**Final report**
**of**
**Major Research Project**

**UGC Reference No F No. 40-357/2011 (SR)**

***Final Report***

**Project Title:**

# Characterization and ultra-structural details of endotoxin induced laminitis in buffalo calf model

**University Grant Commission (U G C)**
**Bahadur Shah Zafar Marg,**
**New Delhi-110 002; India**

***Principal Investigator***

**Dr. R S Sethi,**
Professor,
GADVASU, Ludhiana
Punjab-141004

**UNIVERSITY GRANTS COMMISSION BAHADURSHAH ZAFAR MARG NEWDELHI-110 002**

**Annual/Final Report of the work done on the Major/Minor Research Project.**

| | | |
|---|---|---|
| 1. | Project Report | **Final** |
| 2 | UGC Reference No. & Date | **F No. 40-357/2011 (SR)**<br>**15-11-2011** |
| 3. | Period of the report | 1/7/2011 to 31/03/2015 |
| 4. | Title of the Project | Characterization and ultra-structural details of endotoxin induced laminitis in buffalo calf model |
| 5a. | Name of the Principal Investigator | Dr. R S Sethi |
| 5b. | Department and University/ College where the project has undertaken | College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana |
| 6. | Effective date of starting of the project | **01/07/2011** |
| 7. | Grant approved and expenditure incurred during the period of the report: | Total amount approved Rs: 9,77,032/-<br>Total expenditure Rs.: 9,62,598/- |

7c. Report of the work done: (Please attach a separate sheet)

| | |
|---|---|
| i | Brief objective of the project:<br><br>1. To establish the gross and histopathological changes in hoof corium during endotoxin induced laminitis in buffaloes<br>2. To elucidate the ultrastructural changes in claws during endotoxin induced laminitis in buffaloes<br>3. To investigate pathological process involved in endotoxin induced laminitis in buffaloes<br>4. To understand the process critical for initiation of an inflammatory cascade in hoof during endotoxin induced laminitis in buffaloes |
| ii | Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication |
| | **Morphological changes:**<br>The hooves of buffaloes calves (N=8) were collected from the slaughter house. The tissue samples from the sole-heel junction, abaxial wall, white line and coronary region were fixed in 10% Neutral buffered formalin solution. These tissues were processed by acetone benzene method to obtain paraffin blocks. The paraffin sections of 5µm thickness were obtained on Poly_L_Lycine coated clean glass slides. These paraffin sections were stained with haematoxylin and eosin for routine histopathology and morphology of the bovine hoof and with Periodic acid Schiff staining method to record observations on the integrity of the basement membrane.<br><br>**Sole:**<br>The papillae of the sole of buffalo calf were large and unbranched (Fig 1a). The cells of the normal papillae showed centric nuclei. The arteriole and other blood vessels in the corium of sole appeared normal.<br><br>The laminitis hoof showed disappearance of normal histoarchitecture of papillae. The papillary layer became almost flat (1b). The artery and arteriole of the sole corium showed |

hypertrophy of the tunica media, proliferation of the tunica intima and fibrosis of the tunica adventitia. (1c). The mono nuclear inflammatory cells were observed in the sole corium. (Fig. 1d).

**Abaxial Wall:**

Histology of normal hoof of buffalo calf revealed that stratum lamellatum of abaxial wall was composed of primary laminae as the secondary laminae were absent (Fig 1e). Further, thedDermal corium was seen interdigitating between primary laminae. At the base of the laminae the cells were cigar shaped and even in length, but as migration up the laminae occurred they became more oval in appearance (Fig 1f). The nuclei of the cells stained deeply with haematoxylin and eosin and the nucleoli were usually situated at the periphery of the nucleus. The basement membrane of the laminae was intact, without any discontinuation or retraction and showed positive PAS reactivity. The keratogenous zone was observed in the wall of the lamina.

However, the laminitis hoof showed discontinuation in the basement membrane and retraction of laminae (Fig. 1g). Epidermal laminae showed hyperplasia (1g inset). There was disappearance of keratogenous zone from wall of the lamina, however dermal and epidermal laminae were intact.

**Immunohistochemistry:**

The immunohistochemical techniques for macrophages, TNF-α, IL-1β, IL8, TLR4 and TLR9 were standardized. Briefly, the sections were first deparaffinized with different steps in xylene, dehydrated in different concentration of ethanol, incubated with 3% $H_2O_2$ for 20 min to quench endogenous peroxidase. Following incubation with $H_2O_2$ the sections were processed through antigen retrieval with either exposure to pepsin (2 mg/ml 0.1N HCl) or the microwaving of the sections in Tris EDTA buffer (pH 9.0). The slides were incubated in dark chamber with 1% BSA and the sections were stained with primary antibodies against vWF (Rabbit polyclonal vWF; M0616; Dako; dilution 1:600), macrophage (mouse monoclonal macrophages; MCA874G; AbD Serotec; dilution 1:50), IL8 (Rabbit polyclonal IL8; H-60; sc7922; dilution 1:100), IL-1β (Rabbit polyclonal IL-1β; H-153; sc7884; dilution 1:100),

TNF-α (goat polyclonal TNFα; N-19; sc1350; dilution 1:100) and TLR9 (mouse polyclonal TLR9; Imgenex; dilution 1:100) to identify immunopositive endothelial cells, macrophages, IL8, IL-1β, TNF-α and TLR9 cells, respectively. The incubation with primary antibodies was followed by appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Polyclonal rabbit anti goat; P0449; dilution 1:800; DAKO A/S, Denmark, Polyclonal goat anti mouse; P0447; dilution 1:800; DAKO A/S, Denmark and Polyclonal goat anti rabbit; P0448; dilution 1:800; DAKO A/S, Denmark). The reaction was visualized using a color development kit (SK4600, Vector laboratories, USA). The sections were counterstained with heamatoxylin. Immunohistochemical controls included emission of primary antibodies and staining with only secondary antibody and staining with anti-von Willebrand factor antibody to stain the endothelial cells. The emission of primary and/or secondary antibodies did not result any colour development in the hoof sections. The large blood vessels of normal hoof appeared normal with intact endothelial cells (Fig. 3a), however the blood vessels of the inflamed hoof showed eruption of endothelial cells (Fig. 3b). The sole corium of inflamed hoof showed inflammatory reaction and was infiltrated by mononuclear inflammatory and large cells. The large cell showed immunopositive reaction for macrophages (Fig.3c). However, occasional or no macrophage was detected in the normal hoof. The inflammatory cells showed immune-reactivity for IL-8 (Fig. 3d), IL-1b (Fig. 3e), TNF-a (Fig. 3f) and TLR-9 (Fig. 3g).

**Expression of TLR4 mRNA:**

Tissue samples from control and inflamed hooves stored in RNA later solution at -80°C were used for detection of expression of mRNA of TLR4 by Real Time PCR. About 100 mg of frozen hoof tissue was homogenized using magnetic beads and total RNA was extracted from all the samples using Trizol (Ambion, Life Technologies, USA) method following manufacturer's instructions. The quality as well quantity of the resulting RNA was assessed by spectrophotometrically by Nanodrop (Thermo Fisher) and also by visualizing the ribosomal RNA bands via agarose gel electrophoresis. The RNA samples having $OD_{260/280}$ range from 1.9 to 2 were quantified and were used to reverse transcribed. The concentration of total RNA varied between 1500-3800 ng/ µl in different samples. The amount of total RNA

used for cDNA synthesis was adjusted to 400 ng/ µl for each sample.

Total RNA was reversed transcribed into cDNA using a Revert aid cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instruction. The Real time PCR was performed using ABI 7500, ABI Life Technologies, USA. The real-time PCR reaction was carried out using TaqMan chemistry (Table 1) in duplicates and ß-actin was used as endogenous control. The ingredients of 20µl final volume PCR mixture was containing 1X TaqMan master mix (TaqMan universal master-mix II with UNG), primer-probe mix (900 nM of each primer and 200nM of probe) and template cDNA (2µl). The real time amplification was conducted with the following steps: 2 min of uracil inactivation phase (50°C), 10 min of initial denaturation (95°C), followed by 40 cycles (15 sec each) of denaturation (95°C), and finally, annealing coupled with extension (60°C for 1 min). Threshold Cycle (Ct) values were calculated using the SDS software v.2.3 (Applied Biosystems, Life technologies, USA) with automatic baseline settings at threshold of 0.2. Results were standardized to control vs. exposed animals and given as relative fluorescence over control mRNA level (fold difference) after correction for expression of ß-actin.

**Quantitative RT- PCR:**

The cDNA from hoof tissue was used for quantitative RT-PCR analysis for the expression of TLR4 genes in the control and inflamed hoof samples by using SYBR Green PCR kit (Qiagen) as per manufacturer's instructions. The Beta- Actin was used as reference housekeeping gene. The reaction was performed using primer pair: 5'-TGCTGAGTTTCTGATCCATGC-3' and 3' TGGCTAGGACTCTGATCATGG- 5'for TLR4. The Real time PCR was performed using ABI 7500, ABI Life Technologies, USA. Results were standardized to control- exposed animals and given as relative fluorescence over control mRNA level (fold difference) after correction for expression of Beta-Actin. The inflamed hoof showed almost six fold increase in the mRNA expression of TLR4 compared to normal hoof (Fig 3h).

**Transmission Electron Microscopy:**

Hoof tissues (corium) collected from buffalo calves were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer solution at pH 7.2 at 4ºC for 8-12 hours and processed for transmission electron microscopy. Briefly tissue samples fixed in 2.5% gluteraldehyde were rinsed in 0.1M phosphate buffer solution (pH 7.2) with 3 changes at 4ºC. The samples were post fixed with 2% osmium tetraoxide, dehydrated followed by embedding in the epoxy resin. The transmission electron facility at Punjab Agricultural University, Ludhiana, Punjab was used to observe the ultrastructural details. The sole of normal hoof showed unbranched papillae (Fig. 4a, b), however the inflamed hoof showed retracted papillae (Fig 4c).

**Conclusions**

It's the first data on the immunolocalization of macrophages, various pro-inflammatory cytokines, TLR4 and TLR9 in the normal and inflamed hoof of buffalo calves. The data suggest that there is increase in the immunopositivity for various pro-inflammatory cytokines along with TLR4 and TLR9 in the inflamed buffalo calf hoof indicating that activation of TLRs during the inflammation of hoof in the buffalo calf.

| | | |
|---|---|---|
| iii | Has the progress been according to original plan of work and towards achieving objectives if not, state reasons | The 3rd objective was modified and more number of slaughter house samples were included. Further mRNA expression of TLR4 gene by Real Time PCR was also included. |
| iv | Please indicate the difficulties, if any, experienced in implementing the project | NA |
| v | If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis) may please be sent to the Commission on a separate sheet: NA | |
| vi | If the project has been completed, please enclose a summary of the findings of the study. Two bound copies of the final report of work done may also be sent to the Commission: | |

| | |
|---|---|
| | Two copies of the reports attached |
| vii | Any other information which would help in evaluation of work done on the project. At the completion of the project, the first report should indicate the output, such as (a) Manpower trained (b) Ph. D. awarded (c) Publication of results (d) other impact, if any<br><br>Manpower trained: One<br><br>Publications<br><br>1. **Sethi R S**, Nidhi, Mukhopadhyay CS, Randhawa S S, Singh J, Singh O, Verma Ramneek and Singh S S. 2016. Immunolocalization of pro-inflammatory cytokines in normal and inflamed hoof of buffalo calves. *International Journal of Advanced Research* **4 (5):** 604-613.<br><br>2. Sethi RS, Nidhi Kumari, Singh O, Randhawa SS and Singh SS. 2012. Histological and immunohistochemical studies on the hoof of buffalo calf. Presented in the proceedings of XXVII annual convention of IAVA and National Symposium on "Advances in Applied Anatomy of Domestic and Wild Animals-an interdisciplinary approach for Animal Health and Wealth" organized by Department of Anatomy, College of Veterinary Science, Mannuthy, Thrissur, Kerala |

**PRINCIPAL INVESTIGATOR**
**(SIGNATURES WITH SEAL)**

**DIRECTOR COLLEGE OF ANIMAL BIOTECHNOLOGY**
**(SIGNATURES WITH SEAL)**

**DIRECTOR OF RESEARCH**
**(SIGNATURES WITH SEAL)**

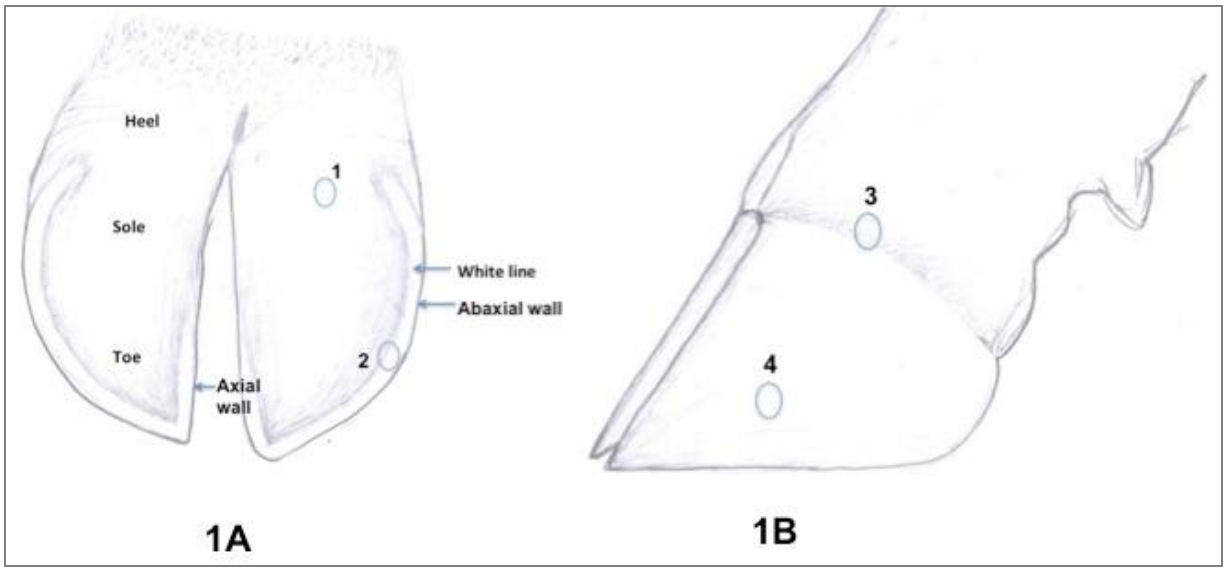
**Legend to the figures**

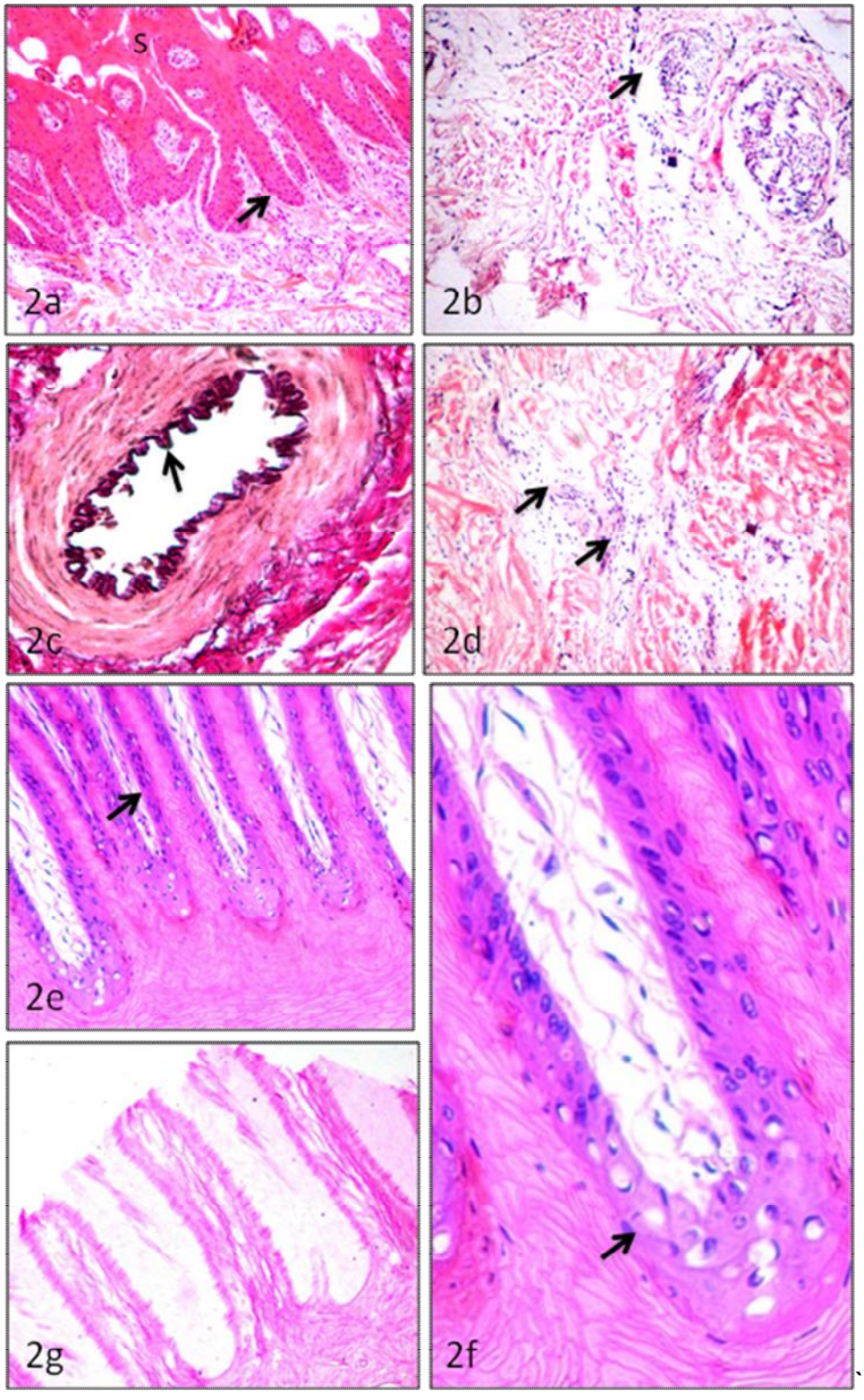
Fig. 1: Drawing of solar (A) and abaxial (B) view of buffalo hoof showing sites of sample collection viz. sole heel junction (1), white line (2), coronary region (3) and abaxial wall (4).

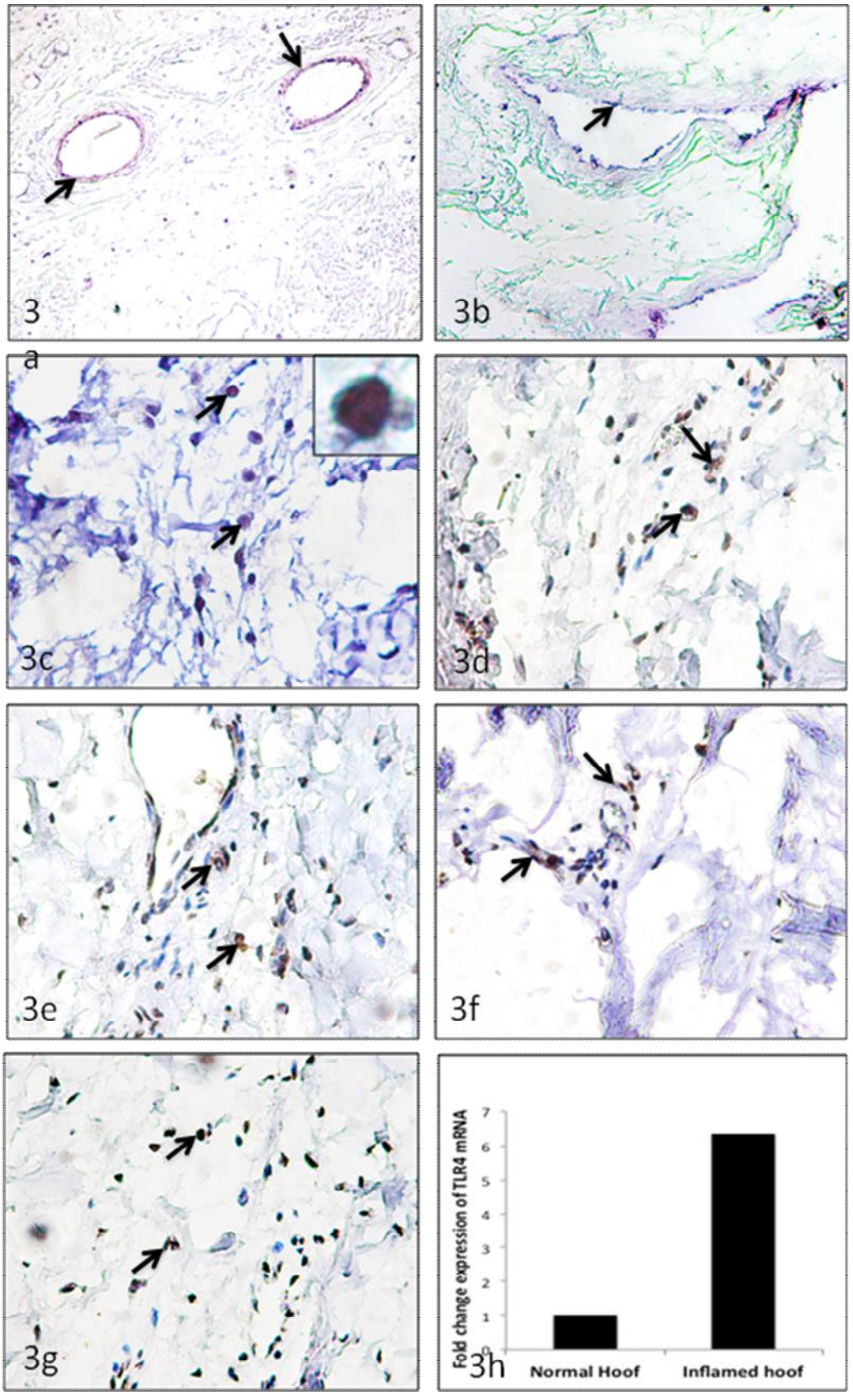
Fig 2: Sole papillae in normal hoof showing dermal papilla (*), epidermal papilla (arrow) and stratum spinosum (s). (Fig 2a); the inflamed hoof showing flat papillary layer (2b), thickening of the endothelial cells (arrow) and tunica media (*) (2c) and infiltration of the mono nuclear inflammatory cells (arrows) in the sole corium (Fig. 2d); normal dermal and epidermal laminae with intact basement membrane (arrow) and keratogenous zone (stars) (2e); inflamed hoof showing loss of basement membrane (arrow) and keratogenous zone (star) along with hyperplasia of epidermal laminae (inset); Fig 2a-b, 2d-e and inset; HE X20; Fig 2c: Von Gieson X 20; Fig 2f: PAS X 20.

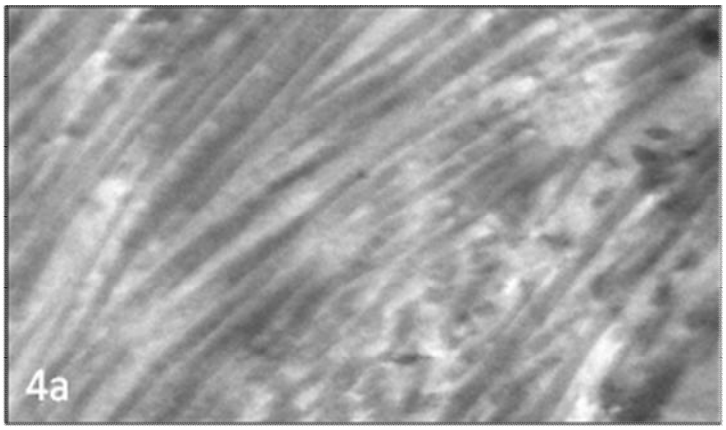
Fig 3: Hoof samples stained without primary antibody did not show any colour development (Fig 3a), VWF staining was localized in the endothelium of the blood vessels (Fig. 3b), sole corium of inflamed hoof showing presence of immunopositive macrophage (Fig. 3c), IL-8 (Fig. 3d), IL-1b (Fig. 3e), TNFa (Fig. 3f) and TLR-9 (Fig. 3g); fold change expression of TLR4 mRNA (Fig. 3h).

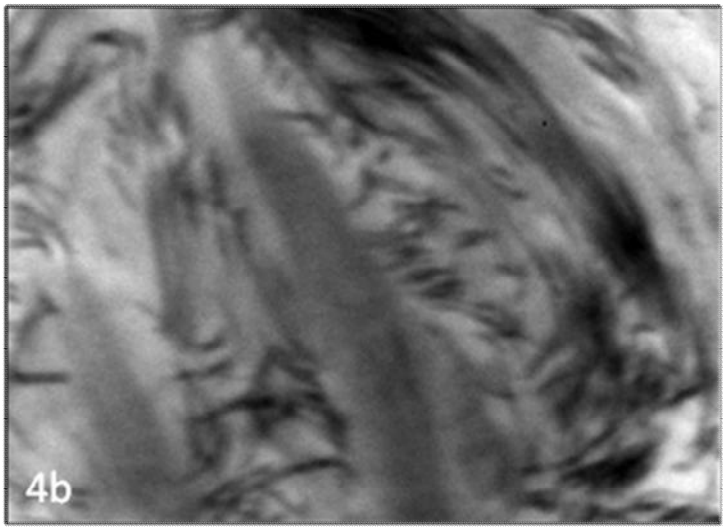
Fig4. Ultrathin section of normal hoof showing unbranched papillae of the sole (4a,b) and retracted papillae of the inflamed hoof (4c).

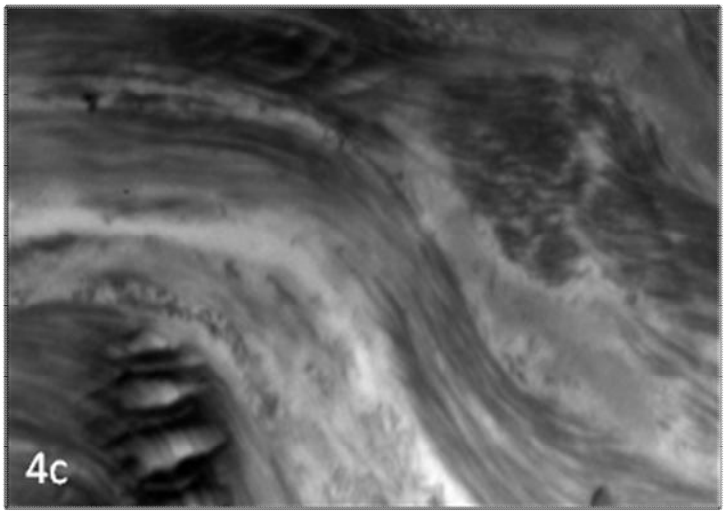

Heel
Sole
Toe
1
2
White line
Abaxial wall
Axial wall
3
4
1A
1B


s
2a
2b
2c
2d
2e
2f
2g


3
3b
a
3c
3d
3e
3f
3g
3h
Fold change expression of TLR4 mRNA
0
1
2
3
4
5
6
7
Normal Hoof
Inflamed hoof


4a


4b


4c