxygen. In addition, catalase activates nitrous oxide (NO)-induced sperm capacitation, which is a complex mechanism involving H_2O_2.

Conclusions

The rationale for antioxidant therapy in infertile males should be based on a high oxidative stress status. The dosage of the antioxidant is a critical point, particularly because investigators do not know the required level of ROS for physiological purposes. The dose and duration of treatment are of vital importance. Evaluation of OS status and use of antioxidants is not routine in clinical practice. The immediate need is to simplify and validate the evaluation of ROS and oxidative stress status so that it can be performed routinely without the use of sophisticated equipments. Also, it is important to establish reference values for ROS above which antioxidants could be used for male infertility treatment. The dose and duration of these antioxidants should also be determined and standardized. With the increase in the use of ART procedures there should be an effort to develop optimum combinations of antioxidants to supplement sperm preparation media.

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In vitro maturation: the current challenges in buffalo

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Maturation is the potential of oocyte to get fertilized, developed into blastocyst and later on viable offspring (Trounson et al 2001, Sirard et al 2006). The maturation is initiated by resumption of meiosis from the arrested dictyate stage of prophase-1. Even after the aspiration of oocyte from follicle meiosis is resumed suggesting the presence of meiotic-inhibitory factors in the follicular fluid. This follicular environment not only has significant effect on oocyte maturation but also influence the success of fertilization and subsequent embryonic development.

In vitro maturation (IVM) is first and the most crucial step for the success of *in vitro* embryo production (IVEP) (Brackett and Zuelke, 1993). The developmental competence of embryo largely depends upon the quality of maturation of oocyte. Even though the in-vitro maturation of above 90% is claimed by most of researchers, but yet, the blastocyst per cent hardly touches to 40-50 percent in cattle and rarely upto 30% in buffalo (Presicce 2007, Manjunatha et al 2008). This alarms us to understand once again the *in vitro* maturation in detail and to optimize the IVM system for buffalo remains a challenge till date. The process in IVM can be divided into nuclear maturation and cytoplasmic maturation. The oocyte nuclear maturation get accomplished in many species whereas the cytoplasmic maturation appears to be compromised resulting in reduced developmental competence of embryo.

Nuclear maturation: Nucleus of mammalian oocytes is arrested at the dictyate stage of prophase-I. Nuclear maturation is the resumption of meiosis from prophase-I through metaphase-I, anaphase, telophase and again rearrest at metaphase-II. This resumption is indicated by germinal vesicle breakdown (GVBD) while the indicator for completion of nuclear maturation is release of polar body I (PBI; Santos et al 2006, Barakat and Alhimaidi 2012), however, it doesn't indicate the degree of cytoplasmic maturation. Thus, the extrusion of PBI doesn't mean the complete maturation of oocyte and the cytoplasmic maturation should be accessed separately.

Cytoplasmic maturation: Oocyte cytoplasmic maturation includes the oocyte capacity to complete nuclear maturation, fertilization, and early embryogenesis and thus provide a foundation for implantation, initiation of pregnancy, and normal fetal development. Currently, no defined method exists to pinpoint the completion of cytoplasmic maturation of oocyte. However, there are certain changes in the morphology of oocyte organelles, their

inter-relationships and functions but these changes are poorly defined and are expected to occur for the storage of enough mRNA, proteins, substrates and nutrients that are required during nuclear maturation, fertilization and early embryonic life before genomic activation of embryo (Meirelles et al 2004). These changes during cytoplasmic maturation mainly involve the mitochondria, lipid droplets and the cortical granules. Completion of cytoplasmic maturation is poorly defined by the localization of cortical granules to just inside the ooplasmic membrane. These modulations in oocyte organelles could be the reason why *in vivo* matured oocytes have superior developmental competence over *in vitro* matured, but the changes are yet to study in buffalo.

For buffalo, different types of maturation media like TCM-199, MEM, Waymouth, Ham's F-10, RPMI, CRlaa, CR2aa etc have been used but TCM-199 is commonly preferred for IVM in buffalo. Oocytes undergo only partially maturation in absence of serum. The addition of sera to the maturation medium promotes the rupture of germinal vesicle and facilitates the oocyte maturation (Sanbuissho and Threlfall 1990). Further, the Fetal calf serum (FCS) has added advantage over bovine estrous serum (BES) or any other adult animal serum during maturation and fertilization as the former contains some unidentified growth promoting components (such as Fetuin) that are absent in the serum of adult animals. Also, FCS lacks components (hormones and immunoglobulins) present in the adult serum that retard the *in vitro* development of cells (Mochizuki et al 1991). However, due to sanitary considerations, serums may be successfully replaced by high molecular weight polymers (Ali and Sirard 2002) for the surfactant effect (such as polyvinyl alcohol, polyvinyl-pyrrolidone), and showed varying effect on maturation; or may be replaced by commercial products such as Ultroser G (Lonergan 1992).

The buffalo oocytes with intact layers of cumulus cells have better maturation over the oocytes with less intact layers of cumulus cell or are denuded. Researchers tried oocytes maturation alongwith one or other somatic cells or cell lines and observed improved maturation rate suggesting these cells secrete factors which support oocyte maturation (Das et al 1997).

Addition of various hormones like follicle stimulating hormones (FSH) pregnant mare serum gonadotropin (PMSG), luteinizing hormone (LH), estradiol 17-β (E_2), either alone or in combination improved the maturation rates significantly (Totey et al 1992). Tatemoto and Terada (1998) reported that FSH stimulation may play in the complex mechanism of chromatin condensation leading to meiotic resumption in the oocytes and LH in the medium enhanced expansion of cumulus cells (Gliedt et al 1996), whereas estradiol improved the fertilizing ability of bovine oocytes cultured *in vitro*. Supplementation of maturation media with growth factors like epidermal growth factor (EGF), insulin like

growth factor-1,2 (IGF-1, IGF-2), transforming growth factor (TGF), fibroblast growth factor (FGF) etc in different concentrations found to improve the oocyte maturation rate. These may act as autocrine/ paracrine manner or as a signalling factor in resumption of meiosis. They may also stimulate the DNA or protein synthesis and thus enhance the maturation rate. Oocyte particularly of pig and buffalo have high lipid content and are more sensitive to oxidative damage (Boni et al 1996), so the antioxidants like cysteamine and β-mercaptoethanol increase the intracellular concentration of glutathione and thus mitigates the oxidative stress to improve the maturation rate. Some other antioxidative molecules (cystein, vitamin A, C, E), divalent cations chelators (EDTA, taurine, hypotaurine, transferrine) or ROS scavengers (superoxide dismutase, catalase) could be added to maturation medium (Blondin et al 1997). Buffalo follicular fluid (BFF) contain gonadotropins, estradiol, progesterone (Totey et al 1993), transforming growth factor-β (TGF-β), inhibin (Palta et al 1996), IGF-2, EGF and TGF (Auckland et al 1992) and these hormones and growth factors play critical role in the maturation of oocytes. BFF from small follicles (<3 mm) had significantly higher potential of developing buffalo oocytes to embryonic stages *in vitro* than from the medium (3-8 mm) and large (>8 mm) follicles (Nandi et al 2002). Similarly, the oocytes harvested from 5-10 mm follicles have better developmental competence over the oocytes extract from other follicular sizes. The highest embryo production rate was obtained when the follicles were >7 mm following FSH superstimulation combined with shorter coasting (FSH starvation) durations of 44 to 68 h and extending the coasting period from 68 h reduced the cumulus cell expansion and nuclear maturation (Dadarwal et al 2014). The dominant follicle, as well as large subordinate follicles, is generally healthy or very slightly atretic and their oocytes preserve good developmental competence (Vassena et al 2003). Follicular atresia begins with signs of apoptosis in granulosa cells then in cumulus cells and finally in the embedded oocyte (Zeuner et al 2003). However, in cattle it was studied that higher developmental competence was achieved in bovine cumulus-oocyte complexes with slight signs of apoptosis in the outer layers of the cumulus (Blondin and Sirard, 1995, Feng et al 2007) but no such study was traced in buffalo. It is well established that healthy cumulus cells are required for the success of oocyte cytoplasmic maturation and fertilization; therefore, maintaining the viability and functionality of cumulus cells is likely to be another important challenge for IVM success. GDF-9 and BMP-15, two members of the TGF-family factors are able to protect cumulus cells against apoptosis (Hussein et al 2005). Recent studies tried the leptin (Amir et al 2014), LIF (Xianhong et al 2014), melatonin (Tian et al 2014), Lysophosphatidic Acid (Dorota et al 2014) etc in different *in vitro* concentrations to optimise the buffalo oocyte maturation. A few latest genomic studies (Mihm et al 2008, Yadav et al 2014) have focused on the follicular status in correlation with gene expression. They demonstrated that dominant follicles were positively associated with enhanced expression of mRNAs in granulosa cells with genes involved in cellular survival, regulation

of proliferation, prevention of apoptosis or DNA damage, and RNA synthesis. On the other hand, subordinate follicles were positively associated with enhanced expression of mRNAs in granulosa cells for genes that were associated with cell death and/or apoptosis.

Although successful progenies of buffalo had been produced from IVM/IVF, still the major challenge in IVEP remains the *in vitro* maturation of oocytes with optimal developmental competence. Active research in several directions may help to overcome these challenges in near future. Identifying the sources of variations between IVM systems or between different laboratories is essential to optimise the efficiency of IVEP. Also, the development of specific culture regimes for buffalo capable of supporting IVM/ IVF and IVC to the blastocyst stage is highly desirable. The best of *in vitro* maturation could be obtained just the need is to simulate the *in vivo* conditions.

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Role of antioxidant therapy in reproduction

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The generation of prooxidant molecules called free radicals or reactive oxygen species (ROS) are product of aerobic metabolism. Free radicals are unstable and highly reactive. They become stable by acquiring electrons from nucleic acids, lipids, proteins, carbohydrate reactions resulting in cellular damage and disease. There are two major types of radical species:

1. Reactive oxygen species (ROS)
2. Reactive nitrogen species

ROS are of three major types: Superoxide O_2^-, Hydrogen peroxide H_2O_2, Hydroxyl OH. ROS has been implicated in more than hundred diseases (Evans et al, .2004, Gibson et al., 2004 and Madamanchi et al., 2005). They have a physiological and pathological role in the female reproduction. Free radicals can influence the oocysts, sperms and embryo in their microenvironments. ROS is involved in oocyte maturation ovarian steroidogenesis, corpus luteum function and luteolysis.

There is a complex interaction of the prooxidant and antioxidants , resulting in the maintenance of intracellular homeostasis. Whenever there is an imbalance between the prooxidant and antioxidants, a state of oxidative stress is initiated. Oxidative stress affects both implantation and early embryo development which determines the successful pregnancy. Imbalance between the production of ROM and their safe disposal can initiate oxidative chain reaction and lipid peroxidation (Miller et al., 1993). These reaction, if not controlled may cause extensive tissue damage which can effect membrane permeability and enzyme function. Antioxidants such as vitamin E, Vitamin C, ß carotene and enzyme like superoxide dismutase (SOD) glutathione peroxidase are critical for body defence against extensive production of ROM. Insufficient dietary antioxidants may lead to low reproductive performance by peroxidative damage to steroidogenic enzymes (Staats et al., 1988). Vitamin E is the main biological chain breaking antioxidant essential for growth, reproduction, prevention of various diseases and integrity of tissues (Quereshi et al., 1997). The activity of glutathione peroxidase in the blood of dairy cattle was associated with the incidence of anoestrus or subestrus (Jukola et al., 1996). Selenium is known to increase the antioxidant action of vitamin E.

Antioxidants: Under normal conditions, scavenging molecules known as antioxidants convert ROS to H_2O to prevent overproduction of ROS. There are two types of antioxidants:
Enzymatic antioxidant which are also known as natural antioxidants. They neutralize excessive ROS and prevent damaging the cellular enzymes ie dismutase, catalase, glutathione peroxidase(GPX) and glutathione reductase which causes reduction of hydrogen peroxide to water.

Non-enzymatic antioxidants are known as synthetic antioxidants and dietary supplements ie vitamin C, vitamin E, Selenium, Zn, Taurine, Hypotaurine, glutathione, beta-carotene and carotene. Vitamin C helps recycle oxidized vitamin E and glutathione. Glutathione is present in the oocyte and tubal fluid and has a role in improving the development of the zygote beyond the 2 cell blastocyst.

Reproduction and Oxidative stress: many studies have shown involvement of ROS in the follicular fluid environment, folliculogenesis and steroidogenesis (Behrman etaal 2001). Cells have developed a wide range of antioxidants system to limit production of ROS, inactivate them and repair cell damage. The pathological effect are exerted by lipid damage, inhibition of protein synthesis and which affect the physiological functions like oocyte maturation, ovarian steroidogenesis, ovulation, implantation etc. ROS are double edge weapon which serve as key signal molecules in physiological processes but also have a role in pathological process.

O**xidative stress and female infertility:** infertility in cow is defined as the inability to produce live healthy calf every year, in 13-14 months in buffaloes and when female fail to conceive following 12 or more months of unprotected sex in ladies. Pathophysiology of oxidative stress in female reproduction: oxygen toxicity is an inherent challenge to aerobic life. ROS can modulate cellular functions. The oxidant status can influence early embryonic development. ROS may also play a major role both in the implantation and fertilization of eggs. Oxidative stress is involved in defective embryo development and retardation of embryo growth (Agrawal et al 2003)

ROS and the ovary: Oxidative stress marker such as superoxide dismutase (SOD) Cu-Zn superoxide dismutase, Mn superoxide dismutase, gluththione peroxidase by immunohistochemical localization. All the follicular stages have been examined for SOD expression including primodial, primary, preantral and non dominat antral follicle. ROS may have a regulatory role in oocyte maturation, folliculogenesis, ovarian steroidogenesis and luteolysis. Antioxidant enzymes neutralize ROS production and protect the oocyte and

embryo. A burst of placental oxidative stress during establishment of maternal circulation may cause early pregnancy loss (Jauniaux et al 2000).

Oxidative stress and assisted reproduction: assisted reproductive technique involves the direct manipulation of the oocyte , sperm or embryo outside the body. There may be many sources of ROS in an IVF including oocytes, cumulus cell mass, spermatozoa. Low levels of ROS play a beneficial role in IVF.

How to overcome oxidative stress in infertility/ ART: considerable interest has been shown in the use of antioxidants to overcome the adverse and pathological results OS. Oxidative stress can damage oocytes in developing follicle, oocytes and spermatozoa. Os can be overcome by

- Reducing generation of ROS
- Increasing the amount of antioxidants available.

Current evidence supports the use of systemic antioxidants for management of selected cases of male infertility (Agarwal et al., 2004). Increased generation of ROS in semen affects sperm function especially fusion events associated with fertilization and leads to infertility (Sikka 2001). The effect of vitamin C supplementation reported: pregnancy rates were higher in the treatment group than in the controls (Henmi et al., 2003). Similarly the concentration of antioxidants was lower in miscarriages and luteal phase defects than in healthy women (Vural et al., 2000). Effect of exogenous administration of vitamin E and selenium during prepartum period on postpartum reproductive performance of buffaloes was found to improve postpartum ovarian activity. Injection of E care Se containing 500mg of vitamin E and 15 mg of selenium, two weeks before expected date of calving led to early initiation of postpartum ovarian activity (27.2±5.66 vs 51.6± 6.99 days) early exhibition of first postpartum heat (38.6± 7.38 vs 53.5± 4.81 days). Duration of first progesterone rise of over 1ng ml^{-1} of plasma was significantly ($P<0.05$) larger in treated animals. Conception rate was significantly ($P<0.05$) higher and service period was significantly ($P<0.05$) less in treated buffaloes. Vitamin E and selenium, both act as antioxidants the former being a fast acting antioxidant. It maintain the integrity of membrane phospholids against oxidative change (Di-Mascio et al., 1991). Selenium is an integral part of glutathionperoxidase enzyme. This enzyme helps in the removal of free radicals in the body thus, minimizing oxidative stress. Thus vitamin E and selenium administration in prepartum buffalo may result in better conception rate.

Oral supplementation with 3500 IU vitamin E alone or in combination with 14 mg selenium as sodium selenite per week per animal for two months in anestrous buffalo heifer resulted in significant decrease of endogenous erythrocytic MDA levels as compared to control

group (Nayyar et al., 2005). The results for erythrocytic lipid peroxidation has been expressed in terms of malondialdehyde (MDA) content. The levels of endogenous erythrocytic MDA were significantly lower in group supplemented both with vitamin E and selenium as compared to Vitamin E group at 1st, 2nd and 3rd week after supplementation.

Vitamin E and selenium deficiency increases the lipid peroxidation in ruminant calves (Wolsh et al.,1993). Vitamin E interact directly with lipid peroxides in plasma lipoproteins and cell membranes to neutralize them (Pironi et al., 1998). Supplementation of vitamin E alone increase erythrocytic glutathione peroxidase activity but decreased the activity of superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase.

Onset of postpartum ovarian activity and fertility status of buffaloes treated with vitamin E and selenium

Parameter	**Treatment group**	**Control group**
First postpartum oestrum (days; Mean ± SE)	38.6 ± 7.38	53.5 ± 4.81
First postpartum progesterone rise (days; Mean ± SE)	27.2 ± 5.66	51.8 ± 6.99
Duration of first progesterone rise (days; Mean ± SE)	12.6 ± 2.38	6.33 ± 2.43
No of buffaloes having onset of Luteal activity <30 days postpartum >30 days postpartum	 3 (60) 2 (40)	 1 (16.6) 5 (83.4)
No of buffaloes showing oestrum Within 45 days postpartum After 45 days postpartum	2 (40) 3 (60)	1 (16.6) 5 (83.4)
Overall service period (days; Mean ± SE)	102.0 ± 24.7	136.0 ±22.9
Conception rate	80 %	50 %

Erythrocytic lipid peroxidaton (n mol MDA/mg Hb) in anoestrus buffalo supplemented with vitamin E and selenium

Group	**Before supplementation**	**After supplementation**			
		1st week	**2nd week**	**3rd week**	**4th week**
Control Gp	368.9 ± 4.16	374.2 ± 1.37	369.2 ± 1.32	368.0 ± 9.0	366.6 ± 1.44
Vitamin E Gp	385.0 ± 19.3	294.3 ± 6.09	258.7 ± 10.22	239 ± 12.0	224.2 ± 4.16
Vitamin E + Selenium Gp	374.2 ± 3.94	264 ± 12.59	239 ± 10.2	215 ± 10.1	210.3 ±10.20

Erythrocytic antioxidant enzyme activities in anoestrus buffalo supplemented with vitamin E and selenium.

Parameters	Control group	Vitamin E group	Vitamin E plus Se gp
Superoxide dismutase (U/mgHb): Before, After	10.30 ± 0.049	10.17 ± 0.026	10.21 ± 0.055
		8.72 ± 0.194	8.45 ± 0.208
Glucose-6-phosphate Dehydrogenase (µ mol-gHb): Before, After	3.18 ± 0.011	3.21 ± 0.215	3.18 ± 0.167
		2.16 ± 0.135	2.07 ± 0.225
Glutathione peroxidase (U/mg Hb): Before, After	13.12 ± 0.01	13.37 ± 0.264	13.20 ± 0.223
		14.68 ± 0.20	16.33 ± 0.75

Erythrocytic SOD activity decreased significantly in supplemented group of vitamin E and Vitamin E & selenium group as compared to control group of anoestrus heifer at 4 week of supplementation (Nayyar et al., 2005). SOD disproportionate superoxide to hydrogenperoxide, which is metabolized in the intracellular compartments by selenium dependant GPX .the increased activity of erythrocytic SOD in anoestrus heifer could be attributed to physiological upregulation of this enzyme in an attempt to diminish the superoxide radical challenge. Supplementation of vitamin E & selenium might be responsible for relieving the load of oxidative stress in anoestrus heifer, thus lowering the erythrocytic SOD activity in anoestrus heifers supplemented with vitamin E and selenium.

Erythrocytic activity of G6PD decreased significantly in supplemented group with vitamin E and selenium. A number of species can upregulate the activity of G6PD in an attempt to mitigate the effects of peroxidative challenge. Supplementation of vitamin E and selenium has lowered the erythrocytic G6PD along with decrease in lipid peroxidation thus relieving the oxidative stress, which was observed earlier too in postpartum anoestrus buffaloes supplemented with vitamin E and selenium (Anita et al., 2004). Activity of GPX in erythrocytes increased significantly in supplemented group. Selenium and vitamin E used separately are able to combat the oxidative stress; used together, they are even more efficacious. Nutritional supplement containing vitamin E, iron, zinc, selenium had significant increase in ovulation and pregnancy rates (Agarwal et al., 2005). Fertilization and embryo development in vivo occurs in an environment of low oxygen tension during ART , it is important to avoid embryos to ROS. During culture, low oxygen tension is more effective at improving the implantation and pregnancy rate. Amino acids added to the IVF media also have antioxidant properties. Adding ascorbate during cryopreservation reduces hydrogen peroxide.

Conclusion

Oxidative stress and free radicals have important roles in modulating many physiological functions in reproduction. Reference values of ROS, minimum safe concentration or physiological beneficial concentration have yet not been defined. Most of the published studies on oxidative stress are either observational or case control studies. Treatment strategies of antioxidant supplementation directed towards reducing OS may be advised when specific aetiology cannot be identified as in idiopathic infertility. Thus supplementation of selenium along with vitamin E can be employed successfully to improve antioxidant status as well as reproductive performance in critically ill dairy animals suffering from parturient complications.

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Endocrinological interventions to augment livestock fertility

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Livestock serves as one of the main pillars of India's agrarian economy, food and nutritional security and livelihood. Its ownership is highly egalitarian and the growth potential is highly pro-poor. India possesses the highest cattle population of around 199 million in the world (15% of the total world's cattle population). Buffaloes with 105 million population form a third of the total cattle and buffalo livestock bovine population and contribute more than 55% of the milk. Farmers prefer buffaloes over cattle, particularly in Northern and North-West India as it is a triple purpose animal contributing milk, draught and meat as compared to the dual purpose native cattle. The buffalo milk also fetches higher price to the farmer due to higher fat content. It can utilize poor quality roughages and is capable of adjusting to wet conditions better than cattle.

Sheep and goat are also important livestock species in India, especially in the arid/semi-arid and mountainous areas. While sheep are mostly reared for wool and meat, goat provide both milk and meat. Backyard pig farming systems is also practiced as part of the mainstream farming in Kerala, Goa, North-Eastern States and by socially weaker sections and tribals in Jharkhand solely for meat. The domestic demand for livestock products is going to increase substantially in the years to come. Additionally, there is a good export potential for livestock products. In order to meet the domestic and export demand the production from livestock sector need to be targeted for rapid growth.

Good reproductive performance is essential for efficient livestock production. Livestock improvement programs should aim to increase reproductive efficiency to the extent that this can be justified economically. The females must grow rapidly to attain sexual maturity, initiate estrous cycles, ovulate and be mated by fertile males or inseminated with viable semen at the proper time for producing offspring. Improved buffalo and zebu cattle production in particular, could significantly enhance the economy and living standards of farmers in India. There are at least 30 different zebu breeds in India. Sahiwal, Red Sindhi, Gir, Kankrej and Tharparkar are predominant dairy breeds. The average lactation yield of these breeds is around 1800 litres in a 305 days lactation period. However, most of the cattle in India are of the nondescript types which yield very little milk. In an effort to increase milk production, cross breeding of zebu with exotic breeds has been carried out. The average milk production over a 305 days lactation period of milch buffaloes ranges from 1500 – 2500

litres. Better adaptation of buffalo and zebu to tropical climates and disease resistance ensures their place in the future of world agriculture facing the challenge of global warming due to climate change. Perusal of Livestock Census of 1992, 1997 and 2003 reveals that there has been a significant decline in cattle population from 204.58 million in 1992 to 198.88 million in 1997 and 185.13 million in 2003. Interestingly, there has been a consistent increase in buffalo population from 84.21 million in 1992 to 105.34 million in 2007 indicating preference for buffalo rearing among farmers. With an overall 127 million tonnes of milk production in 2011-12 from cattle, buffaloes and goats and a per capita milk availability of 290 g/day the Indian Dairy scenario is constantly looking ahead and promises to take greater strides in making Dairying more remunerative to the farmer. However, there are serious bottlenecks in our quest for making livestock rearing a profitable venture. An important issue is flagged under.

Anestrous and repeat breeding in buffaloes and bovines are two of the most serious reproductive problems affecting 30-40% of the total cattle and buffalo population. On a conservative estimate the country is losing 20-30 million tonnes of milk annually on account of anestrus and repeat breeding in cattle and buffaloes which translates to a loss of nearly Rs. 50000 crores annually. At a micro level, each missed heat is a missed opportunity. For each heat missed the farmer incurs a loss of milk production of 21 days, in addition to bearing the feeding cost for animal maintenance. This tantamounts to about Rs. 3300. Artificial insemination (AI), which is a normal practice in cattle, is not as successful in buffalo, especially in hot summer months, because of the weakness of oestrus symptoms and the variability of oestrus length, which make oestrus detection very difficult. The usual weak symptoms of estrus in the normal breeding season (September to February) become even weaker during hot months of summer (Prakash 2002). The incidence of silent heat among buffaloes was lowest in December (10.5%) while the peak was seen in the hot summer month of April (70%; Prakash et al. 2005). Failure to detect estrus and time of onset of estrus in buffalo considerable percentage of oestrous cycles are left uncovered resulting in increase of unproductive period which adversely affect economics of livestock production.

Several studies have attempted to understand the reproductive physiology of buffalo and the factors affecting its behaviour. These have been adequately reviewed (Madan and Prakash, 2007). During the last two decades, considerable attention has been focussed on reproductive endocrinology, with the aim of developing models to improve reproductive efficiency, particularly when using controlled breeding techniques.

The basic goal of all production oriented animal reproduction techniques is to increase the proportion of females that have oestrous cycles and get pregnant in a reasonable time. The

important fact is that the increase of animal production over the last two decades has been mainly due to the increase in the number of livestock, while productivity per head has generally remained static. This is in my opinion an alarming fact, especially when we consider that it will probably be very difficult to reduce human population growth to any extent in the near future. India has the highest population of bovines and buffaloes in the world. For every four human beings there is a cow or a buffalo also occupying land space and resources as well. We cannot continue in the future to increase livestock numbers parallel to human population growth. The arguments behind this are that animal production is expensive. About three to ten times the amount of energy or protein of plant origin is needed to produce protein or energy of animal origin.

More than 80% of our bovine population is still non-descript and the breed improvement programs have hardly taken off. Conception rate for artificial insemination (A.I.) is very poor. A.I. facilities are not available at the farmers' doorsteps. Semen available for A.I. is not of required quality. There is shortage of qualified para-veterinarians. We have learnt a lot from our past failures. Farmers must be provided incentives to use A.I. program. An A.I. program should always be an essential part of an integrated livestock development programme for obtaining high yielding livestock, and hence, should never be implemented on its own.

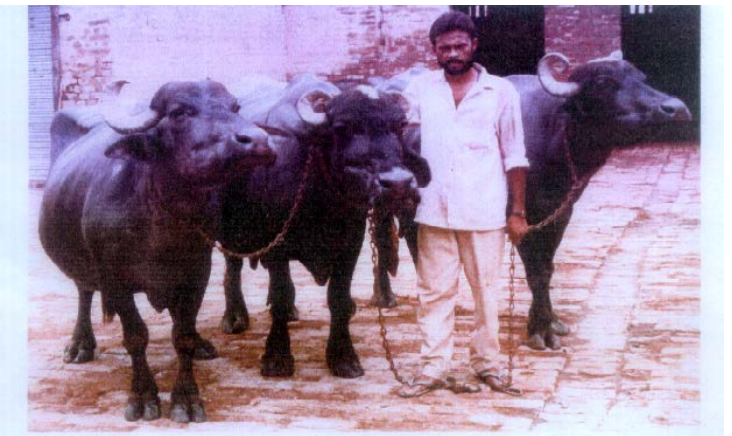
Reproductive technologies

The various potential reproductive technologies, which hold promise, are listed below.

Field application of progesterone determination for fertility augmentation: Since progesterone is secreted from the corpus luteum, determination of progesterone in body fluids such as plasma and milk is a good marker for determining the functional status of tissue. Of the steroid hormones known to be synthesized by the bovine ovary, it is the varying concentrations of progesterone in body fluids which has up to present yielded most information of ovarian functions. Progesterone determination in plasma or milk can therefore serve as a valuable diagnostic tool in buffaloes for:

- Accurate estrus confirmation and hence correct timing of A.I.
- Diagnosis of pregnancy/non-pregnancy 20-24 days post A.I.
- Identifying ovarian conditions such as acyclicity, silent heat, and cystic ovarian disorders.
- Control of certain biotechnological manipulations like embryo transfer, estrus synchronization, parturition induction etc.

Suitable treatment can be administered for anestrum, repeat breeding and cystic ovarian conditions, if the animals are systematically monitored for ovarian activity by milk or blood plasma progesterone determinations.



Three anestrus buffaloes belonging to Sh.Suba Singh, resident of village Phusgarh, Karnal District which were monitored by milk progesterone assay and were subsequently treated for successful pregnancies.

Pregnancy confirmation through oestrone sulphate determination: Oestrone sulphate has been found to be quantitatively one of the major oestrogens in the milk and blood plasma of pregnant, lactating cows and buffaloes. During the first half of pregnancy its concentration increases gradually in these animals so that after 110 days of pregnancy it is present in all milk samples taken from pregnant cows and buffaloes, whereas it is low or undetectable in non-pregnant animals. These results adequately suggest the practical applicability of using estrone sulphate estimations in body fluids (milk or blood) for confirmation of pregnancy and fetal viability in bovines after 110 days post-insemination since it is low or undetectable in non-pregnant animals. Under normal circumstances a pregnant cow exhibits an exponential increase in estrone sulphate levels in blood and milk with advancing pregnancy which is indicative of fetal viability. Undetectable estrone sulphate concentrations in milk during second trimester or advanced pregnancy could indicate embryonic loss, fetal death or mummification of fetus.

Estrous behavior: Reproductive efficiency among large ruminants is greatly dependent upon the detection of estrus. This is even more important with reference to small herds managed under tropical or subtropical environments because high air temperatures shorten the duration of estrus and lower its intensity as demonstrated under controlled environments in cattle. The intensity of estrous behavior in tropical buffaloes has been found to be much less than cows. The usual weak symptoms of estrus in the normal breeding season

(September to February) become still weaker during the hot months of summer. Among Murrah buffaloes we have recorded diurnal patterns of estrous behavior with 59% of estruses detected between 10pm and 6am. The maximum occurrences of various heat symptoms were seen in the winter months of November to February while the lowest occurrences were during March to August in a selected group of buffaloes observed throughout the year. Out of the 8 major symptoms of estrus, 5 symptoms that are, vulval engorgement, frequent urination, bellowing, bull mounting and restlessness contributed to 85 percent of the total observations. Mucus discharge, licking of the female by the bull and chin resting by the bull were minor symptoms. During the summer months frequent urination was the most prominent heat symptom recorded.

In another study, the incidence of silent heat occurrences throughout the year was determined in buffaloes by milk progesterone monitoring with the objective of studying the influence of changing environmental temperatures on heat occurrences. Out of a total of 292 estruses detected by milk progesterone monitoring 108 estruses (37%) went unobserved. The incidence of silent heat was lowest in December (10.5%) while the peak was seen in April (70%). There was a gradual decline in incidence of silent heat occurrence from May onwards. Due to the high incidence of silent heat, large numbers of buffaloes are left unbred and contribute substantially to a high service period in this animal (139 days among 89 buffaloes in this study).

Season of calving had a profound influence on the service period. We observed that the mean service period of animals calving from December till June was more than 140 days and was significantly higher than mean service period of animals calving in the months of July to November (<110 days). The high service period of buffaloes in the former group of animals was attributed to the high incidence of silent estrus, which the animals would exhibit in the summer months once they commence cycling postpartum. The effect of different seasons on both the resumption of ovarian activity and embryo survival may be a function of temperature and/or photoperiod.

Timing of Insemination, Estrus Synchronization and Timed A.I

We investigated the timing of ovulation following spontaneous estrus which information is essential for correct timing of AI in buffaloes. On the basis of our observations we also suggest the following A.I. timings in buffaloes exhibiting spontaneous estrus.

Timing of Insemination in buffalo

- Double Insemination
- First Insemination – 24 h after onset of heat.
- Second Insemination – 36 h after onset of heat.

Scientists the world over are now working on developing new estrus synchronization protocols which can reduce the ovulation time window post synchronization so as to practice insemination at a fixed time thereby obviating the need for heat detection which is a serious problem especially in buffaloes. an estrous synchronization protocol (ovsynch protocol) in cattle has been developed recently; it makes the use of a combination of GnRH - $PGF_{2\alpha}$ - GnRH injections which has been reported to considerably narrow down the ovulation time to a range of 24 hours to achieve the maximum conception rate with set time artificial insemination.

Ovsynch

Our innovative research work has yielded a promising technology which could help augment fertility in buffaloes by bringing anestrus animals into cyclicity and/or making them pregnant. This technology named ovsynch protocol involves the combination of GnRH-$PGF_{2\alpha}$-GnRH injections regime to synchronize estrus and ovulation. An injection of GnRH (receptal; 10µg intramuscularly) is administered at a random stage of estrous cycle (or anestrus or repeat breeding condition) followed by an injection of $PGF_{2\alpha}$ (lutalyse; 25mg intramuscularly) 7 days later. Ovulation is synchronized by a second injection of GnRH (receptal; 10µg intramuscularly) given 2 days after $PGF_{2\alpha}$. The animals are then inseminated at a fixed time of 12 h and 24 h post second GnRH injection. The first injection of GnRH induces ovulation of dominant follicle and causes emergence of a new follicular wave. The $PGF_{2\alpha}$ injection induces regression of the spontaneous and / or GnRH induced *corpora lutea,* and the second GnRH injection synchronizes the time of ovulation of the dominant follicle of the follicular wave that began growing after the first GnRH injection. Buffaloes are subjected to set time artificial insemination after synchronized ovulation. The animals are inseminated if they do not settle and return to estrus during the subsequent cycle.

This technology viz. ovsynch protocol for estrus synchronization and set time A.I. was initially successfully demonstrated in cycling (Paul and Prakash, 2005) and non-cycling anestrus/repeat breeding buffaloes (Roy & Prakash, 2009a, 2009b) in the NDRI farm. Subsequently in association with the KVK of the institute the technology has also been tested on village buffaloes. Initially a limited preliminary field trial conducted in the villages, 24 buffaloes became pregnant out of 60 (40%) while another 28 anestrus buffaloes became cyclic (47%) thus making a healthy 87% positive response for augmenting fertility among anestrus buffaloes. In another exhaustive trial on village buffaloes we obtained 67 pregnancies out of 131 buffaloes while another 38 anestrus buffaloes becoming cyclic giving an overall succeed rate of 87% again (prakash et al. 2008).

Cost of treatment: the cost of ovsynch treatment (calculated for the second trial on 147 buffaloes of which data was available from 131 buffaloes) was Rs 520 per animal (cost of drugs Rs 450 + 15% miscellaneous expenses which includes petrol, labour, and disposables etc). Total expenditure incurred in the project for the treatment of buffaloes was Rs 76,440. On an average, the buffaloes which became pregnant were non-pregnant for 276 days before treatment. Assuming a feeding cost of Rs 50 per animal per day (this study is a few years old) the total loss to the farmers was Rs 9.2 lacs. Assuming an average production of 5 litres of milk/d, the milk production loss incurred by farmers amounts Rs 16.65 lacs taking the total losses to the farmers to Rs 25.9 lacs. Hence the cost benefit ratio of technology works out to be 34 times without taking into account the loss of calves and labour costs during this period.

Procedure: Ovsynch protocol involves the combination of GnRH - $PGF_{2\alpha}$ - GnRH injections regime to synchronize estrus and ovulation. An injection of GnRH (Receptal; 10µg intramuscularly) is administered at a random stage of estrous cycle (or anestrus condition) followed by an injection of $PGF_{2\alpha}$ (Lutalyse; 25mg intramuscularly) 7 days later. Ovulation is synchronized by a second injection of GnRH (Receptal; 10µg intramuscularly) given 2 days after $PGF_{2\alpha}$. The animals are then inseminated at a fixed time of 12 h and 24 h post second GnRH injection. The first injection of GnRH induces ovulation of dominant follicle and causes emergence of a new follicular wave. The $PGF_{2\alpha}$ injection induces regression of the spontaneous and / or GnRH induced *corpora lutea,* and the second GnRH injection synchronizes the time of ovulation of the dominant follicle of the follicular wave that began growing after the first GnRH injection. Buffaloes are subjected to set time artificial insemination after synchronized ovulation. The animals are inseminated if they do not settle and return to estrus during the subsequent cycle.

Heatsynch

During the last few years the estrus synchronization protocol called heatsynch in buffaloes has been developed which makes use of a combination of GnRH-$PGF_{2\alpha}$-Estradiol benzoate injection followed by fixed time AI. Estradiol benzoate is a less expensive hormone in place of second GnRH injection of Ovsynch protocol. The major advantages of Heatsynch are reduced hormone costs and somewhat easier scheduling and implementation, since all injections and A.I. are at 24 and 48 h interval in cows. We investigated the efficacy of Heatsynch protocol in buffaloes in summers and winters and evaluated the interrelationships of hormones (progesterone, estradiol and LH) during the critical periovulatory (Mohan et al. 2009, Mohan and Prakash, 2010). After making detailed laboratory investigations we examined its potential for ameliorating infertility in buffaloes belonging to farmers' herds. To study the feasibility of heatsynch protocol application for fertility improvement in buffaloes belonging to farmers' herds, trials were conducted on

anestrus buffaloes in 11 villages of Karnal district in collaboration with the KVK of NDRI. The buffaloes were selected on the basis of being at least six months anestrus or repeat breeders (anestrus or repeatbreeding ranging from 6 months to 2 years or more). The animals were treated as per the protocol and inseminated twice. In the 11 trials conducted in a total number of 285 buffaloes in different villages the results obtained through Heatsynch protocol were very encouraging in terms of enhancing fertility. The success rate in terms of pregnancy establishment was 48%. It is pertinent to mention here that the heatsynch protocol has been initiated after a thorough basic research on buffaloes at the Institute level involving studies on endocrine changes, timing of ovulation and success rate in the farm animals in summer & winter subjected to heatsynch protocol on an experimental scale.

Doublesynch

Studies on dairy cows have demonstrated that the success rate of the Ovsynch and Heatsynch protocol is dependent on the estrous cycle stage at the onset of the protocol. For example, the intiation of the Ovsynch protocol between days 13 and 17 or early in the estrous cycle (days 2–4) led to a reduced pregnancy rate. Other studies have established that GnRH induced follicular turnover or induction of a new follicular wave is the most efficient if ovulation is induced in response to the first GnRH treatment and that resetting the follicular development. can produce a new dominant follicle containing an oocyte with greater potential fertility.

Several strategies employing hormonal treatment before initiating the Ovsynch protocol have been utilized to minimize the proportion of cows in the above-mentioned problematic stages and to maximize the proportion of cows in favorable stages of the estrous cycle. However, these strategies have several disadvantages:

Either pregnancy rates are limited, or the protocol requires a long period of time to complete. Recently, Cirit et al. (2007) developed a new synchronization method that includes the administration of an additional $PGF_{2\alpha}$ injection 48 h before beginning the Ovsynch protocol. They named this new protocol the Doublesynch (the abbreviation of double synchronization) protocol, as it resulted in synchronized ovulation after both the first and second GnRH treatments. Öztürk et al. (2010) confirmed the pregnancy rate success (increased by 43% compared with the Ovsynch protocol) of the Doublesynch protocol in both cyclic and anestrus cows. In a study conducted on buffaloes Mirmahmoudi and Prakash (2012) the following results were obtained:

The pregnancy rates were 60% using TAI following doublesynch application on cycling buffaloes, 55% for anestrus buffaloes, in comparison to 27.3% for cycling buffaloes inseminated following spontaneous estrus. The overall pregnancy success rates after the

Doublesynch protocol in both cycling and anestrus buffaloes increased by 30.8% compared to spontaneous estrus (58.1% vs. 27.3%). The study demonstrated that the Doublesynch protocol followed by TAI significantly ($P < 0.005$) enhanced the pregnancy rate in cycling and anestrus buffaloes in comparison to untreated controls during the low breeding season (summer).

Final Remarks: Scope for scientist-industry interaction for improving productivity

Although several techniques have been developed for enhancing production and reproduction in livestock unfortunately most of these have not yet been adopted at the field level in a big way which is imperative if they are to make an impact towards increasing milk production and thereby enhance farmers' incomes. One main reason for research efforts not reaching the farmers doorsteps is the lack of interaction between farmers, scientists and the industry. Till date, there are no known indigenous manufacturers of hormonal drugs for therapeutic applications as described in this text. While the knowledge is available to produce them, effective mechanisms need to be set in motion to ensure an effective dialogue between the scientists and industry to produce them indigenously.

Uses of Diagnostic Haematology and Cytology in Reproductive Tract Diseases

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Diagnostic Haematology

Hematology is the study of formed blood elements including erythrocytes, leukocytes and thrombocytes. Hematologic testing essentially consists of ascertaining the relative number (or amount), size, and volume of the respective cellular elements in the blood. This seemingly simple information can yield critical information about disease states, including those affecting the reproductive system of animals.

RBC: The RBC or "red cell count" may be expressed as the number of erythrocytes per cubic millimeter (mm^3 or mL) of plasma. This measurement is made by examining a blood sample on a grid under a microscope and literally counting the number of cells over a given area. A typical, normal value for a healthy adult is anywhere between 4.1 million and 5.6 million cells/mL.

The RBC may be diminished or elevated in certain disease states. Obviously, if there are bleeding disorders or other loss of blood elements, the RBC will be reduced. This is referred to clinically as anemia. There are numerous etiologies in anemia; most commonly these include loss of blood (e.g. trauma, surgery), nutritional deficiencies, or bone marrow dysfunction. In cases where there is an excess of red blood cells, such as in certain malignant bone marrow disorders, the appropriate term is polycythemia. Both of these conditions can have ocular manifestations.

Hb: The Hb is a direct measurement of the hemoglobin in a sample of blood and is indicative of the overall volume of erythrocytes. To perform this test, the red blood cells in the sample are lysed and the liberated hemoglobin is measured spectrophotometrically.
Levels of hemoglobin are important in diagnosing certain anemias and polycythemias. Typically, the Hb value follows the RBC value, but it is possible for these to be inconsistent in certain hematologic disorders. Normal hemoglobin values are generally between 12 and 18 g/dL.

MCV: The mean corpuscular volume represents the average size of the red blood cells within a sample. Again, this is typically a calculated measurement today, rather than a direct observation [MCV = (Hct/RBC) X 1,000]. This test yields a great deal of information

when taken in context with other hematologic findings. The MCV may be altered in various disorders. For example, it is elevated in folic acid deficiency, vitamin B12 deficiency, hemolytic anemia, cirrhosis of the liver, or chronic alcoholism. When the MCV is high, we say that the condition is "macrocytic", indicating larger-than-average erythrocytes. The MCV may also be diminished in some patients – this is representative of a "microcytic" disorder, or a situation in which smaller-than-normal erythrocytes prevail. Conditions in which the MCV is decreased include chronic iron deficiency. Less commonly, a low MCV may be indicative of severe chronic infection, polycythemia, or lead poisoning. Occasionally, the MCV may be normal even though other findings (such as the RBC or HgB) are abnormal. This further helps to define "normocytic" anemia, such as is seen in renal failure or acute blood loss. The MCV is a very small value, as one might imagine, and is measured in cubic micrometers or units called femtoliters (fL). Normal values are generally between 80-100 fL.

MCH: The mean corpuscular hemoglobin is an estimate of the amount of hemoglobin present in an average red blood cell. Like MCV, the MCH is calculated from other CBC values [MCH = (HgB X 10)/RBC]. Because the MCH tells us about the amount of hemoglobin pigment in the cells, it helps us to differentiate "hypochromic" anemias (i.e. those with low MCH values) from "normochromic" and "hyperchromic" disorders (i.e. those with high MCH values). Examples of hypochromic anemias include iron deficiency and thalassemia, while hyperchromic states are noted in folic acid or vitamin B_{12} deficiency. Hypochromic anemias are also often microcytic, whereas hyperchromic conditions are commonly macrocytic. The MCH is measured in picograms, which is one trillionth (10^{-12}) of a gram. Normal values for this test are roughly between 25 and 35, depending on the laboratory.

MCHC: The mean corpuscular hemoglobin concentration is very similar to the MCH, however the MCHC describes the proportion of hemoglobin in an average erythrocyte, rather than the absolute value. It is calculated by the following equation: [MCHC = (HgB X 100) / Hct]. The MCHC is expressed as a percentage, with normal values between 31 and 36%.

Platelet Count: The platelet count represents the number of thrombocytes, or platelets, per cubic millimeter of blood plasma. There are normally between 150,000 and 450,000 platelets in each microliter of blood. Low platelet counts denote a condition known as thrombocytopenia, which is typically associated with bleeding disorders. High platelet counts define thrombocytosis, usually indicative of bone marrow disorders but sometimes secondary to infections, cancer, or splenectomy.

WBC: The WBC or "white cell count" is expressed as the number of leukocytes per cubic millimeter (mm^3 or mL) of blood plasma. Most healthy adults have between 4,000 and 11,000 cells/mL. By convention, the WBC is expressed in units of 1,000. Therefore, doctors may refer to this value as (for example) "5.5," which is normal, or "16.2," which is elevated. When the white cell count is elevated, we describe this clinically as leukocytosis. Leukocytosis may occur in a variety of disorders, however we generally think of infectious or autoimmune disease in patients with a high WBC. Leukemia, a malignant proliferation of white blood cells, also presents with a grossly elevated white count. A diminished WBC is expressed clinically as leukopenia. Leukopenia generally results from immune deficiencies (e.g., AIDS) or from bone marrow disorders in which insufficient white cell production occurs. Aplastic anemia is one such condition (red cells and platelets are also reduced in this disorder).

Differential: Most laboratories typically also perform a "Diff," or WBC differential count, as part of the CBC (some labs may however require that the ordering physician specify this on the Rx, e.g., "CBC w/ Diff"). This important test helps to identify the various types of white cells present in the blood. Two different measurements are reported in the Diff – a percentage (%) and an absolute value (cells/mm3). In general, there are five distinct forms of white blood cells, each of which perform a different function and is implicated in different conditions. These include:

- Neutrophils, also known as polymorphonuclear leukocytes (PMNs), are the most prevalent form of white blood cell. They are macrophages that selectively seek out and destroy invading organisms, most commonly bacteria and other single celled pathogens, such as Chlamydia. In a normal patient, neutrophils comprise about 50-70% of the white cell count, or 2000 to 7500 cells/mL. An elevated Poly count usually suggests a bacterial infection.

- Lymphocytes are the second most common form of white blood cell. There are two main types of lymphocytes. B-lymphocytes are responsible for binding antigens and producing specific antibodies. T-lymphocytes help recruit other white cells (macrophages) to infection sites, attack virus-infected and possibly tumor cells, and also help enhance the production of antibodies by B cells. Lymphocytes normally constitute 20-40% of all white cells, or 1000 to 4000 cells/mL. Lymphocyte counts are elevated in cases of viral infection, pronounced allergic or toxic reactions, and also in leukemia.

- Monocytes are derived from the cells that line vascular and lymphatic channels (reticuloendothelial cells). Their function is to phagocytize dead or damaged cells, as

well as those organisms not suppressed by neutrophils or leukocytes. About 3-7% of white cells are monocytes. Normal absolute values range from 100 to 1000 cells/mL. Monocytes may be elevated in acute bacterial and viral infections, as well as in chronic infections such as malaria or tuberculosis. The count may also be abnormally high in connective tissue disorders including rheumatoid arthritis, sarcoidosis, and inflammatory bowel disease.

- Eosinophils are involved in both allergic disorders and parasitic infestations. These white cells contain numerous toxins that are effective in neutralizing parasites; unfortunately, mast cell degranulation also stimulates eosinophil chemotaxis, and the toxic chemical barrage is launched against innocent bystander tissue as part of the allergic response. Ordinarily, eos comprise about 1-3% of the total white count, from zero to 400 cells/mL in the normal patient. The eosinophil count is characteristically elevated in allergic rhinitis, atopy and asthma, as well as atypical infections such as trichinosis, toxocara and leprosy.

- Basophils are the least prevalent of the white cells, representing <1% of the total WBC. They function in an auxiliary capacity to mast cells in allergic responses, essentially releasing similar mediators (e.g. histamine, bradykinin) and spurring production of pro-inflammatory cytokines. Normal basophil counts range from zero to around 200; elevated levels are seen in allergic disease as well as some viral infections (e.g. Varicella), some inflammatory diseases (e.g. inflammatory bowel disease), and Hodgkin's Lymphoma.

Factors influencing DLC: Age (calf in 1st week -low levels of lymphocytes and higher neutrophils, swine at birth 70% neutrophils, young dog more lymphocytes), breed (cattle, sheep, goat and pig lymphocytes always more), exercise (neutrophilia), emotional state (neutrophilia).

Shift to left: Excess number of young forms of the granulocytic series cells in peripheral circulation.

Regenerative Shift to left: TLC elevated above normal, associated with immature forms of neutrophils in the peripheral circulation.

Degenerative Shift to left: TLC normal or below normal, associated with below normal number of mature neutrophils and the presence of immature neutrophils, many of which show toxic changes.

Shift to right: Abnormal number of hyper segmented neutrophils or aged cells

Diagnostic Cytology

Veterinary practioners are faced with difficulties in diagnosis of diseases in day-to-day working. The challenges in diagnosis are usually to achieve accuracy, cost and speed and these objectives can be achieved by diagnostic cytology particularly in complex reproductive disorders.

Diagnostic cytology is the study of cellular changes for disease diagnosis or to determine the etiology or studying bodily responses. Cytological specimens are collected in different ailments like cutaneous and subcutaneous lesions, enlarged lymph nodes, suspected splenic or hepatic pathology, body cavity effusions, upper and lower respiratory tract diseases, synovial fluid analysis, cerebrospinal fluid (CSF) analysis, urogenital diseases like prostatic/kidney aspirates, urine sediment examination, staging of oestrus cycle e.g. vaginal cytology, bone marrow evaluation. In addition cytology is also indicated whenever diagnosis cannot be made on clinical examination, radiographic evaluation and ultrasonography.

Diagnostic cytology is a very useful tool for rapid diagnosis of neoplastic and non-neoplastic diseases /conditions. The technique bridges the disciplines of Pathology, Immunology, Histology and Microbiology. The cytological interpretation is valuable in establishing a diagnosis, identifying the diseases process, directing therapy, predicting the prognosis and determining the procedures /interventions to be followed next. The technique poses little risk to the patient and is almost non-invasive, therefore stands a great scope for diagnosis and prognosis of animal diseases in the laboratory as well as at field level. By augmenting the cytology with modern biomolecular methods, further improvements in diagnostic efficacy and in understanding the intricate disease mechanisms or identifying subtle or scarce etiologies can be precisely achieved. Cytologic evaluation can also be correlated with other clinical or laboratory findings /data, so as to arrive at definitive disease diagnosis and tracking down the epidemiology of diseases and their forecasting.

However, the cytological technique for the diagnosis of disease(s) in Veterinary practice is in preliminary stage in India, and therefore stands a great scope for further expansion.
Since the mid-1960s, when the first reports of cytology appeared in the veterinary literature, cytology has become an extremely useful diagnostic aid in veterinary medicine. Cytology has many advantages over histopathology:

- Cytology samples can be easily obtained pre-operatively, often without general anesthesia and sometimes even without sedation, and can be used to screen patients for more comprehensive diagnosis.
- Fine needle aspiration cytology is less costly than surgical biopsy in both sample collection and laboratory analysis.

- The fine needle aspiration procedure is less likely to result in adverse effects when compared to tissue biopsy.
- Because less sample processing is required, cytology results are available sooner than histopathology results.
- 'Quick' checking for recurrence of local malignancies or regional lymph node metastases.
- Pathologic micro-organisms involved in microbial infections of various organs (e.g., canine and feline leprosy, subcutaneous mycoses, bacterial prostatitis) diagnosed initially by cytology have a greater chance of being cultured successfully.
- Techniques are being developed whereby the aspiration of neoplastic lymphoid cells from suspected malignant lymphomas can be immunocytochemically phenotyped into T & B cell populations either directly from FNAC smears of lymph nodes or via flow cytometry – thereby avoiding costly and sometimes contraindicated general anaesthesia to perform an incisional/excisional surgical biopsy.
- Cytology aspiration of particularly 'hard to get to' organs (e.g., pancreas, heart base), can be successfully sampled with the use of ultrasound guided techniques.

Limitations: However, cytology also has its limitations. As the cells/material being evaluated are 'outside' their normal environment, an assessment of cellular organisation, arrangement or architecture is often not possible by cytology. Therefore, adequate sample collection and preparation is of considerable importance when it comes to cytological interpretation, as this will provide the pathologist with as much information as possible on which to base an interpretation.

Collection tips and advice for cytology specimens by fine needle aspiration cytology

- Avoid blood contamination. Possibly use the non-aspiration needle biopsy technique for soft, highly vascular and small lesions.
- Do not prolong the period of aspiration (should take less than 30 seconds) and make smears immediately after collection to optimize cell preservation.
- Attempt 2-3 separate collections (if the lesion/mass is large enough).
- Make 2 slides from each collection (see preparation of slides).
- If cell yield appears poor via FNAB, use a larger needle and syringeand/or increase the amount of negative-pressure within the syringe.
- Material within the hub of the needle is usually sufficient and further sampling often results in unwanted blood contamination.
- Should blood appear in the syringe during FNAB, stop the procedure immediately and start again with a new needle and syringe.

Preparation of Slides

The aim of slide preparation for cytological evaluation is to achieve a monolayer of well-preserved cells. This can be achieved by several methods:

Squash Preparation (A misnomer?)

This is the preferred method for preparing slides from needle biopsies, FNAB and scrapings. Material collected by fine needle biopsy or scraping is placed towards one end of a 'frosted edge' glass slide (the region adjacent to the frosted region of a slide is preferred). A second slide is aligned perpendicular to the first and is allowed to rest on the slide containing the expelled material. This slide is then gently and smoothly drawn over the length of the first slide whilst concurrently rotating them from a perpendicular to a parallel position. This results in the simultaneous preparation of two smears. Avoid physically squashing the material, which causes excessive pressure, leading to cell rupture and a non-diagnostic preparation. Often the fluid material will 'suck' the second slide down onto the first and together with the weight of the second slide, further downwards pressure is not required.

Methods for preparing cytological smears

a) Needle/Starfish Preparation: Material collected by fine needle biopsy is placed in the center of a glass slide and the needle is used to drag/tease the material outwards – in multiple directions - to produce a star/starfish shaped smear with multiple projections. Many areas of the smear will be too thick for evaluation, however, there are usually multiple cell monolayer regions present on the smear that should be acceptable for cytological assessment.

b) Blood Smear Technique: Aspirated material may contain enough blood and/or liquid to allow smearing of the material in a similar fashion to that used to make a peripheral blood smear. The material is expressed towards one end of a 'frosted edge' glass slide (again, the region adjacent to the frosted region of a slide is preferred) and the short edge of the spreader slide is placed in front of the sample. The spreader slide is tilted to an angle of approximately 45 degrees, pulled backwards into the material and once the material has dispersed along the width of the spreader slide, the spreader slide is smoothly, steadily and rapidly slid forward. The smear ends with a feathered edge of material. As a general rule, the more material placed on the specimen slide, the slower the spreader slide is slid forward and the more acute the angle between the spreader and specimen slide, the longer the smear will be.

Collection and preparation of slides from fluid samples

Fluid samples are obtained from sampling body cavity effusions, cysts, joints, cerebrospinal fluid, seromas, urine and when performing various types of washes (e.g., bronchoalveolar lavage, transtracheal wash). Volume permitting, fluid aliquots should be collected into EDTA-containing tubes and sterile/plain tubes. Smears should also be made at the time of sampling. EDTA prevents coagulation and therefore allows for accurate cell counts to be performed when required. EDTA helps preserve cell morphology during transit to the laboratory, however, morphology is best preserved by making smears at the time of sampling. This is particularly true with respect to urine cytology as prolonged contact with urine often causes severe cellular swelling and degeneration. Making smears at the time of sampling also helps better determine the relevance/significance of certain cytological features such as erythrophagocytosis and the presence of intra-cellular bacteria (phagocytosis of both red cells and bacteria by leukocytes may occur post collection during transit). EDTA is bacteriostatic and culturing from EDTA-containing fluid is generally not advised. Concurrent submission of a plain fluid sample collected into a sterile container, allows for culture to then be performed if initial cytology indicates that this may be warranted.

Smears from cloudy, highly cellular, well-mixed fluids can be made directly via the blood smear technique or line smear technique. If there are any floccules of particulate matter grossly visible in the fluid at the time of collection, then these should be included in the smears as well. Aliquots of clear or slightly turbid fluids should be concentrated via centrifugation to increase the cellularity of prepared smears. Following centrifugation, the majority of the supernatant is decanted, the pellet (cellular material) is then resuspended in the minimal remaining supernatant, and smears are made via either the blood smear or line smear techniques. The line smear technique is similar to the blood smear technique; however, the spreader slide is abruptly stopped and lifted off the specimen slide prior to creating a feathered edge, resulting in a higher concentration of cells present in the terminal line, than within the remainder of the smear.

Interpretation of cytologic specimen

Epithelial tumors exfoliate relatively easily and the smears prepared from fine needle aspirates and/or impressions are moderately cellular with cells organized in groups or clusters. These cells from epithelial tumors are round to columnar or squamous and have distinct and sharply defined cytoplasmic borders and, at times clearly showing cell-to-cell junctions. Cytologic diagnoses are made for most of these tumors on the basis of typical cytomorphology.

Cluster of cells showing asynchronous nuclear and cytoplasmic differentiation with occasional big cells with small perinuclear vacuoles, tadpole cells and phagocytic squamous cells are the diagnostic cytologic findings in squamous cell carcinoma. Basal cell tumor is diagnosed by the characteristic clumps of cells composed of small and uniform epithelial cells forming cords or ribbon like arrangement. Basosquamous cell carcinomas are characterized by 2 different cell populations resembling cells of basal cell as well as squamous cell origin. Hepatoid gland adenomas are diagnosed with the distinctive cytomorphology of large, round to polyhedral and hepatocyte-like cells, occurring in groups.

Unlike epithelial tumors, FNAB smears prepared from most of the mesenchymal tumors are poorly cellular. Cells from most mesenchymal tumors are spindle shaped, with cytoplasmic extensions, besides ill defined cytoplasmic borders. The cytomorphology is found efficient for the definitive diagnoses of lipomas, liposarcoma, osteosarcoma, fibrosarcomas, leiomysarcoma and hemangiopericytoma. Cytologic diagnosis of lipoma is based on the characteristic oily aspirates, and the presence of large cells with eccentric nucleus and clear cytoplasm outlined by a thin membrane. The characteristic findings of osteosarcoma are the presence of flame-shaped cells with highly basophilic moderate cytoplasm, prominent Golgi zone, and multinucleated giant cells or osteoclasts. The distinctive cytologic features of liposarcoma are the presence of well exfoliated polyhedral or roundish cells with abundant lightly basophilic cytoplasm, variably sized clear cytoplasmic vacuoles, anisocytosis and anisokaryosis along with prominent nucleoli. The diagnosis of hemangiopericytoma is based on the characteristic findings of moderate to high cellularity, the presence of multiple, small, clear cytoplasmic vacuoles, binucleated and crown cells. The fibrosarcomas and leiomyosarcomas exfoliate poorly and are identified on the basis of typical cytomorphology.

Round cell tumors or discrete cell tumors are also common neoplasms diagnosed in Veterinary practice. For the round cell tumors, cytomorphology is sufficiently distinctive to differentiate the cell types to reach specific diagnosis. The presence of several more or less uniform sized discrete cells with a round nucleus, moderately abundant pale blue cytoplasm and distinct cytoplasmic vacuoles are the characteristic findings for the diagnosis of TVT. The diagnosis of mastocytoma is based on the characteristic cytomorphology of abundant metachromatic (purple) cytoplasmic granules that sometimes obscure nucleus. Replacement of the normally present heterogeneous population of mature lymphocytes of lymph nodes by monomorphic population of immature lymphoid cells is the characteristic cytologic finding of lymphomas. The aspirates in lymphoma are dominated by moderately pleomorphic lymphoblasts i.e. large cells having a rim of basophilic cytoplasm that does not encircle the nucleus completely.

Melanocytic tumors are also diagnosed frequently by cytology. The characteristic cytologic findings for malignant melanomas are the presence of large number of round to spindle shaped pleomorphic cells having abundant cytoplasmic melanin granules in most cases.

In nutshell, diagnostic cytology is a rapid and effective technique in the diagnosis of specific diseases /conditions, particularly in discriminating neoplastic conditions from non-neoplastic lesions and is reported to have a very high sensitivity and specificity as well as positive and negative predictive values. However, the success of diagnosis in a given pathological condition depends upon appropriate selection of lesions of the disease or condition, correct cell retrieval method, fixation, staining and above all the experience and personal expertise of the cytopathologist. As per the experience of the authors, multiple cytologic sampling techniques along with complete history and clinical or laboratory findings are highly useful for definitive cytologic diagnosis.

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Current trends in obstetrical treatments for enhancing subsequent survival rates and fertility in cattle and buffalo

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Parturition is the most critical event in the life of a female that begins the productive phase. Any mishap or obstruction in the normal process of parturition can be life threatening for the calf, mother or both or lead to enormous reduction in the productive capability of the animal. Besides the uterine involution may be delayed, infections flare up and the onset of reproductive activity is hampered. It thus is of paramount importance to have a normal or nearly normal puerperium to have optimum production and reproduction. Uterine diseases threaten animal wellbeing, reduce milk production and cause infertility; making dairy farming unprofitable.

Postpartum uterine diseases such as retained placenta, metritis, endometritis and pyometra are inflammatory conditions mostly associated to bacterial infection. Bacterial contamination of uterus is ubiquitous following parturition and can lead to harmful infection and inflammation. Establishment of infection and development of pathological conditions depends on the balance between local immune defenses and the nature of bacterial invasion.

Pre-evaluation of a case before instituting any treatment is very important. This includes thorough anamnesis, clinical examination and decision regarding the procedure to be adopted. Depending upon severity, the dystocia due to mal-presentations can be handled by fetal mutations, fetotomy and/or caesarean section. Most of dystocia cases can be delivered through mutations while a few need fetotomy for successful delivery. It is dismemberment, dissection or cutting the fetal parts inside the uterus to reduce its size and achieve per-vaginal delivery of the fetus. Cesarean section is the last resort to deliver the fetus in cases of narrow pelvis, incorrectable fetal mal-presentations, delayed cases of uterine torsion, fetal monstrosities, incomplete dilatation of cervix and vaginal and cervical tumors. Major post-operative complications of cesarean include peritonitis, adhesions of the uterus with surrounding viscera and wound dehiscence. Moreover, the future fertility of the cesarean operated cases remains guarded due to uterine adhesions.

Parturition is highly stressful and dystocia further enhances the degree of stress. The animal at this stage is exposed to different kind of stressors like physical, behavioral, and transport, if she has to be transported for any specialized medical aid. Different stressors have different effects that vary with the nature, duration (acute/chronic) and intensity

(mild/moderate/severe) of the stressor. Species, sex, steroidal status, stage of reproductive cycle, general health, nutrition, season of the year and prior exposure to stressful stimuli can alter the responses. Stressors mainly have adverse effect on reproduction. Many have effect on a single critical event of the estrous cycle, e.g. confinement, shearing, foot shock, hypoglycemia can disturb the LH secretion. In contrast, some stressors target different event of the estrous cycle, like heat stress results in suppressed dominance of large follicle, lowered steroidogenic activity and low progesterone production and compromised endometrial function. All of these adverse reactions are through disrupted hypothalamic-pituitary-gonadal axis. Commonly observed infections of the uterus may influence the hypothalamic GnRH pulsatility. At pituitary level, pituitary responsiveness to GnRH is reduced while at ovarian level estradiol production prevented in response to gonadotropin stimulation. Hence it becomes paramount to alleviate the effects of stress or to prevent the occurrence of stress to have a normal reproductive activity at any stage. Following abnormal parturition, altered body metabolism (later discussed) and shock often compromises survivability depending upon the degree of stress. Thus, attempt should be to prevent dystocia or handle it in such a way to minimize trauma for normal production.

Classic events that take place after parturition like involution of uterus and regeneration of endometrium and onset of ovarian activity are directly related to uterine environment. Continuity of reproduction depends upon involution of uterus; delay in involution impairs fertility by delaying estrus and ovulation while incomplete involution prevents fertilization during the first few weeks after parturition. Uterine involution usually is completed within 45 days; however, this period varies with many factors. It should progress as a normal consequence to expulsion of uterine contents but age, genotype of the dam, immune status of the dam at parturition, season of calving; energy status and complications at calving are some of the factors that may influence the involution of uterus. Attempts to enhance the uterine involution and the interval to first post-partum estrus have yielded variable results. Administration of hormones like GnRH, steroids, PGF2 alpha or pre-partum administration of vitamin E and selenium has been tried. Two shots of PGF2 alpha given at a gap of one week after parturition, enhanced uterine immunity and shortened the period to uterine involution and enhanced fertility parameters. Also GnRH administered between days 15-20 was beneficial in enhancing fertility in dairy cows.

Dystocia and post-partum uterine infections delay uterine involution and post-partum fertility. Puerperal metritis was more frequent in buffaloes with calving difficulty (87.5%) as compared to those having normal calving (20%). Several types of bacteria have been isolated from the uterus after normal and abnormal parturitions. The common isolates are *E coli, A pyogenes, Pseudomonas, Proteus sp., Streptococci and Klebsiella sp*. The pathogenecity and the effect vary with the degree of stress the animal suffers from.

Corynebacterium sp. was more common in cases that developed fetid pururlent discharge after handling of dystocia. Recently anaerobes have also been isolated from the uterus in abnormal parturitions that may not respond to routine drugs thus prolonging the phase of uterine pathology. *Fusobacterium* and *Bacteroides* sp. have been isolated from dystocia affected buffaloes. Data from organized farms have shown that more than one third animals affected with dystocia were culled and of the remaining onset of ovarian activity was significantly delayed. Administration of PGF2 alpha and sensitivity based antibiotics post-partum helped to clear the infections quickly and reduce the period of onset of reproduction. Strengthening of uterine immunity by infusing immune-modulators like *E. coli* lipopolysaccharide (LPS) in the uterus @ 300 microgram dissolved in saline or buffer have been found to rapidly the clear the uterine infections post-partum.

Stress, as mentioned above, is a critical link at parturition for production and reproduction post-partum. Although enhanced glucocorticoids help maintain body homeostasis, yet prolonged and abnormal rise may adversely affect body metabolism, body defense and production. Animals with marginally adequate/deficient adrenal function require supplementation of steroids during periods of stress; higher doses being necessary in severe illness and major surgeries. Administration of steroids after handling of dystocia significantly reduced cortisol levels by day 1 and improved survival over untreated controls due to improved body metabolism and homeostasis.

Oxidative damage due to excessive production of free radicals has also been observed in dystocia affected buffaloes. Altered levels of Malondialdehyde (end product of lipid peroxidation), superoxide dismutase and glutathione peroxidase were recoded in such animals. Exogenous administration of anti-oxidants like vitamin E and selenium, which act synergistically to break down the free radicals, have proved beneficial to improve the survival and production following abnormal parturitions in buffalos.

Critical evaluation of animals with abnormal parturition and puerperium is a must to institute proper treatment. This should include all possible parameters that may influence the body metabolism and homeostasis. Though, hematological parameters do not vary much and fluctuate within normal limits, yet, neutrophilia is a consistent finding around normal parturitions. However, in dystocia leucopenia due to neutropenia and lymphopenia was observed that may be due to endotoxemia, shock and higher catecholamine levels. Immature neutrophils with band cells dominating the leucogram followed by metamylocytes and very few mature cells were observed for the first time in dystocia affected buffaloes. The phogocytic ability of the neutrophils is also affected following dystocia. The killing capacity of neutrophils, as studied in *in-vitro* experiments, was lower following dystocia than normal calving suggesting lowered immunity and proneness to

infections. Thus, proper evaluation of animals should be done and treatment given so as to prevent possible complications.

A mention about metabolic derangements around calving will be important for rational therapeutic management of complicated cases. Circulating AST and Total Bilirubin levels can make good indicators of liver function in large ruminants. Elevated AST and TB levels in dystocia affected animals may indicate liver damage associated with transition metabolic disturbances. Negative energy balance and excessive mobilization of lipid reserves is a common feature in postpartum dairy animals. The latter often leads to hepatic accumulation of triglycerides which is associated with inflammatory tissue damage (fatty liver disease). Puerperal metritis is suggested to exacerbate liver damage in the transition cow/buffalo. This may reflect effect on feed intake, energy balance and lipid mobilization as well as a more direct effect of the underlying infectious and inflammatory process. Elevated circulating concentration of urea, a waste product of protein breakdown, can indicate pathological conditions in the kidney. As such, estimation of BUN has become a useful parameter in the assessment of renal function.

A respiratory –metabolic acidosis develops at parturition in most of animals due to hypoventilation and carbon-dioxide retention. However, following dystocia, mild metabolic alkalosis develops due to stress and trauma incidental to dystocia. Depending upon duration of problem and degenerative changes, tissue ventilation may vary and determine the recovery of the case. Allostatic changes in response to stressful stimuli release catecholamines and cortisol that enhance gluconeogenesis in liver and inhibit glucose utilization in extra hepatic tissues thus elevating circulatory glucose levels. The levels of non-esterified fatty acids (NEFA), an index of general stress and lipid mobilization, are elevated on the day of calving while its levels are much higher in dytociac buffaloes. Plasma protein levels did not vary much around parturition, but the author has recorded significantly lower protein and plasma immunoglobulin concentrations in buffaloes that died following obstetrical manipulations than those that survived indicating a severe physiological derangement. Removal of stress and shock besides control of infections post-partum are important managemental criteria that help to improve reproductive efficiency especially in complicated cases. Hormonal treatments to enhance uterine involution and ovarian activity are recommended to have a nearly normal puerperium.

Thus, postpartum uterine infections are a major cause of infertility and economic loss in dairy production systems. Both suppressed and exaggerated immune responses to bacterial contamination of the uterus following calving can contribute to the development of deleterious inflammatory conditions which impair reproductive functions. The latter is attributable to the effect of bacterial and inflammatory products on various aspects of the

hypothalamic –pituitary-ovarian- uterine interaction proceeding reestablishment of normal reproductive capacity.

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Metritis-Mastitis syndrome in bovines

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Metritis and mastitis in dairy cows are serious diseases, because they can adversely affect both production and reproduction. Metritis-mastitis syndrome commonly reported in sows; occasional reports are available for cattle also. It was Zebracki and Lubieniecki (1971) who gave first evidence of relationship in uterus and udder in dairy cows. The relationship between udder and uterus has been documented on many aspects as discussed below:

Etiological Interactions

Several workers could isolate identical microorganisms from uterus and milk samples of same cows simultaneously. It is observed that organisms of genital tract origin are one of the important causes of mastitis in post-calving period, and it is advised to control genital tract infections to prevent post-calving intramammary infections. In an experimental study, Rubstov (1971) infused 0.1 ml of uterine discharge in to teat of 25 cows, mastitis developed in 80% of cows. These workers further observed that 09 cows infused intrauterine with antibiotics could cure subclinical mastitis in 08 cows. Gudimova (1986) found that 10 cows infected in udder with *Streptococcus agalactiae* developed catarrhal endometritis due to same bacteria. Esmat and Badr (1996) studied 127 dairy cows with purulent uterine discharge post-calving for mastitis. The 87 (68.5%) had acute mastitis and 23 (18.1%) had subclinical mastitis. A Study on association of clinical mastitis and metritis in 5300 dairy cows by Silva Luiz Antônio Franco da et al (2004) showed that 128 (2.42%) cows had only metritis, 165 (3.11%) only clinical mastitis, and 89 (1.68%) both metritis and clinical mastitis. Other workers (Ghavi Hossein-Zadeh et al. 2011) found retention of placenta at calving as one of the significant risk factors for developing clinical mastitis in dairy cows with OR=9.45.

Functional Interactions

Rubstov (1973) observed that massage of udder in cows elicited an increased intensity and frequency of uterine contractions. Similarly, Oxytocin administration did not result in intensification of uterine contractions in cows with acute mastitis but increased uterine tone and amplitude of uterine contraction was observed in cows with healthy udder (Rauluszkiewiez 1983). On the other hand, the cows with acute endometritis produced less milk and had significantly lower levels of α-lactalbumin, β-lactoglobulin and α-casein in milk (Baranova et al. 1995). The administration of intrauterine antibiotics increased milk yield by 10% and milk quality improved.

Mastitis and Reproduction

Preliminary field evidence regarding effect of mastitis on reproductive efficiency in cows was given by Kiryushina 1980. He found that service period in cows is related to degree of mastitis; the post-calving conception period increased from 58 days in cows with no quarter infection to 126 days in cows with all the four quarters infections. Moore et al.1991 suggested that clinical mastitis may indirectly impair reproductive performance in dairy cows due to alteration of inter-estrus intervals and shortening of the luteal phase (premature luteolysis). Barker et al. (1998) showed that the onset of clinical mastitis before first AI increased days to first service and days open, but did not affect services per conception. The same researchers reported that when clinical mastitis occurred between first AI and conception both days open and services per conception increased significantly compared to uninfected cows. Schrick et al. (2001) showed that cows with mastitis before first service had an extended number of days to first AI, increased days open and services per conception compared to uninfected cows. Risco et al. (1999) reported that cows with clinical mastitis within the first 45 days of gestation were at 2.7 times greater risk of abortion within the next 90 days than uninfected cows. Similarly, Chebel et al. (2004), following evaluation of health records of 1400 Holstein cows, showed that the occurrence of clinical mastitis within the time frame of the day of AI to pregnancy reconfirmation was associated with increased pregnancy loss, such that cows having clinical mastitis were 2.8 times more likely to lose their pregnancy than those not experiencing mastitis. In a retrospective study, Santos et al. 2004 found that cows that experienced clinical mastitis prior to first postpartum AI and cows that experienced clinical mastitis between first postpartum AI and pregnancy confirmation had extended days open. Moreover, culling rate was increased in cows with mastitis compared with uninfected cows. Furthermore, cows with mastitis, anytime in lactation, had a greater incidence of abortions.

Ahmadzadeh et al. 2010 in a study predicted exponential lines for the proportion of cows that remained non-pregnant during 224 days postpartum for healthy and clinical mastitis cows. The estimated rate of decline (5 non-pregnant cows over time) for mastitis group was significantly lower than that of healthy cows, indicating a smaller proportion of cows became pregnant over time in the mastitis group compared with cows in the healthy group. For instance, by 160 days postpartum, a higher proportion of cows in mastitis group were still open as compared with uninfected cows (48.5 vs. 33.5%, respectively). Therefore, it appears that mastitis, either prior to or after first postpartum AI, increases culling rate and decreases reproductive efficiency in dairy cows. Thus, economically the effect of mastitis on reproduction is alarming. The depending on stage of lactation, loss of income for each day a cow remains non-pregnant over 100 days in milk (DIM), has been estimated at $US 0.42 to $US 5.00 per day (French and Nebel, 2003). Various studies have indicated that

regardless of experimental design, breeds, and locations, the numbers of days open in cows with mastitis were 22 to 49 days longer than uninfected cows. Assuming the additional cost per day open over 100 DIM is approximately $US 2.00 per day, the estimated additional monetary loss for a cow with mastitis is between $US 44 to 98.

Possible factors responsible for metritis-mastitis syndrome

How Metritis Affects Mastitis

(i) Functional relationship as suggested above

(ii) Infection from uterus to udder by Extraneous route i.e. discharges from vulva, Heamatogenous route: Perineal vein is considered shortest route to carry infected blood directly from genital organs to udder. The establishment of infection in udder is further promoted by reduced speed of blood flow in udder necessary for transport of blood constituents to glandular epithelium of udder for milk formation. Also around calving, venous pressure of mammary veins is high which leads to development of udder edema that facilitates setting up infection in udder.

(iii) Metabolic events: Workers have demonstrated that metabolic events associated with energy insufficiency i.e. increased fat metabolism and serum lipoprotein metabolism increased risk of metritis, retained placenta and mastitis. Similarly, high lactation demand for calcium post-calving results in hypocalcemia that decreases the muscle tone in uterus and teat sphinctor resulting in retained placenta and mastitis.

(iv) Decreased Immunity around peri-parturient period: Why immune system is depressed around calving, is not fully cleared. Some workers consider estrogens and glucocorticoids (cortisol) that increase during peri-parturient period responsible for it. It has been observed that around parturition, ability of lymphocytes to respond to mitogens and to produce antibodies gets impaired. Also the neutrophils had impaired ability to ingest and kill bacteria.

How Mastitis Affect Reproduction

How mastitis affects reproductive performance is not completely understood, although possible mechanism(s) have been theorized. Mastitis results in elevated levels of cytokines including tumor necrosis factor-α (TNF-α), and nitric oxide (NO) and a variety of interleukins (Hansen et al., 2004). Intra-mammary infusion of *E. coli* endotoxin (LPS) and *Streptococcus uberis* resulted in increased concentrations of PGF2α, TNF-α, and NO in blood or milk. The increased PGF2αin infected cows may cause premature luteal regression

and (or) may have detrimental effects on embryonic development and quality, causing increased embryonic loss, and consequently, increased services per conception and days open. Shuster and Kehrli (1995) reported that infusion of *E. coli* into the mammary gland of dairy cows resulted in elevated levels of cytokines (interleukin-1) in milk. It has been suggested (McCann et al., 1997) that cytokines may block follicle stimulating hormone (FSH) action and the pulsatile secretion of luteinizing hormone (LH). Both LH and FSH are important for follicular growth and maturation, ovulation, and progesterone and estrogen synthesis. Moreover, LH and FSH are involved in oocyte maturation, cumulus cell expansion, and nourishment of the oocyte (Zuelke and Brackett, 1994). Therefore, mastitis could influence reproductive function by altering LH and FSH activity and (or) function, thus affecting preovulatory follicular development, oocyte maturation and (or) steroidogenesis. In contrast, acute clinical mastitis induced by Gram-positive toxin caused both immediate and carryover attenuation of preovulatory follicle steroid concentrations and low mRNA expression of LH receptors. Thatcher et al. (1997) and Lucy (2001) suggested that premature luteal regression during the first month of gestation would likely result in decreased conception rates or increased pregnancy losses in lactating dairy cows. Chebel et al. (2004), in a study investigating embryonic mortality between 31 and 45 days after AI, reported an increase in the incidence of pregnancy loss when clinical mastitis occurred between AI and pregnancy confirmation. The fact that bacterial products such as LPS, and elevated cytokines can affect embryonic development *in vitro* support the theory that mastitis can increase the incidence of embryonic loss.

Management of mastitis

Mastitis needs no introduction; rather it introduces itself due to its frequent occurrence and heavy economic losses. The current annual economic losses due to mastitis in India have been estimated to be Rs. 7165 crores which included Rs. 503 crores for Punjab state alone. The disease generally occurs in two forms; subclinical and clinical. The average incidence of subclinical mastitis has been found to be 49% in cows and 33% in buffaloes. Similarly, clinical mastitis is prevalent in 7% of cows and 4% buffaloes. Besides this, 17.28% of the cows and 8.54% of buffaloes were suffering from various udder and teat lesions such as udder/teat warts, bovine ulcerative mammilitis, udder impetigo, teat chaps etc. These lesions pre-dispose the animal to mastitis and cause a great discomfort at milking and hence markedly decrease the milk yield.

Implications of Mastitis

Effect of mastitis on yield and quality of milk and milk products

Subclinical mastitis though in apparent causes 10-25% loss in milk production whereas in clinical mastitis there may be total loss of milk. The disease besides causing direct

economic losses to the farmer in the form of decreased milk production, have great public health significance from consumer point of view. The presence of mastitis causative organisms and antibiotic residues in milk following therapy of mastitis poses a major threat to the consumer health. Another important and inevitable fact is the adverse effects of mastitis on the compositional and keeping quality of milk and milk products. Mastitis results in increase in the somatic cell count (SCC) and bacterial load of milk. The European Union has set up a threshold of 400, 000 cells/ml for the milk to be from healthy quarter of a cow. Whereas, this limit is set at 100, 000 cells by the German Medical Veterinary Association. The high SCC in mastitis milk has lipolytic effect on fat and there is increased tendency for rancidity of milk and milk products. Rancid milk with acid degree value > 1 could be detected at 400, 000 cells/ml. Also, the mastitis milk with total bacterial count of more than 100 000 cfu/ml could release hydrolytic enzymes, which spoil the milk and milk products. It has been also observed that mastitis milk inhibits the growth of starter bacteria and results in decreased cheese production. In mastitis milk good things such as lactose, casein, butterfat, solids not fat, calcium and phosphorus decrease while undesirable milk components such as lipase, whey proteins, immunoglobulins, sodium and chloride increase. The milk loss in lactation with the increasing SCC is being graded as under:

Milk SCC (Cells/ml)	Milk Loss in lactation
< 3 Lac	Nil
5 Lac	6.0%
10 Lac	10.0%
20 Lac	16.0%
40 Lac	24.5%

Effect on hygienic quality of milk

Internationally, hygienic milk production forms an important component of milk payment systems, and usually milk from producer should have (i) TBC < 0.1 million/ml (ii) SCC < 400,000 cells/ml. and/or (iii) penicillin levels not more than 0.004 µg/ml. In India standards of milk quality are lacking. Limited data available for few farms shows that cattle farms in Punjab usually represent bulk tank milk SCC of 0.7 to 1.5 million cells/ml, and it is not uncommon for our dairy plants to receive raw milk with TBC of 5 to 10 million/ml and high levels of drug residues. The contamination of milk at the farm starts from within the udder. Raw milk as it leaves the healthy udder normally contains very low number of microorganisms (usually < 1000 cfu/ml) while a cow with mastitis has the potential to shed in excess of 10 million bacteria per ml. If the milk from one cow with this bacterial count comprises 1% of the bulk tank milk, the total bulk tank count, disregarding other sources, would be 100, 000 per ml. An increase in the SCC of milk serves as supportive evidence that an increase in the bulk milk bacteria count is caused by the mastitis pathogens. The

50% of violations with respect to bacterial count of milk result from mastitis. A linear relationship exists between the bulk tank milk SCC and the percent of quarters infected with major pathogens; infected quarters being about 25% at bulk tank SCC of 7,50,000 cells/ml. The presence of antibiotic residues in milk following therapy of mastitis is another important factor affecting the quality of raw milk. The relative risk of drug residues in milk increases to 7.1 fold for SCC > 700 $\times$ 10^3cells/ml. It has been reported that 60% of violations with respect to drug residues in milk occur due to mastitis therapy and in milk with SCC of > 400 $\times$ 10^3 cells/ml. Thus, the Bulk tank milk SCC may be used to monitor the prevalence of mastitis in dairy herds, as an indicator of raw milk quality to processors; and as a more general indicator of the hygiene and animal welfare at the farm.

Etiology of disease

The disease is mainly caused by bacterial organisms, which are categorized into two groups; the contagious organisms such as *Staphylococcus aureus* and *Streptococcus agalactiae* which frequently present on teat and udder skin of animal, and transmitted form one animal to another animal at the time of milking through milking utensils, milker's hands and cups of milking machine. The other group comprised environmental organisms such as coliform and *Streptococcus uberis*, which are frequently present in dung, animal bedding, manure, soil, feed stuffs, uterine discharges and urine etc., may be transmitted to animal at any time, even in-between the milkings.

Diagnosis of mastitis

In its clinical form, disease may be diagnosed well by the classical signs of inflammation and visible alterations in milk consistency, colour and appearance etc. The changes in levels at which certain components in the mammary secretion are present are commonly employed in identifying the disease at its subclinical level. A variety of diagnostic tests for mastitis are available which differ markedly with respect to sensitivity, specificity, simplicity, rapidity and cost. Among these, Bromothymol blue (BTB) card, Sodium lauryl sulphate (modified California mastitis test) and Electrical conductivity tests are simple and economical tests that can be performed as cow-side tests at the field level.

Sodium Lauryl Sulphate (SLS) test: It is based on the principle that reagent ruptures somatic cell releasing cellular proteins (DNA) that results in gel formation, and depending upon the degree of gel formation the reaction is scored as 0, Trace, 1, 2 and 3. Thus, this test gives the indirect estimate of milk somatic cell count.

Electrical conductivity (EC) test: The ions in milk conduct electricity, such that any change in concentration of ions is reflected as a change in conductivity. Dissociated, inorganic salts such as sodium, chloride and potassium are the main contributors to conductivity. In

mastitis there is damage to tight junctions between epithelial cells and an increased permeability of the blood capillaries occurs. As a result Na^+ and Cl^-, which are higher in extracellular fluid, pour into the lumen of alveolus and in order to maintain osmolarity K^+ and lactose levels decrease proportionately. The alterations of the concentrations of Na^+, K^+ and Cl^- in mastitis without a concomitant change in osmotic pressure are the main reason for the higher EC of mastitis milk. A relative difference of 0.5 mS/cm or more in the conductivity between any two quarters of a cow is taken as evidence for mastitis. The EC could be measured by digital conductivity meters, which are easily available in the market. Even, hand-held battery operated digital conductivity meters are available for use as cow-side test.

Bromothymol blue card (BTB) card test: It is based on the principle that in mastitis, the pH of milk rises due to entry of bicarbonate salts from blood into milk. Depending upon the health status of quarter and hence pH, the color of the dye changes from yellow (normal) to greenish-yellow (+), green (++) and blue (+++) when a drop of quarter milk is placed on the card.

Treatment of mastitis

In vitro testing of milk samples revealed that drug sensitivity pattern of mastitis organisms goes on changing. At present gentamicin, amoxicillin-sulbactam and Ceftriaxone-sulbactam are found much effective *in vitro* drugs in Punjab. So, treatment should be given preferably based on culture and sensitivity test particularly in mild cases. In acute or per acute cases, there is no time for these tests, so the therapy in such cases is based on the past data of herd infection and sensitivity reports. However, before starting therapy in such cases, the milk sample should be invariably taken and put to culture sensitivity so that the therapy may be changed if needed in the light of sensitivity report. Moreover, it may also be made clear that there is no surety that *in vitro* sensitivity determination will correlate with the *in vivo* treatment results. For example, enrofloxacin that shows high *in vitro* sensitivity and is pharmacologically considered to distribute well in the udder clinically proved to be less efficacious against staphylococcal mastitis because of its inability to kill intracellular organisms. On the other hand, amino-glycosides (gentamicin and neomycin) that are considered to have poor distribution in the udder, *in vivo* proved very much effective in treatment of clinical mastitis. The organism involved in mastitis also affects the efficacy of treatment. Streptococci respond well, staphylococci less and coliform are difficult to treat due to severe per acute reaction. However, enrofloxacin could be best recommended for treatment of per acute mastitis caused by coliform. For taking specific therapy, clinical mastitis is generally divided into three forms viz., per acute, acute and chronic form.

(i) *Peracute mastitis:* It is generally caused by coliform and it occurs commonly around calving but may develop at any time during lactation. The disease is usually sudden in onset: the cow may appear normal at one milking and at the next milking shows pronounced signs including anorexia, rise of temperature, depression, shivering and rumen stasis. Inflammatory signs in the udder may be minimal at this time and swelling may be detectable only after the udder is milked out. Later, the quarter is swollen and hard, the teat may be thickened, oedematous, hot to touch and sensitive. In the early stages, the milk may appear normal or faintly watery. Subsequently it may be serous and contain tiny particles. In severe cases it may become blood tinged. Recommended therapy includes:

- Removal of bacteria, toxins and inflammatory exudates from the mammary gland by frequent milking, even oxytocin injections (20-30 IU I/M) may be given for complete milking out.
- Appropriate antibacterial therapy to start with systemic administration that may be later (after 12-24 h) supplemented with suitable intramammary infusion.
- Fluid therapy; dextrose saline solution (10-20 L in first hour, up to 60 L in severe cases) to restore vital body fluids, dilute toxins and counteract acidosis. Even 5% sodium bicarbonate (150-250 G) with first 3-5 L of fluid may be given.
- Systemic glucocorticoids, Dexamethasone @ 1-3 mg/kg IV or IM once or may be repeated after 8-12 hours.
- Calcium borogluconate 20% @ 500 ml IV to counteract hypocalcaemia induced by endotoxin. Administer with care as such therapy may have damaging effects on the heart in animals that are in shock.
- Aspirin 30 grams P.O. reduces pain and inflammation, and restores appetite
- Antihistaminic drugs and multivitamins

(ii) *Acute mastitis:* In this form there is no systemic reaction. Primarily changes are observed in milk, which may contain flacks, become watery or thick. The udder may become swollen and hard. The line of treatment includes use of antibacterial drugs plus calcium and multivitamin therapy. The combination therapy i.e. intramammary plus parenteral works well than the alone parenteral or intramammary. The important recommendations in mastitis therapy are (i) Use antibacterial on need for recommended time i.e. at least for 5 days (ii) Use appropriate dose and dosing interval (iii) Stick to the recommended milk withdrawal times.

(iii) *Chronic mastitis:* A case is considered chronic when (i) there is formation of fibrotic cord inside teat canal (ii) there is thick pus discharge, not responding to treatment (iii) there is frequent reoccurrence of mastitis in the same quarter. The treatment/surgery of chronic mastitis is not rewarding. Rather such cases should be isolated from the milking herd or the

affected quarter may be permanently dried-off by producing a chemical mastitis. Infusing 30-60 ml of 3% silver nitrate solution or 20 ml of 5% copper sulphate solution can do it.

Control of mastitis

Mastitis control is a comprehensive program that includes good milking and environment hygiene, use of properly functioning milking equipment, application of teat dipping, proper identification and treatment of mastitis cows, use of dry therapy and sound nutritional program. Worldwide, many dairy farmers have adopted these procedures for mastitis control. The important features of a successful mastitis control programme are:

- Minimising the source of infection
- Elimination of existing udder infections
- Prevention of new intramammary infections (IMI)
- Increasing the udder resistance to mastitis

(i) *Minimising the source of infection:* It can be achieved by maintaining optimal Environmental and Milking Hygiene; segregation and prompt treatment of clinical mastitis cases, culling of carriers and drying off of chronically infected quarters. The adoption of hygienic measures depends upon the epidemiology of the causative organisms. For example in case of contagious organisms, which are transmitted from one to another animal through the milking equipment and milker's hands, proper washing of udder, cleanliness of milker's hands/milking machine clusters in between each milking and post-milking teat dipping in germicidal solution will reduce the infection to a great extent. On the other hand, for the organisms that come from the environment e.g. to prevent coliform mastitis animal environment should be kept clean by frequent removal of dung, proper drainage, and adequate milking and feeding space should be provided.

(ii) *Elimination of existing udder infections:* It is achieved by Dry therapy. The dry therapy is done at the end of lactation (after last milking) with a long acting antibiotic intramammary preparation that maintains effective drug concentration for 6-8 weeks i.e. throughout the dry period. It not only eliminates the subclinical infections of previous lactation but also prevents new IMI and increases the milk production by about 8-10%. In addition it improves the milk quality at calving and prevent the occurrence of clinical mastitis cases during dry period and around calving.

(iii) *Prevention of new intramammary infections (IMI):* It is achieved by Post milking teat dipping. The teats of all the lactating cows and dry cows (during first 10-14 days of dry period) are dipped regularly after every milking in a germicidal solution. The recommended teat dips arr Iodine (0.5%) solution + Glycerine @ 12-15% of iodine solution and Chlorhexidine (0.5%) solution + Glycerine @ 06% of chlorhexidine solution

The iodine teat dip is found best as it treats various types of teat lesions and injuries also.

(iv) *Increasing the udder resistance to mastitis:* Future trends in mastitis control are aimed at increasing the immunity of udder to mastitis pathogens. This can be achieved by use of non-specific (cytokines/ proper nutrition) and specific (vaccination) immunomodulators.

(a) Cytokines: Cytokines include interferons, interleukins, colony stimulating factors (CSF), and a variety of other proteins that modulate the activity of immune cells and thus enhance the phagocytic cell functions in the udder. It has been shown that interferon treated cells exhibit significantly more phagocytosis and intracellular killing of *Staphylococcus aureus.* Interleukins enhance the production of local antibodies and accelerate the involution process that will further promote resistance to mastitis during the dry period. Similarly, the granulocyte-macrophage CSF significantly increases the chemo tactic and bactericidal activities of mammary gland neutrophils.

(b) Nutrition: Even slightest deficiencies of certain vitamins (Vit E, C, A and ß-carotene) and micro-nutrients (Cu, Se, Zn, Co) are reported to have detrimental impact on the efficient functioning of immune system. Vitamin A is involved in maintaining a functional epithelium that provides a physical barrier to the entrance of pathogens. ß-carotene also referred as pro-vitamin A enhances the immune function and disease resistance. Zinc supplementation prevents the infection by strengthening the skin and stratified epithelium (keratinocytes) of teat canal. The biological role of Cu is exerted through a number of Cu containing proteins including ceruloplasmin and superoxide dismutase (SOD). These proteins protect the host tissues from membrane oxidation by acting as antioxidant and scavenging oxygen free radicals produced during the inflammatory response. Similarly, vitamin E and the Se containing enzyme glutathione peroxidase (GSH-pX) also act as integral part of the antioxidant system. Studies have shown that supplementation of cows during dry period and around calving (first 8-10 weeks) with the following nutrients per head per day proved beneficial in preventing mastitis.

(i) Vitamin 53000 IU + Beta- carotene 300 mg
(ii) Zinc-methionine (180-360 mg Zn, 360-720 mg methionine)
(iii) Copper @ 20 ppm i.e. about 200 mg
(iv) Vitamin E 1000 IU during dry period and 500 IU for lactating cows
(v) Selenium is recommended as 3 mg during dry period and 6 mg during lactation

(c) Vaccination: The effective immunization against mastitis has been a goal of mastitis researchers for many years. But, the nature of disease creates a number of unique challenges for the production of successful immunity against mastitis. Commercially, few

mastitis vaccines are currently available in developed world for immunization against mastitis caused by *Staphylococcus aureus* and *E. coli.* Several studies have evaluated these; the outcomes have been inconsistent and confusing to interpret. However, it is generally accepted that *S. aureus* vaccine have limited ability to prevent new infections and clinical mastitis cases. The best use of the vaccine is the reduction of chronic infections rather than prevention of new infections. The use of vaccine against coliform mastitis has been considered efficacious even though the rate of intramammary infection is not significantly reduced in vaccinated animals but because they significantly reduced the severity of clinical disease. Farmer may expect mastitis vaccine to eliminate existing infections, prevent new mastitis cases and reduce the severity of mastitis. While these expectations seem reasonable, it is unlikely that any one vaccine will be able to achieve all of these outcomes. So, we may expect role of vaccination as one the component of mastitis control programme, but alone its use may not be giving much encouraging results.

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Emerging Significance of Colour Doppler Ultrasonography in Reproduction

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Ultrasound is any sound frequency above the normal hearing range of the human ear; i.e. greater than 20,000 Hz. At Colorado A & M College, now Colorado State University, back fat thickness was measured on beef cattle with a "somascope" ultrasonic unit (Temple et al 1956). This technology was later used to evaluate the fat and muscling of all the steers in the 'Quality Beef Contest' at the International Livestock Exposition in Chicago,IL in 1960, 1961, and 1962. The first use of ultrasound as a diagnostic aid in veterinary medicine was for the detection of pregnancy in sheep. Subsequently, more veterinary applications emerged with a review of the literature up until 1986 identifying 492 references. Fifty percent of these related to large animal applications with farm animal reproductive examinations accounting for 25%.

Before ultrasound, the techniques used in studying patterns of follicular development involved histological evaluation of ovaries of animals killed at various stages during the estrous cycle, or marking of follicles with ink, followed by serial laparoscopy. In contrast, the development of ultrasonic probes that can be used transrectally to visualise ovaries has opened up new possibilities for examining the dynamics of follicular growth and regression (Fortune et al 1991) and provided a means for repeated, direct, non-invasive monitoring and measuring of follicles within the ovary (Griffin and Ginther 1992). This has immensely contributed to our understanding of ovarian physiology and development of several protocols for synchronization of estrus and ovulation.

An emerging facet of ultrasonography that has the potential to further enhance diagnostic and predictive capabilities for clinical veterinarians and the depth and breadth of hypothesis testing for animal and veterinary research scientists is Doppler imaging of blood flow. Doppler ultrasound adds blood-flow information to the B-mode image giving more insight about anatomy and function of organs. The applications of color Doppler ultrasound to study animal reproduction have enhanced the scientific merit of research and represent a technological breakthrough that has revolutionized knowledge of reproductive biology.

The Doppler principle was first hypothesised by Johann Christian Doppler and presented as a paper before the Royal Bohemian Society of Learning in 1842. Once scientifically verified in 1845, it formed the basis for the development of Doppler ultrasound (White et al

1982). When an ultrasound beam encounters a moving object such as a red blood cell in vascular flow, the frequency of the returning echo is altered. An increase in frequency occurs when the object is travelling towards the transducer; this is known as positive Doppler shift. An object travelling away results in reduced frequency and a negative Doppler shift. The measurement of these alterations in the returning echo allows the direction and velocity of the flow encountered to be determined (Abelson and Balin 1972). The Doppler technology has had an increasing impact in human medicine and science for more than two decades but is in its infancy for cattle (Bollwein *et al* 2002, Acosta *et al* 2003) and equines (Acosta *et al* 2004).

Color Doppler ultrasonography has been used in mares to study the ovarian haemodynamics, early embryonic mortality and fetal sexing (Acosta et al., 2004,b, Bollwein et al 2002). In cattle and buffaloes, color Doppler has been used to study the blood flow to ovarian structures (Acosta et al,.2003, Herzog et al,.2011, Varughese et al,. 2014) and uterus (Honnens et al,. 2008).

Ovarian vasculization/haemodynamics

Color flow Doppler (CFM) ultrasonography helps to understand that the physiological status of a follicle (e.g., dominant, subordinate, growing, regressing) or corpus luteum that cannot be determined during a single B Mode ultrasound exam. B Mode ultrasonic imaging aids in distinguishing anatomical attributes of a structure but confers little information regarding physiological or endocrine status. For example, ovarian cysts can be categorized by anatomical attributes such as diameter and presence or absence of luteal tissue; however, no information regarding functionality such as plasma hormone concentrations can be conferred. It is difficult to distinguish between developing corpora lutea and older regressing corpora lutea using B Mode technique (Pieterse et al., 1990).

Vascularization can be detected in the theca externa of each follicle >2.5 mm in diameter. Before follicle selection, there is no difference in the percentage of follicles with detectable blood flow. Following follicle selection, blood flow was less intense in the second largest follicle compared to the largest follicle. In addition, small follicles with detectable blood flow one day before the occurrence of follicle selection, subsequently developed larger diameters than those without detectable blood flow at this time (Gastal et al., 1999) indicating that the maintenance of follicle vasculature and appropriate blood supply is essential for acquiring and maintaining follicular dominance. Studies suggest that the change of the blood supply to an individual follicle closely relates to the dynamics of follicular growth and selection in the first follicular wave in the cow.

Follicle blood-flow assessment by Doppler ultrasonography has been used in mares to

study follicle selection, anovulation during transitional seasons, first versus later ovulations of the year, follicle maturity and proximity to ovulation, oocyte recovery rate, maturity, and quality, effects of circulatory hCG antibodies on follicles and oocytes, age-related effects,and pregnancy establishment. A daily increase in vascularity of the wall of the dominant follicle as it matures and approaches the day of ovulation has ben recorded in mares. Further on the day of ovulation, a few hours before evacuation, an abrupt decrease in blood perfusion in the wall of the preovulatory follicle has been detected. In cattle, it was also characterized that the real-time changes in the blood flow in the theca externa of mature follicles are associated with the LH surge and ovulation. Cows with both spontaneous, and GnRH- induced, ovulation had a clear LH surge followed by ovulation 26–34 h later. Blood flow before the LH surge was only detectable in a small area in the base of the follicle. An acute increase in the blood flow area and velocity was detected at 0–6 h after the onset of the endogenous LH surge, or at 0.5 h after the GnRH injection, synchronous with the initiation of the LH surge. The data confirm the concept that the complex structural and functional changes induced by the LH surge in a mature follicle are closely associated with a local increase in the blood flow within the preovulatory follicle wall.

Color-Doppler ultrasonography also has the potential for judging the status (future ovulatory or anovulatory) of dominant follicles during the transitional period. During the anovulatory transitional season, vascular changes in the follicle walls of both a future dominant anovulatory follicle and a future ovulatory follicle were studied from 25 mm until 7 days after the follicle was 30 mm (Acosta et al., 2004). Blood-flow area was already less for dominant-sized anovulatory follicles than for ovulatory follicles by the time blood-flow determinations began at 25 mm. Color images of an anovulatory follicle and a preovulatory follicle indicated that the atretic follicle was characterized by a lack of detectable blood flow and a progressive decrease in diameter. The preovulatory follicle which was well vascularized with detectable blood flow surrounding the antrum in the base of the follicle ovulated and a corpus luteum developed.

Greater vascularity of the preovulatory follicle has been associated with greater follicle diameter, retrieval rate of oocytes, retrieval rate of mature oocytes, in vitro fertilization rate and pregnancy rate. Based on the percentage of follicle wall with blood flow signals at the time of artificial insemination, the follicles were grouped arbitrarily into greater (≥70%) and lesser (<70%) blood flow percentages. A greater ($P < 0.004$) percentage of heifers in the pregnant group (19/25; 76%) had a greater percentage of blood flow than in the nonpregnant group (2/9; 22%) (Siddiqui et al.2009). A greater percentage of follicle wall with blood-flow signals in the heifers that became pregnant indicated an increased blood circulation from an increase in number and diameter of the arterioles that form a network

encasing the follicle. Diameter of the preovulatory follicle was strongly correlated with vascularization in cows (r= 0.8, P < 0.01) but weakly correlated in buffaloes (r= 0.21, P < 0.01). Varughese et al, 2014 reported that vascularisation to the preovulatory follicle was positively correlated with intrafollicular and plasma concentration of IGF-1 and E in buffaloes and intrafollicular concentration of IGF-1 in cows. However, vascularization was weakly correlated with plasma concentration of IGF-1 and E and there existed no correlation with intrafollicular concentration of E in cows. Animals (cows and buffaloes) with a highly vascularised follicle (> 550 pixel^2) underwent a normal pregnancy, whereas those that had moderately (250-550 pixel^2) and poorly (< 250 pixel^2) vascularised follicle experienced complicated pregnancy or remained non-pregnant, respectively. Animals with moderately vascularised follicles had complicated pregnancy and the most common types of complicated pregnancies seen were IUGR, late embryonic death, early fetal mortality, high-risk pregnancy or a combination of IUGR and early fetal mortality. However, a poorly vascularised follicle led to non-pregnant animals in majority of the cases (Varughese et al 2014).

The Doppler technology shows the blood flow in the wall, and the area of the blood flow therefore gives a clearer view of the thickness of the wall in cysts. This makes the choice for therapy easier (Matsui and Miyamoto, 2009). In mares 85.7% of anovulatory follicles become luteinized and have a thick, highly vascularized luteal wall and the remaining 14.3% of anovulatory follicles remain as non-luteal and little-vascularized structures.

In the early corpus luteum (CL), the blood flow (area and velocity) gradually increased in parallel with the increase in CL volume and plasma progesterone concentration from day 2 to day 5, which are correlated during the growth period (Herzog et al, 2010) indicating active angiogenesis. The growth occurs very rapid and is induced by insulin like growth factor (IGF). The development of the corpus luteum and its functions are dependent on blood supply (Acosta and Miyamoto, 2004). After the development of the corpus luteum, there is a static phase in the luteal size. In this period the progesterone production still increases, as does the blood flow to the corpus luteum.

With spontaneous luteolysis, the blood flow surrounding the CL increased on days 17–18 in cows, followed by a decrease in plasma progesterone concentration one day later (Shirasuna et al 2004 and Miyamoto et al 2005). The progesterone levels also decrease rapidly during regression phase, and is correlated to the blood flow (Herzog et al, 2010). The decrease in progesterone may be caused by cytokines which are originated from fibroblasts, endothelial and epithelial cells of the corpus luteum. They inhibit the LH-stimulated progesterone production of the luteal cells. IGF1 is a luteotropic factor and stimulates the progesterone secretion. TNF and IL1b elevate the IGF-binding protein and

therefore the availability of IGF is less. Coincidentally, plasma PGFM concentrations drastically increased as luteal blood flow increased on days 17–18, strongly suggesting that pulsatile release of PGF 2α from the uterus stimulates the increase in luteal blood flow (Shirasuna et al 2004 and Miyamoto et al 2005). These results represent the first direct evidence in any mammalian species that the luteal blood flow acutely increases before plasma progesterone concentration declines.

Luteal blood flow was found to be superior to luteal size as an indicator of highprogesterone concentrations. This may be attributed to (a) the correlation during each phase of the estrous cycle between progesterone concentration and luteal blood flow (LBF); (b) the similarity of the relative changes of progesterone and LBF for most of the days, which is specifically true for the luteal regression phase; (c) the higher changes of LBF throughout the cycle over those of luteal size (i.e., the maximum of LBF is fourfold higher on Day 14 compared with Day 4, whereas lutealsize increases only by 100%). The close correlation between LBF and progesterone emphasizes that a high luteal blood supply represents an important precondition for the secretion of progesterone. The blood delivers steroid precursors for production of hormones (Janson et al 1981), accordingly, vascularization precedes progesterone synthesis (Acosta et al 2004). The majority of the steroidogenic cells of the CL are in close contact with the capillaries (Reynolds et al 1992). The spreading of capillaries appears to be induced by growth factors and is further potentiated locally by angiotensin II supporting the synthesis of progesterone CL, thereby causing vasodilation and triggering the luteolytic cascade leading to the decrease of progesterone. The rapid decline of progesterone during the luteal regression phase is well known from other studies (Kastlic et al 1990 and Ribadu et al 1994) and is explained by the dramatic reduction of luteal cells during luteolysis (i.e., the proportion of luteal cells decreases between Day 12 and Day 18 by about 40%)(Lei et al 1991). The subsequent, pronounced decline of LBF indicates that the availability of steroid precursors may be a rate-limiting factor for progesterone production by the remaining luteal cells.

Uterus blood flow

Bollwein et al 2012, found that uterine blood flow of all estrous cycles followed a consistent cyclic pattern with a high negative correlation between the two blood flow parameters ie time-averaged maximum velocity (TAMV) and resistance index (RI) ($r = -0.93$; $P < 0.0001$). The highest RI and lowest TAMV values occurred on Day 0 (ovulation) and Day 1, followed by constant medium levels for both parameters between Day 3 and Day 13 ($P > 0.05$). Minimum RI and maximum TAMV values were observed on Day -3, Day -2 and Day -1. However, assuming that factors such as cardiac performance, vessel compliance, blood viscosity and perfusion pressure in the uterine artery remain constant, a decrease in the resistance index and an increase in time-averaged maximum velocity would

be associated with a proportional increase in flow and helpful in detection of stage of the estrous cycle.

Endometrial vascular perfusion was studied by Silva et al 2004 by studying color flow signals in endometrium from 0-16 days from ovulation in pregnant and non pregnant mares. It was found that between 12-15 days endometrial perfusion was noticeably higher in the horn with the embryo than the opposite horn. Highest endometrial perfusion was present locally round embryo. It may be helpful in distinguishing the early vesicle from an endometrial cyst (Silva et al 2004) as uterine perfusion is reduced with uterine cysts. Colour flow signal in the endometrium some 2-3 days before embryo can be visualized which may be due to some interaction of endometrial vessels close to embryonic disc. Uterine blood flow increases during the second week of pregnancy. Low resistance (0.85 in early pregnancy to 0.55 in mid pregnant then remain stable upto full term) and high volume uterine blood flow (150ml/min at 14 day to>18500ml/min at term) is present in pregnant mares in late pregnancy. Slightly reduced uterine artery blood flow in older mares is also observed. No effects on uterine artery blood flow in case of experimentally induced ascending placentitis (Ousey et al, 2012).

The mean RI values were lower in the middle uterine artery ipsilateral to the fetus compared to contralateral artery in every stage of pregnancy ($P < 0.05$) and the RI values decreased continuously during the first 8 months of gestation and remained fairly constant or slightly higher thereafter ($P > 0.05$) in buffaloes. TAMV increased from the first to second trimester and then increased by 1.3–1.8 fold during the last trimester (8–10 months) of pregnancy for the ipsilateral artery, while the contralateral artery had a much slower increase of 1.1–1.2 fold. The volume also increased fairly continuously with a 1.6–1.8 fold increase from 8 months until term ($P < 0.05$) in both ipsilateral and contralateral arteries and with a greater increase in ipsilateral artery. The difference in the volume through the ipsilateral and contralateral artery was low in the 2nd month (1563.8 mL/min), after which it started increasing until the 5th month (3543.01 mL/min) and thereafter remained fairly constant till the 8th month. In the last month, the difference rose to 6192.34 mL/min between the ipsilateral and contralateral blood vessels . There was an increase in the diameter of the arteries leading to a decrease in resistance to blood flow. The RI in the ipsilateral artery was lower compared to the contralateral artery in most of the months of gestation, which may be due to the proportionate increase in the diameter of the blood vessel (Varughese et al 2013).

In the case of metritis and pyometra, the contents of the uterus may vary in appearance as anechogenic fluid in black, to the presence of echogenic material floating in a black background, to a purulent exudate that is echogenic in appearance and similar to the

surrounding tissue, or isoechogenic. In such conditions, it is important to seek other essential signs (RI/PI/TAMV/Volume of blood/Notch index) that would confirm a diagnosis of pregnancy, which are possible with color Doppler ultrasound.

Overall color flow Doppler ultrasonography adds lot of information to images received by B-mode and helps to diagnose various conditions more accurately.

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Cytokines expression for prediction of endometrial health in bovines

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Endometritis is one of the major cause of sub-fertility in bovines. Mostly bacterial in origin, it often occurs after calving. At the time of calving, the vulva/vestibule are relaxed and cervix is dilated thus making a continuous passage from vulva to uterus. Immediately after parturition negative pressure gets created in uterus which leads to suction of vaginal fluids into uterus. These fluids are often contaminated and hence microorganisms get entry into the uterus. Sheldon et al (2008) reviewed that after parturition almost 100% animals have bacterial contamination of uterus (Fig 1).

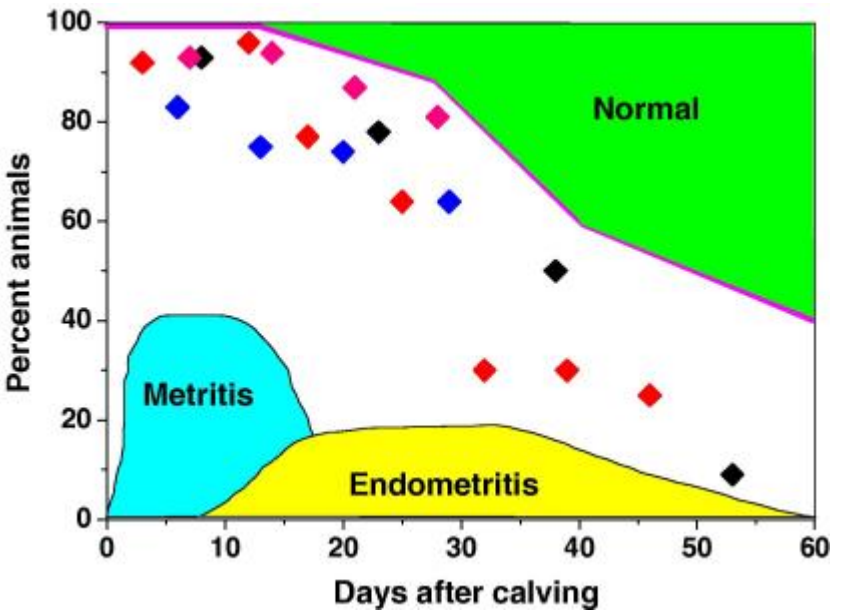

Fig 1. Pattern of uterine infection after calving (♦ indicate percent animals with bacteria in uterus)

Depending upon the immunity of the animals almost 60% animals are able to clear the infection by two months after calving, however in rest it leads to endometritis. Once endometritis gets established till breeding, it is bound to affect the conception rats and hence days open. So it becomes important to predict the development of endometritis, so that it can be prevented or treated well in time so as to maintain acceptable inter-calving

interval. There are number of ways to predict the development of endometritis and its prognosis, it this article the role of cytokine is discussed.

What are cytokines: Cytokine are the inflammatory mediators. Interleukin (IL)-1, IL- 6, IL-8 are major pro-inflammatory cytokines and IL-10 is the major anti-inflammatory cytokine. The development of postpartum disease depends on balance between pro-inflammatory and anti-inflammatory cytokines apart from balance between innate immunity of individual and uterine infection.

Role of cytokines in uterine health: The innate immunity of an individual involves (i) the perceiving the infection and (ii) eradication of infection (Beutler 2004). The animal response to infection involves the recognition of pathogens and/or their molecules by endometrial Toll Like Receptors (TLRs), release various inflammatory mediators and induction of effector mechanisms targeted at clearing contaminants.

Each infective agent has specific microbial molecules, also known as Pathogen Associated Microbial Pattern (PAMP), These are the ligands which are recognized by the endometrium, against which the animal is to react. If they are not recognized, there will not be any reaction in defense from the animal. For recognition of these ligands (PAMP's), endometrium has receptors called as Pattern recognition receptors (PRR). For example, if E coli infection is there and it produces endotoxin LPS, this LPS will be recognized by PRR, once recognised, the endometrial cell will get activated and cytokines and chemokines will be released from these cells. They will attract PMNL and activate them for phagocytosis.

Most important PAMPs are bacterial cell wall components, and most important recognition receptors (PRR's) are Toll Like Receptors (TLR). TLRs are numbered 1 to 10. Each TLR has ability to recognise a discrete set of ligands (PAMP's), as given in table;

TLRS	**Ligands (PAMPs)**	**TLRs**	**Ligands (PAMPs)**
TLR 1	Bacterial lipopeptide	TLR 6	Lipopeptide, Zymosan
TLR 2	Peptidoglycan,Lipopeptides, Zymosan	TLR 7	Single stranded RNA
TLR 3	Double stranded RNA (viral)	TLR 8	Single stranded RNA
TLR 4	Lipopolysacharide (LPS)	TLR 9	Unmethylated DNA (Bacterial)
TLR 5	Bacterial Flagellin	TLR 10	Unidentified bacterial molecules

Purified populations of endometrial epithelial cells express TLRs 1–7 and 9,and stromal cells express TLRs 1–4, 6, 7, 9 and 10. TLR 4 in particular is up regulated during endometritis and is considered an important mediator of postpartum uterine infection.

Apart from cytokines, various antimicrobial peptides (AMPs) also act as inflammatory mediators. Local tissue and vascular injury associated to microbial invasion of the uterus also induces release of vasoactive, pro-inflammatory and regulatory mediators. Vasoactive mediators cause dilatation and increased permeability of small local blood vessels where as chemokines and pro-inflammatory cytokines activate endothelial cells and leukocytes thereby facilitating the flux of PMNS and immune molecules to tissue sites of microbial invasion and injury. The cytokines IL-1, IL-6 and TNF α also stimulate production of phagocytosis enhancing mediators (Eicosanoiedes, Nitric oxide) and induce a systemic acute phase response involving hepatic acute phase proteins (APPs) production.
A complex feedback system balancing pro-inflammatory mediators (IL 1, IL 6, IL 8, TNF α, etc) and anti-inflammatory as well as tissue repair promoting mediators (IL 10, PG E2 and Complement Regulatory Protein, TGF β) is essential to help put a check on uterine inflammatory response to bacterial contamination in the postpartum cow.

Role of cytokine in predicting endometrial health: Cytokine concentrations or their mRNA expression in endometrium help to predict the impending uterine disease. During precalving period anti-inflammatory cytokine predominate while in post calving period, pro-inflammatory cytokines predominate. Accordingly, the anti- inflammatory cytokine will predict the impending disease and pro-inflammatory cytokines will diagnose the disease and give its prognosis.

Islam et al (2013) estimated IL-10 concentrations in blood of dairy cows in peripartum period (Fig 2) from15 days before calving till 30 days post-calving. The cows suffering from postpartum disease including retention of placenta, clinical metritis, clinical endometritis and delayed involution of uterus had different circulatory concentrations of IL-10, fifteen days before calving.

The cows having high IL-10 concentration (1956.96± 325.30 pg/mL) at day 15 before calving later on suffered from retention of placenta (IL-10 Conc 827.19 ±127.11pg/mL in normal cows). Similarly cows which suffered from clinical metritis after calving had significantly higher IL-10 concentrations at day 15 before calving (2283.01 ± 326.82 Vs 827.19 ±127.11pg/mL) compared to normal cows. The concentration of IL-10 was lower in cows with delayed involution of uterus than other groups of cows at all days of the periparturient period. The cytokine level in cows with delayed involution of the uterus was 328.906 ± 107.19, 263.08 ± 84.92, 415.26± 102.14, and 386.28 ± 111.11 on -15 d, 0 d, 15

d, and 30 d, respectively. The workers concluded that IL-10 can be used as a prognostic marker to identify the cows that are going to develop postpartum disease.

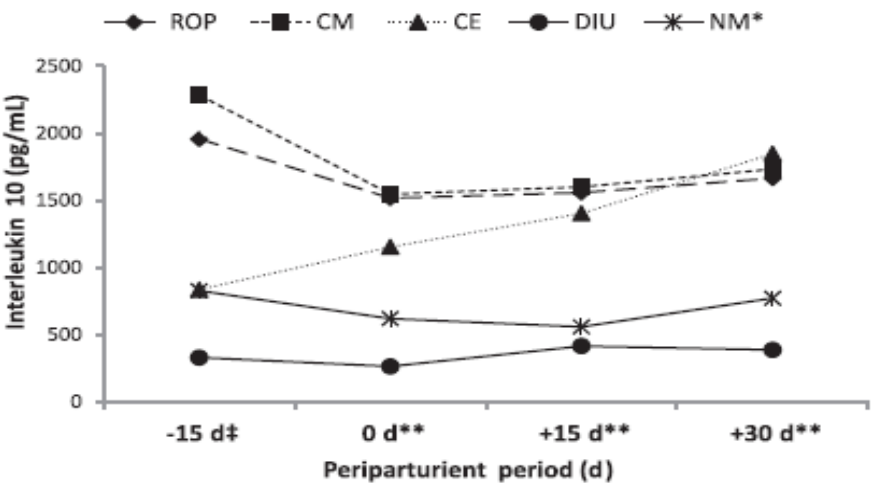

Fig 2: IL-10 concentrations in cows during peripartum period (ROP-retention of placenta; CM-Clinical endometritis; CE-Clinical endometritis; DIU-Delayed involution of uterus; NM-Normal cows)

Lower level of the anti-inflammatory cytokine IL-10 was recorded in mares, which were susceptible to endometritis as compared to resistant ones (Fumuso et al 2007). Since the

Role of cytokine in prognosis of endometrial disease: Pro-inflammatory cytokines can diagnose the disease and predict its prognosis as well. Mares susceptible to endometritis had higher endometrial mRNA expression of the proinflammatory cytokines IL-1, IL-6, IL-8, and TNF-a, compared with resistant mares (Fumuso et al 2007). After calving, cytokines expressions have been studied in normal calving and dystocia affected buffaloes (Urga 2014). The gene expression of pro-inflammatory cytokine IL-6, IL-8 and TNF-α were higher in dystocia affected buffaloes and further the buffaloes which developed postpartum disease including metritis and clinical endometritis. The cytokine gene expression at day seven postpartum could predict the development of clinical endometritis later on (Urga 2014). Since the microbial molecules are recognized through TLRs, some workers have reported increased TLR concentration in cytobrush flushing in cases of endometritis.

In conclusion, the cytokines are indicators of endometrial health and can be successfully used for prediction of occurrence of postpartum disease and its prognosis. The challenge remains to develop a cowside test based on the cytokine concentrations/gene expression.

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