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RESEARCH ARTICLE

Immunolocalization of pro-inflammatory cytokines in normal and inflamed hoof of buffalo calves.

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Abstract

Laminitis is characterized by inflammation of the sensitive laminae of the hoof, loss of lamellar basement membrane and infiltration of mononuclear inflammatory cells in the laminar tissue. Buffaloes commonly suffer from subclinical mastitis resulting insidious economic losses to the farmers in terms of decreased milk yield and weight gain and increased cost of treatment. Effective tools to treat lameness in buffaloes are still lacking due to incomplete understanding of its complex patho-physiology. Moreover, there is paucity of data on the *in situ* localization of macrophages, pro-inflammatory cytokines, TLR-4 and TLR9 in inflamed hoof of buffalo calf. The present study identifies immunopositive endothelial cells, macrophages, IL8, IL-1β, TNF-α and TLR9 cells in the normal and inflamed hoof of buffalo calves. Labeling of PIMs with the anti-human macrophage antibody (MCA874G) demonstrated frequent occurrence of these cells in inflamed hoof. Further, there was increased immunopositive expression of IL8, IL-1β, TNF-α and TLR9 cells in the inflamed hoof of buffalo calf. TLR4 mRNA expression showed almost six fold significant increase ($p<0.05$) in the inflamed hoof compared to normal hoof. We present 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 taken together 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 may contribute significantly to the pathogenesis of hoof inflammation in buffalo calves.

Introduction:-

Buffalo is an economically important animal in India and South East Asia. It contributes to economic well being of millions of small livestock farmers in India by providing meat, milk, manure, and draught power(Das *et al.*, 2008). Lameness in buffaloes results substantial economic losses to the farmers in terms of decreased milk yield and weight

gain and cost of treatment. Although precise economic losses due to lameness in cattle and buffaloes in India are not available, we can get a sense of their magnitude in India from the fact that lesions associated with laminitis were observed in about 50 percent of cattle and buffaloes (Randhawa*et al.*, 2014).
Laminitis is characterized by inflammation of the sensitive laminae of the hoof, loss of lamellar basement membrane and infiltration of mononuclear inflammatory cells in the laminar tissue. It is primarily a peripheral vascular disease manifested by reduced capillary perfusion within the foot, a significant amount of arterio-venous shunting, ischaemic necrosis of epidermal laminae and pain (Singh *et al.*, 1994). Despite increased understanding of the pathogenesis of lameness, the economic damage caused by the disease is still significant. The fundamental reason for this is incomplete understanding of complex patho-physiology of laminitis. Therefore, effective tools to treat lameness in buffaloes are lacking.

Endotoxins, lactic acid and histamines are considered to play significant role in the etiology and pathogenesis of laminitis(Anderson, 1981; Singh, 2001). There is high incidence of laminitis and sole lesions in the dairy animals suffering from endotoxemia associated with metritis, mastitis, carbohydrates overload (Dougherty *et al.*, 1975; Haalstra, 1989)and endotoxin administration (Mortensen *et al.*, 1986; Singh *et al.*, 1994). On the other hand, the local factors such as weight distribution, body and foot conformation, concrete flooring etcindicate some role of local mechanism in the pathogenesis of laminitis (Singh *et al.*, 1993).

Toll-like receptors (TLR) are responsible for innate host responses to pathogens and TLR4 is critical for the ligation and endocytosis of lipopolysaccharides (LPS) released from bacteria during endotoxemia (Elson *et al.*, 2007). Further, TLR9 binds bacterial DNA to activate of cell signaling which further result NF-κB activation and elaboration of various pro-inflammatory cytokines (Palsson-McDermott and O'Neill, 2004). Interleukins (IL) like IL-8 is a potent chemo-attractant for neutrophil recruitment and a reduced expression of this chemokine may underlie the reduced accumulation of neutrophils during inflammation (Gerszten *et al.*, 1999; Kidney and Proud, 2000; Kubes, 2002; Patel *et al.*, 2002). Tumor necrosis factor (TNF) and platelet activation factor also play a significant role in the pathogenesis of laminitis (Anderson, 1981).

Inflammation is one of the key events which has been thoroughly described in the laminar tissue in experimental models of laminitis in horse (Fontaine *et al.*, 2001; Waguespack, Cochran, *et al.*, 2004; Blikslager *et al.*, 2006; Hurley *et al.*, 2006; Belknap *et al.*, 2007; Leise *et al.*, 2011) and dairy cattle (Dougherty *et al.*, 1975; Singh *et al.*, 1993; Singh *et al.*, 1994). It has also been established that macrophages along with endothelial cells are classically sentinel and clearance cells associated with initiating the inflammatory and vascular changes (Gordon, 2007). However, there is no data on the *in situ* localization of macrophages, pro-inflammatory cytokines, TLR-4 and TLR9 in inflamed hoof of buffalo calf, hence, the present study was undertaken.

Materials and methods:-

The normal and inflamed hooves (N=8; each) from buffalo calves were collected from the slaughter house. The tissue samples from the sole-heel junction, abaxial wall, white line and coronary region (Fig. 1) were collected and fixed in 10% Neutral buffered formalin solution. The tissue samples were also chopped and placed in RNA later solution and was stored at -80°C until further use for detection of expression of mRNA of TLR4 by quantitative RT-PCR.

Histopathology:-

These tissues were processed by acetone benzene method to obtain paraffin blocks (Luna, 1968). The paraffin sections of 5µm thickness were obtained on Poly L Lycine coated clean glass slides. These paraffin sections were stained with hematoxylin and eosin for routine histopathology and morphology of the bovine hoof and with Van Gieson elastin stains (Luna, 1968) to demonstrate endothelial lining of the blood vessels. The inflamed hooves were confirmed based on the histopathological observations.

Periodic Acid Schiff Staining:-

Periodic Acid Schiff (PAS) staining was done for demonstration of integrity of the basement membrane. The paraffin sections were incubated at room temperature with 0.5% periodic acid solution followed by Schiff's reagent as per the manufacturer's protocol. Finally the slides were washed under tap water and rehydrated. Basement membrane was stained dark pink.

Immunohistochemistry:-
The immunohistochemistry was carried out as described previously (Sethi *et al.*, 2013). Briefly, the sections were first deparaffinized, dehydrated, incubated with 3% H_2O_2 for 20 min to quench endogenous peroxidase and 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; AbDSerotec; 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. Controls consisted of staining without primary antibody or secondary antibody or both.

Quantitative RT- PCR:-
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. 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 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 (Singh et al., 2015). 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.

Statistical Analysis:-
Data for TLR4 mRNA were presented as mean (±SE) and appropriate transformations were made. The data was analyzed by one way analysis of variance followed by group comparisons by Tukey's test ($P<0.05$).

Results:-
Histopathology:-
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. The inflamed 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 mononuclear inflammatory cells were observed in the sole corium. (Fig. 1d)

Histology of normal hoof of buffalo calf revealed that stratum lamellatum of abaxial wall was composed of primary laminae and the secondary laminae were absent (Fig 1e). Dermal 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 stained deeply with hematoxylin and eosin and

the nucleoli were usually situated at the periphery of the nucleus. The basement membrane of the laminae was intact and showed positive PAS reactivity.

However, the inflamed 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 emission of primary and/or secondary antibodies did not result any colour development (data not shown). 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-1β (Fig. 3e), TNFα (Fig. 3f) and TLR9 (Fig. 3g) in the inflamed hoof.

Expression of TLR4 mRNA:-
TLR4 mRNA expression was observed in normal hoof. However, the inflamed hoof showed more than six (6.37) folds significant increase ($P<0.05$) in the mRNA expression of TLR4 compared to normal hoof (Fig 3h).

Discussion:-

It is the first data on the *in situ* presence of macrophages, immunolocalization of various pro-inflammatory cytokines (IL8, IL-1β and TNF-α), TLR4 and TLR9 in the normal and inflamed hoof of buffalo calves. The information is of potential importance in understanding the complex pathophysiology of the lameness.

The normal hoof showed presence of only primary laminae with interdigitating dermal corium and intact PAS positive basement membrane. There was disappearance of normal histoarchitecture of papillae in the inflamed hoof. The papillae were almost flat along with hypertrophy of the tunica media, proliferation of the tunica intima and fibrosis of the tunica adventitia in the artery and arteriole of the sole corium. Arteriosclerosis with varying degree of proliferation of tunica media of arterioles has also been observed in corium biopsy from digits with sole hemorrhages in four out of five adult buffaloes (Randhawa et al., 2012). Loss of the normal orientation and ovoid shape of basal cell nuclei may also result from cytoskeletal compromise (de Laat *et al.*, 2013). Further, the inflamed buffalo hoof showed discontinuation in the basement membrane and retraction of laminae. The loss of integrity of basement membrane has also been reported previously during laminitis (Pollitt, 1996; Belknap *et al.*, 2007; de Laat *et al.*, 2013). Cytoskeletal compromise results physical disruption of the basement membrane during laminitis that may be due to the role played by matrix metalloproteases in the lamellar breakdown (Johnson *et al.*, 1998; Katwa *et al.*, 1999; Mungall and Pollitt, 1999).

The large blood vessels of normal hoof appeared normal with intact endothelial cells, however the blood vessels of the inflamed hoof showed eruption of endothelial cells. Alterations in endothelial cells have been reported during laminitis in horse (Eades *et al.*, 2007). The end result of altered endothelial function during laminitis may be creation of a pro-inflammatory state (Eades, 2010).

There was occasional immunopositive macrophage in the normal hoof. We have previously immunolabelled pulmonary intravascular macrophages in buffalo lungs by using same primary antibody (Sethi *et al.*, 2011) indicating ability of the antibody to recognize correct epitope in buffalo tissue. Further, the sole corium of inflamed hoof showed inflammatory reaction and was infiltrated by mononuclear inflammatory and frequent large cells. The large cell showed immunopositive reaction for macrophages. The macrophages have been identified in the healthy horse hoof and there was increased population of immunopositive macrophages during of BWE induced laminitis in horse (Faleiros, Nuovo, *et al.*, 2011). Macrophages are extremely dynamic cells with a wide phenotypic heterogeneity (Gordon and Taylor, 2005) and capacity to initiate the acute inflammatory and vascular changes (Gordon, 2007). These cells can assume phenotypes ranging from pro- to anti-inflammatory subtypes depending on their localisation and the type and intensity of the stimulus in pathological situations (Gordon and Taylor, 2005; Mosser and Edwards, 2008; Glaros *et al.*, 2009).

The mononuclear and large infiltrating cells showed increased immune-reactivity for important pro-inflammatory

cytokines like IL-1β, IL-8 and TNFα in the inflamed hoof. Cytokines play a vital role in leukocyte activation and emigration that reportedly plays a central role in laminar injury in equine laminitis (Faleiros, Leise, *et al.*, 2011). There is up-regulation of IL-1β, IL-8 (Belknap *et al.*, 2007) and IL-6 (Boontham *et al.*, 2003) along with COX-2 (Waguespack, Cochran, *et al.*, 2004) and signaling protein NF κB(Waguespack, Kemppainen, *et al.*, 2004) in the developmental period of the black walnut extract (BWE) model of laminitis. Bacterial ligands and other cytokines result expression of IL-8 in various cell types including endothelial cells (Boontham *et al.*, 2003). IL-8 is a key mediator in the neutrophil emigration into the hoof lamellae (Black *et al.*, 2006).

In the present study there was simultaneous increase in immune-positivity for IL-1β, IL-8 and TNF-α in the inflamed hoof specially in the mononuclear infiltrating cells. The increased expression of IL-1β may be downstream of an IL-8 induced neutrophil activation and emigration during laminitis (Fontaine *et al.*, 2001; Black *et al.*, 2006). Further, TNF-α expression in the face of increased IL-1β and IL-6 expression is usually reported in sepsis and other causes of systemic inflammation (Cohen, 2002; Bhatia and Moochhala, 2004). There was significant increase in the mRNA expression of TNF-α along with IL-1β, IL-6, IL-8, P-selectin and L-selectin during carbohydrates overload induced laminitis in horses (Kwon *et al.*, 2013). The increased expression of these pro-inflammatory cytokines suggests involvement of innate immune response.

Toll-like receptors (TLR) play central role in the pathophysiology of inflammation (Devaraj *et al.*, 2011) and regulation of the innate immune response (de Laat *et al.*, 2014). We report the presence of TLR4 mRNA in the hoof of normal buffalo calves. TLR4 protein has also been reported in the lamellar tissue of hoof of healthy horse (de Laat *et al.*, 2014). In the present study, there was significant increase in the mRNA of TLR4 along with increased TLR9 immunopositive reactivity in the inflamed buffalo hoof. TLRs presence indicates that inflammatory pathways activated by TLRs are of potential significance to the lamellar structure (de Laat *et al.*, 2013; de Laat *et al.*, 2014). The data taken together suggest increased expression of pro-inflammatory cytokines along with TLRs in the inflamed hoof. Our study does not explain the mechanism(s) or pathway(s) involved but suggests that activation of TLRs may contribute significantly to the development of pathogenesis of hoof inflammation in buffalo calves which requires further exploration in a laminitis induced model of buffalo calf.

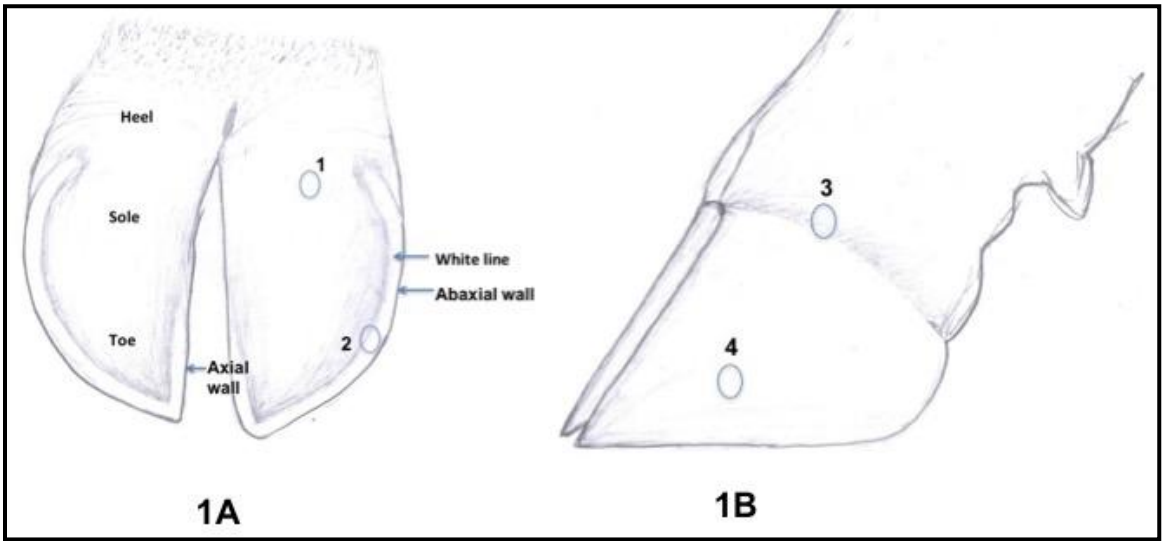

Fig. 1: Drawing of solar (A) and abaxial (B) view of buffalo hoof showing sites of sample collection viz. sole heel junction (1), white line (2), coronary region (3) and abaxial wall (4).

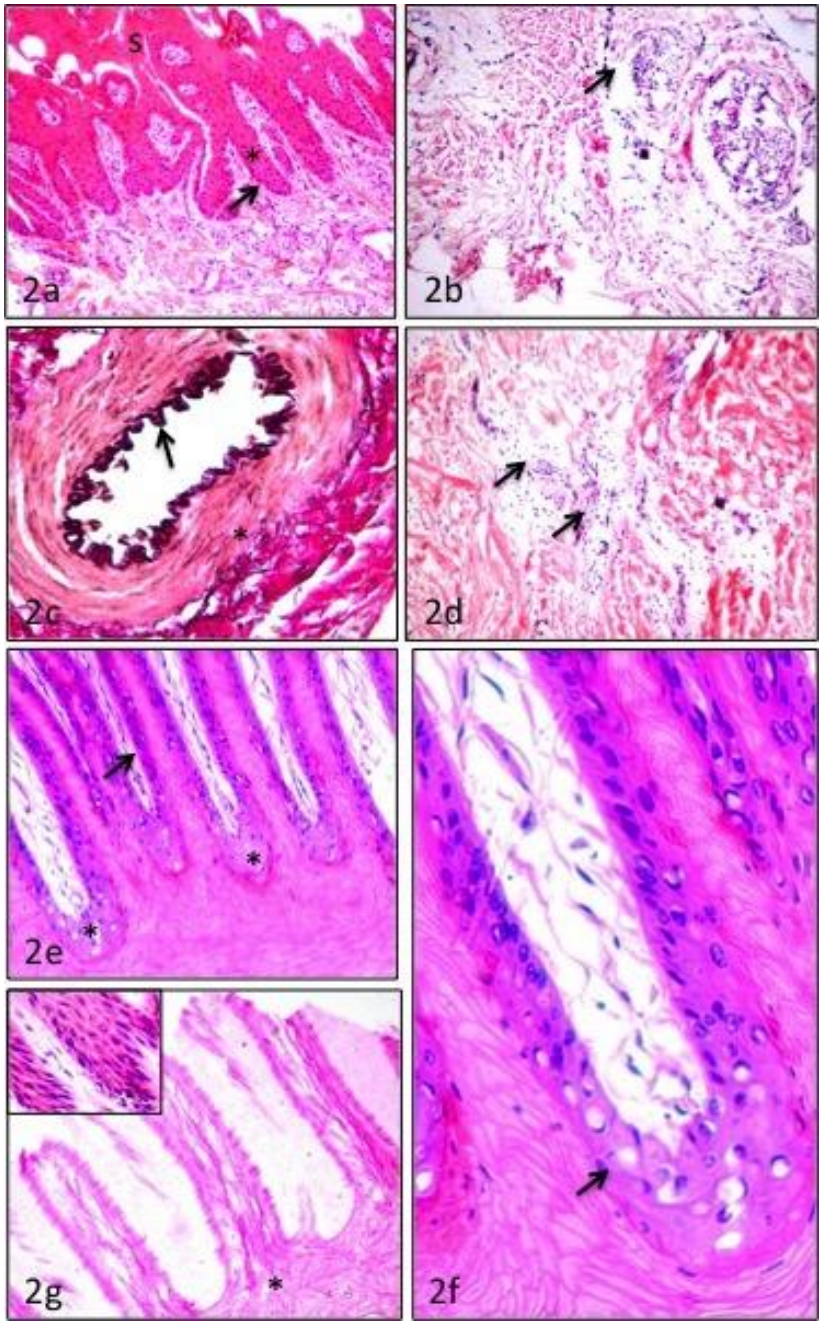

Fig 2: Sole papillae in normal hoof showing dermal papilla (*), epidermal papilla (arrow) and stratum spinosum (s). (2a); the inflamed hoof showing flat papillary layer (2b), thickening of the endothelial cells (arrow) and tunica media (*) (1c) and infiltration of the mono nuclear inflammatory cells (arrows) in the sole corium (Fig. 1d); normal dermal and epidermal laminae with intact basement membrane (arrow) and keratogenous zone (stars) (1e); inflamed hoof showing loss of basement membrane (arrow) and keratogenous zone (star) along with hyperplasia of epidermal laminae (inset); *Original magnifications2a-b, 2d-e and inset; HE X20; Fig 2c: Von Gieson X 20; Fig 2g-f: PAS X 20.*

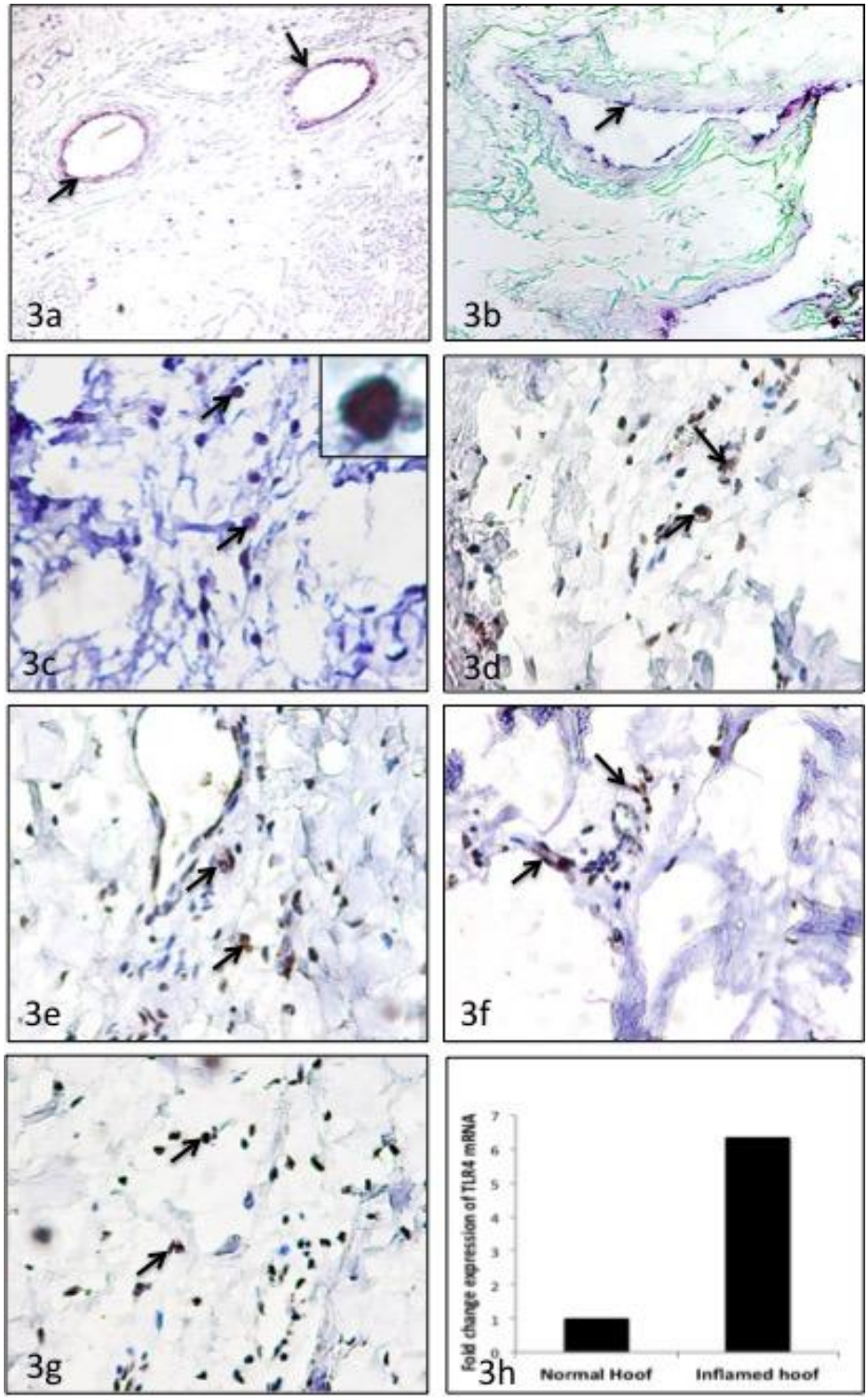

Fig 3: Hoof samples form normal buffalo calf stained with vWF showing vWF immunopositive (arrows) normal endothelial cell lining (3a) and eruption of endothelial cells (arrows) in inflamed hoof (3b); sole corium of inflamed hoof showing presence of immunopositive reaction (arrows) for macrophage (3c), IL-8 (3d), IL-1β (3e), TNFα (3f) and TLR-9 (3g); fold change expression of TLR4 mRNA (3h); *Original magnifications 3a-g 20X; inset 100X*

Table 1: Sequences* (5'to 3') of the Assay by Design (ABD) for the probe-primer mix for TaqMan Assay of the target-gene bubaline TLR4 and the endogenous control ß-actin.

Primer Name	Forward Primer (5'-3').	Reverse Primer(5'-3').	Reporter Sequence (Dye-FAM)(5'-3')
TLR4	Tgagcttcaatgatgtcattaccttagg	gaagatccaggtgttctagttgct	aagcccatgaagtttg
ß-actin	Cacggtgcccatctacga	Gccagccaggtccagac	cccatgccatcctgc

* The above sequences are the proprietary items of the manufacturing agency Invitrogen-Life Technologies (Applied Biosystems Life technologies, USA)

Conclusions:-

It's the preliminary data on the immunolocalization of macrophages, various pro-inflammatory cytokines, TLR4 and TLR9 in the normal and inflamed hoof of buffalo calves. We conclude 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 may contribute significantly to the development of pathogenesis of hoof inflammation in buffalo calves.

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Conflict of Interest Statement:-

There is no conflict of interest.

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