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Collagen Fibril Morphological Study of Medial Patellar Ligament of Stifle Joint Affected with Patellar Luxation in Cattle

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ABSTRACT

Background: Upward fixation of patella is common clinical malady in cattle due to extensive movement of stifle joint during the locomotory activity because of unknown etiology. However, at certain peak point (overstretching of ligament) or due to occupational trauma (wear and tear), ligament become flaccid and weak leading to patellar displacement and lameness. Patellar ligaments play crucial role in stifle joint movement and any functional or structural alterations in the ligaments can lead to acute to chronic lameness in cattle. The present study was carried out to evaluate the structural changes in collagen fibril material in medial patellar ligament in cattle suffering with patellar luxation.

Methods: Tissue specimens of medial patellar ligament of normal (control animal) and affected cattle (luxation of patella) were harvested and preserved for scanning electron microscopy (SEM) and transmission electron microscopic (TEM) study as per standard procedure at RUSKA Laboratories, College of Veterinary Science, PVNRTVU, Rajendranagar, Hyderabad, India.

Result: The incidence of medial patellar luxation was higher in females as compared to male cattle and the duration of lameness was between 3-12 months (7.35±0.56 months) characterized by dragging of toe, outward jerky movement of affected limb, extension of limb and change in appearance of gait during walk and flexion of fetlock. Scanning electron study of normal ligament showed dense, uniformly and compact arrangement of collagen fiber bundles with morphometric thickness was 3.66±0.58 µm. whereas in luxated patella cases, medial ligament showed derangement in collagen fiber bundles. The collagen fibers were arranged irregular and wavy with morphometric thickness of collagen was 2.58±0.03 µm. Electron microscopy of the normal ligament showed presence of thick collagen fiber bundles packed in dense and compact arrangement. Electron microscopy of affected ligament had degenerative changes of the fibers characterized by thin collagen fiber bundles which were loosely and disorganized arranged fibrils. The present study concludes that, luxation of patella was common locomotory disorders in cattle due to derangement in collagen fiber bundles with drastic reduction in thickness of collagen fibers, increased gap between two collagen fibers, broken and overlapping fiber bundles and surface of collagen fibers appeared irregular rough via scanning and electron microscopy analysis.

Key words: Cattle, Collagen, Electron, Fibril, Medial ligament, Morphometry, Patella, Scanning.

INTRODUCTION

In Indian context, marginal farmers at rural area prefer free grazing system to cattle and buffaloes on economic background especially, in western part of Maharashtra. Due to extensive movement of animals during grazing, locomotory system gets adopted with these types of various occupational trauma. However, at certain peak point (overstretching of ligament) or due to trauma (wear and tear), ligaments become flaccid and results into displacement of patella. Patellar ligaments play crucial role in stifle joint movement and any functional or structural alterations in the ligaments can lead to acute to chronic lameness in cattle. Ligaments are specialized connective tissues with very interesting biomechanical properties *i.e.* tensile strength to bear excessive forces. Injury to a ligament results into a drastic change in its ground substances by restoring function through replacing normal tissue with scar tissue (Frank, 2004). This locomotory affection elicits pain in the day to day physical activities of animal which causes reduction in drafting ability of animal and loss of milk production leading to great economic losses of farmers (Alfars, 2007).

Collagen fibril morphology and its organization, its implications for force transmission in ligaments and tendons and concluded that, collagens is the primary structural elements of the extracellular matrix, are the most abundant proteins in tissue such as ligament, tendon, cartilage, bone, cornea and skin. Type I collagen assembles via collagen molecules into collagen fibrils which are long filamentous

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structures, aggregate to form collagen fibres (Nimmi and Harkness, 1988). Microscopic analysis of mature tendon and ligaments and detected no fibril ends which indicated that, fibrils in mature tendon and ligament are either continuous or functionally continuous and concluded that, the force within these tissues is directly transferred through collagen fibrils and not through interfibrillar coupling (Provenzano and Vanderby, 2006).

Transmission electron study of patellar ligament with patellar luxation in dogs and revealed that, dogs affected with patellar luxation exhibited unevenly distributed collagen fibres (103.67±15.58 nm) with marked gaps between the collagen fibres abnormalities (Ueda *et al.*, 2018). The present clinical study was conducted on twenty clinical cases of cattle suffering with patellar luxation due to the micro-structural changes in collagen fibril material of medial patellar ligament of stifle joint via electron microscopy study.

MATERIALS AND METHODS

Out of 38, 20 (52.63%) clinical cases of cattle were presented to the Veterinary College Hospital, KNP College of Veterinary Science, Shirwal, Satara, Maharashtra with history of unilateral or bilateral hindlimb lameness due to acute or chronic upward luxation of patella and they were examined clinically as well as physically to rule out the concurrent causes of Hindlimb lameness during the year 2018-19. All animals were subjected to clinical, physical and surgical intervention (desmotomy) under local infiltration and deep sedation with Inj. Xylazine hydrochloride @ 0.01mg/kg, intravenously. Open medial patellar desmotomy was performed to harvest the 2-3 cm thick medial patellar ligament and preserved in 2.5% glutaraldehyde for histomorphological examination. For control group, the two cases cattle which were presented to the hospital with history of irreparable trauma and they were died during the treatment. The normal medial patellar ligament (2-3 cm thick specimen) was harvested during postmortem and persevered for microscopic study.

The preserved sample were subjected for scanning electron microscopy (SEM) and transmission electron microscopic (TEM) study as per standard procedure described by Bozzola and Russell (1999), Lakshman, (2019) to evaluate the collagen morphology of affected as well as normal medial patellar ligament at RUSKA Laboratories, College of Veterinary Science, PVNR TVU, Rajendranagar, Hyderabad, India.

Scanning electron microscopy procedure

Samples were fixed in 2.5% Glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 hrs at 4°C and post fixed 1% in aqueous osmium tetroxide for 4 hrs. Dehydrated in series of graded alcohols and dried to critical point drying with CPD (EMS 850) unit / vacuum desiccation for 35-45 minutes for complete drying of specimens was carried out. The dried samples were mounted over the stubs with double-sided carbon conductivity tape and a thin layer of heavy metal (gold) was coated over the samples by using an automated sputter coater (Model - JEOL JFC-1600) for 3 minutes and scanned under Scanning Electron Microscope (SEM - Model: JOEL-JSM 5600) at required magnifications as per the standard procedures.

Transmission electron microscopy procedure

The pieces of suitable sizes of medial patellar ligament were collected from normal and affected animal by open surgery. The collected tissue samples were fixed in 2.5% Glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 hrs. at 4°C and washed with PBS for four times each 1 h, then post fixed in aqueous osmium tetroxide for 3 hrs. Later washed with deionised distilled water for 6 times each one minutes, dehydrated in series of graded alcohols, infiltrated and embedded in Araldite resin. Incubated at 80°C for 72 h for complete polymerization. Ultrathin (60 nm) sections were made with a glass knife on ultra-microtome (Leica Ultra cut UCT-GA-D/E-1/00), mounted on copper grids and stained with saturated aqueous Urenyl acetate (UA) and counterstained with Reynolds lead citrate (LC). Longitudinal and transverse section were then examined by TEM (JEM-2100 Electron Microscope JEOL Ltd., Tokyo, Japan).

Statistical analysis

The obtained data analysed with the help of Web Agri Stat Package (WASP), ICAR-CCARI, Goa.

RESULTS AND DISCUSSION

Out of 38 animals, 20 (52.63%) were cattle and remaining 18 (47.37%) were buffaloes which were excluded from present study and not considered for further laboratory investigation. Among these 20 cattle, 6 (33.00%) were them of khillar breed of cattle and 14 (77.00%) were Holstein Fressian crossbreds. In present study, incidence of patellar luxation was higher in cattle than buffaloes in contrary to this Shivaprakash and Usturge (2004) reported that, maximum incidence was noted in bullock (48.58%) followed by buffalo (40%) and cow (8.00%). Among 20 cattle, 17 (85.00%) were females and remaining three (15.00%) were males indicating that the incidence of medial patellar luxation was higher in females as compared to male animals in accordance with Da Silvaa *et al.* (2004) and Shivaprakash and Usturge (2004) findings could be due to female animals has to maintain heavy weight during pregnancy probably which compromises the ligament function of hind limb.

Scanning electron microscopic findings

The SEM observations of ligament sections from normal (control group) animals showed dense and compact arrangement of collagen fiber bundles (Fig 1). The collagen fibers were well organized lengthwise. The morphometric analysis for thickness (diameter) was carried out and mean value was reported to be 3.66±0.58 μm (Fig 2). The gap between two collagen fiber bundles was very less *i.e.* compact in nature. The collagen fibers were uniformly arranged without any pathological conditions like break in the continuity or interwoven pattern. Inflammatory cellular changes were not observed in the ligament sections from normal group. With the help of 5000x of SEM, the surface of collagen fibers appeared uniform, compact as well as uniform lengthwise (Fig 3). The present findings are in accordance with the findings of Nimmi and Harkens (1988) who stated that, well organized and compact collagens, the primary structural elements of the extracellular matrix, are the most abundant proteins in ligament, tendon, cartilage and bone. Scanning electron microscopy of normal anterior cruciate ligament, medial collateral ligament, achillies ligament and patellar ligament as cell source for tissue engineered ligament and found that, these types of cells are highly proliferative cells and can be used in tissue engineered ligament repair (Copper *et al*., 2006).

The observations of SEM of various sections from the affected ligament showed derangement in collagen fiber bundles. The collagen fibers were arranged irregular and wavy. Collagen fiber bundles were not well organized and were loosely attached to each other (Fig 4a and b). The mean thickness of collagen fiber bundle was recorded to be 2.58±0.03 µm (Fig 5) and 3.06±0.07µm (Fig 6) for 2 animals under observation. The gap between two collagen fibers was remarkable (Fig 7) as compared with observations of normal ligament. The collagen fibers appeared divided, broken and overlapping on each other. Collagen fibers were arranged interwoven and interlacing with each other. In certain sites, as per the observations of SEM, presence of erythrocytes and leucocytes (Fig 8) were seen in the

Fig 1: Collagen fiber bundles with dense and compact arrangement in normal medial patellar ligament of cattle. (SEM, X 2000).

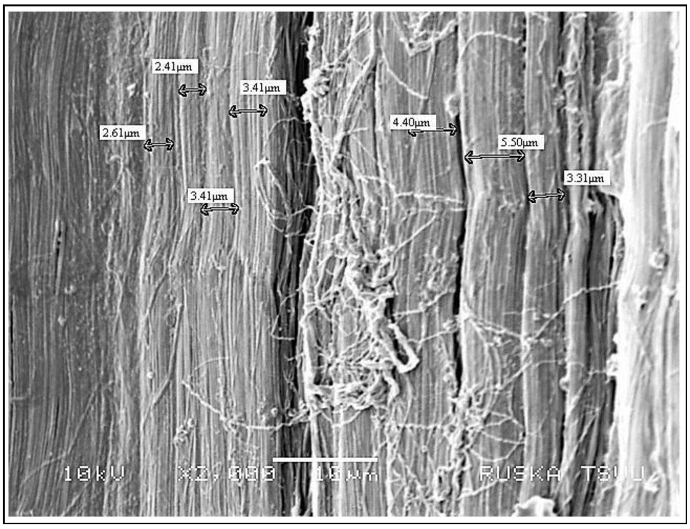

Fig 2: Morphometric analysis for thickness of collagen fiber bundle and thickness in normal medial patellar ligament in cattle (SEM, X 2000).

collagen fibers. Under 5000x of SEM, the surface of collagen fibers appeared irregular, rough and coarse in nature with few fibers released outside from the bundles due to degenerative changes. Overall, present clinical study showed that, animals affected with upward luxation of patella showed significant reduction in thickness of collagen fibrils and fibrils appeared as loosely arranged, rough with disorganized fashion as compared with normal medial patellar ligament and depicted in Table 1.

Frasca *et al*. (1978) studied terminal portion of quadriceps tendon collagen into patellar bone in various species of animal and concluded that, collagen fibril was exposed in calcified tissues. Bayat *et al*. (2003) analyzed ruptured anterior cruciate ligament in dog and found, wavy interlacing collagen fibres and fascicles with irregular network of collagen material as recorded in present clinical study.

In present investigation, majority of collagen fibril was damaged, broken and there was remarkable gap, which might have could cause laxity of medial patellar ligament that predisposed patella to displace from its normal position during movement. Similarly, Provenzano and Vanderby (2006) studied collagen fibril morphology, its organization and its implications on force transmission of ligament and tendon and concluded that, fibrils in mature ligament and tendon are either continuous or functionally continuous and its force within the tissue is directly transferred through collagen fibril and not through inter fibril coupling (proteoglycan bridge).

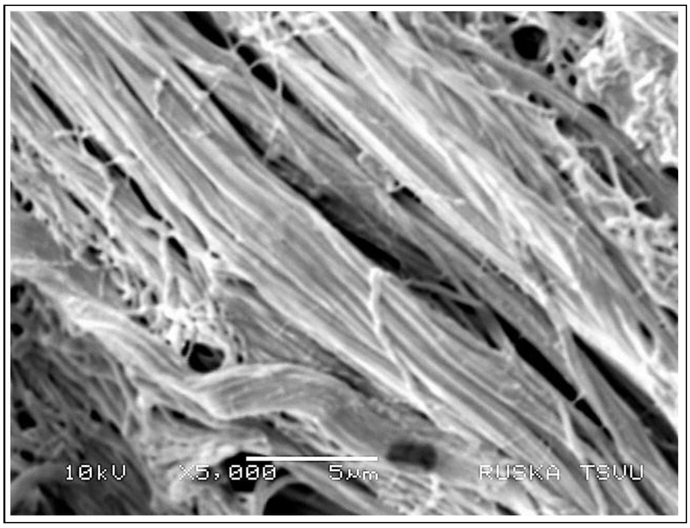

Fig 3: Surface of collagen fibers appeared uniform and compact in normal patellar ligament in cattle (SEM, X 500).

Fig 4(a): Collagen fiber bundles of medial patellar ligament are not very compact but loosely attached to each other in cattle affected with upward luxation of patella (SEM, X 2000).

Transmission electron microscopic findings

Electron microscopy of the ligament sections from normal (control group) animals showed presence of thick collagen fiber bundles packed in dense and compact arrangement (Fig 9). Collagen fiber bundles were uniform, homogenous and well organized (Fig 10). Surface area of collagen fibers was uniform and smooth in nature. There was no remarkable gap between the collagen fiber bundles with diameter of 0.22±0.003 µm (Fig 11).

Affected ligament characterized degenerative changes of the fibers, presence of thin collagen fiber bundles which were loosely arranged. The ligament fiber bundle showed disorganized fibrils (Fig 12) and heterogeneity of size and diameter in collagen fiber bundles. There was remarkable gap between collagen fiber bundles. Surface area of collagen fiber bundles were rough and uneven with coarse in appearance (Fig 13). The diameter of collagen fiber was measured and was found to be 0.13±0.007 µm (Fig 14) and was compared with normal medial patellar ligament and depicted in Table 2.

In this study, measurement of thickness of collagen fiber was followed in normal and affected medial ligament in conferring with Frank *et al,* (1989) who measured diameter of collagen microfibrils in growing and matured rabbit to assess the growth. Patellar ligament in dog suffering with patellar luxation showed uneven distribution of collagen fibril (103.67±15.58 nm) with marked gap between collagen fibril indicating cellular damage (Ueda *et al*., 2018). In present study, twenty clinical cases of cattle suffering with luxation

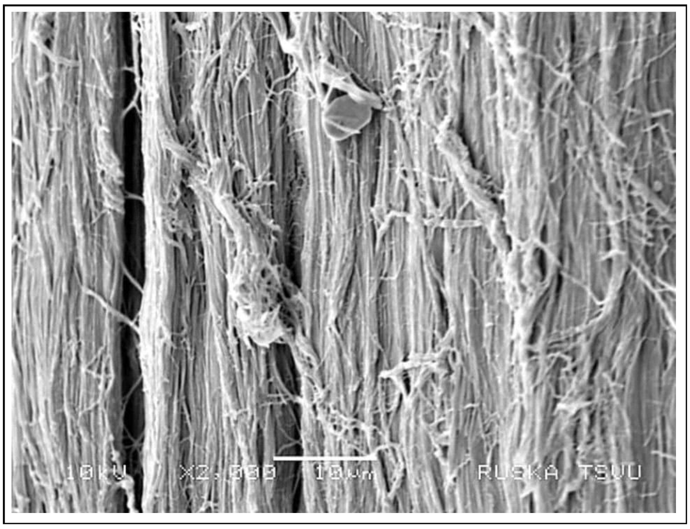

Fig 4(b): Collagen fiber of medial patellar ligament are irregular and arranged in loose and wavy manner from each other (SEM, X 2000).

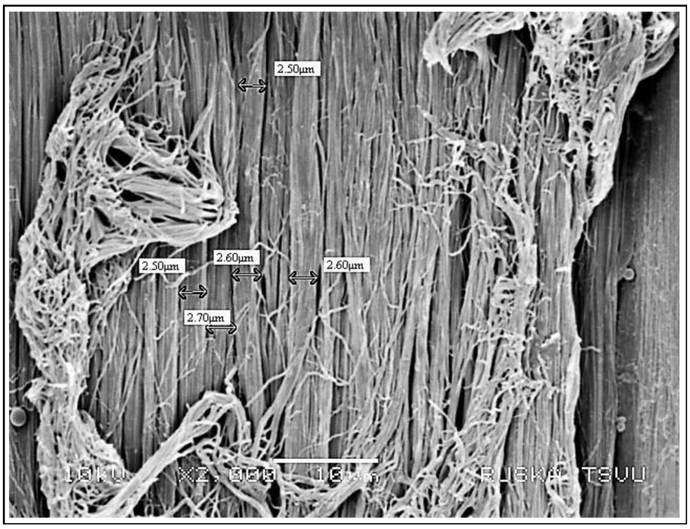

Fig 5: Morphometric analyis for thickness of collagen fiber bundle and thicknes in affected medial patellar ligament of cattle (SEM, X 2000).

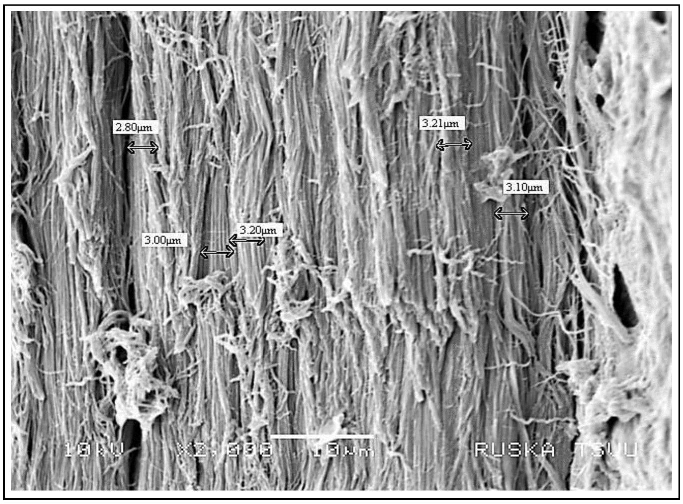

Fig 6: Morphometric analyis for thickness of collagen fiber bundle and thicknes of medial patellar ligament in cattle (SEM, X 2000).

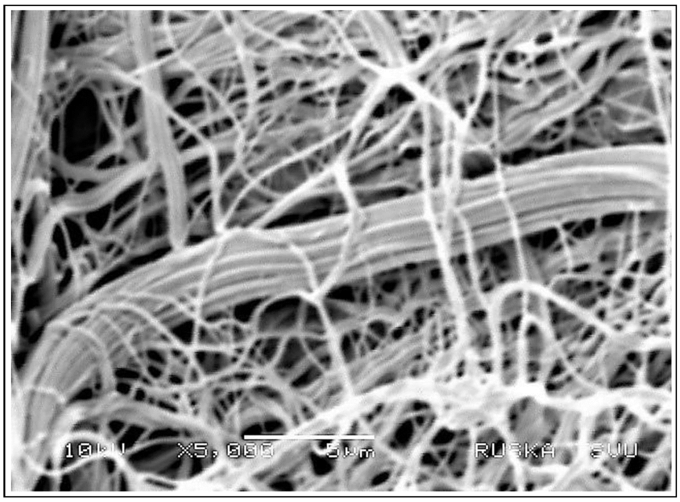

Fig 7: The gap between two collagen fiber was remarkable in medial patellar ligament in cattle (SEM, X 5000).

Fig 8: Presence of leucocyte seen in the collagen fibers in medial patellar ligament of cattle (SEM, X 5000).

of patella underwent medial patellar desmotomy *via* open method and affected medial patellar ligament showed microscopic changes characterized by derangement in collagen fiber bundles with drastic reduction in thickness of collagen fibers, increased gap between two collagen fibers, broken and overlapping fiber bundles and surface of collagen fibers were appeared to be irregular and rough *via* scanning and electron microscopy study.

Table 1: Average mean (±SE) thickness of collagen fiber bundles recorded during scanning electron microscopic study of normal (control) and affected (luxated patella) medial patellar ligament in cattle.

No. of field view	Normal medial patellar ligament (control)	Affected medial patellar ligament (case 1)	Affected medial patellar ligament (case 2)
1	2.41 µm	2.50 µm	2.80 µm
2	2.61 µm	2.50 µm	3.00 µm
3	3.41 µm	2.60 µm	3.10 µm
4	4.40 µm	2.60 µm	3.20 µm
5	5.50 µm	2.70 µm	3.21 µm
Mean±SE	3.66±0.58 µm	2.58±0.03 µm	3.06±0.07 µm

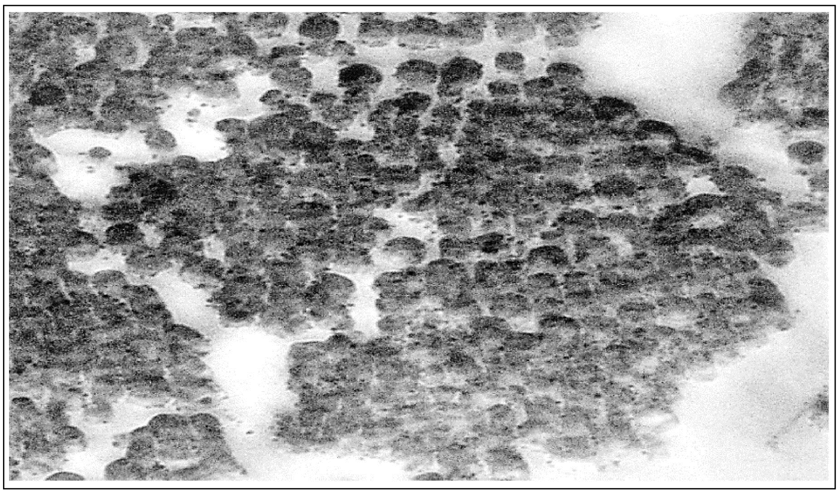

Fig 9: Micrograph cross section of normal medial patellar ligament in cattle showing presence of thick collagen fiber with dense arrangement and uniform morphology of bundles (TEM, X 5000).

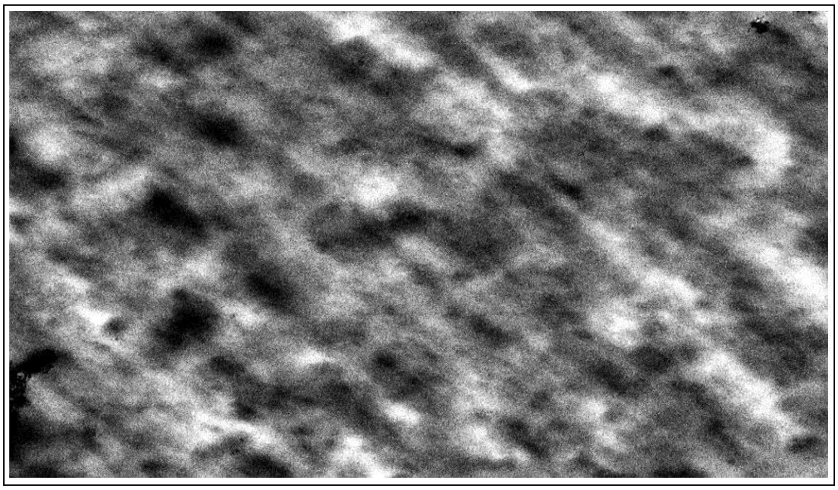

Fig 10: Micrograph longitudinal section of normal medial patellar ligament in cattle showing uniform and homogenous collagen fiber bundle (TEM, X 3000).

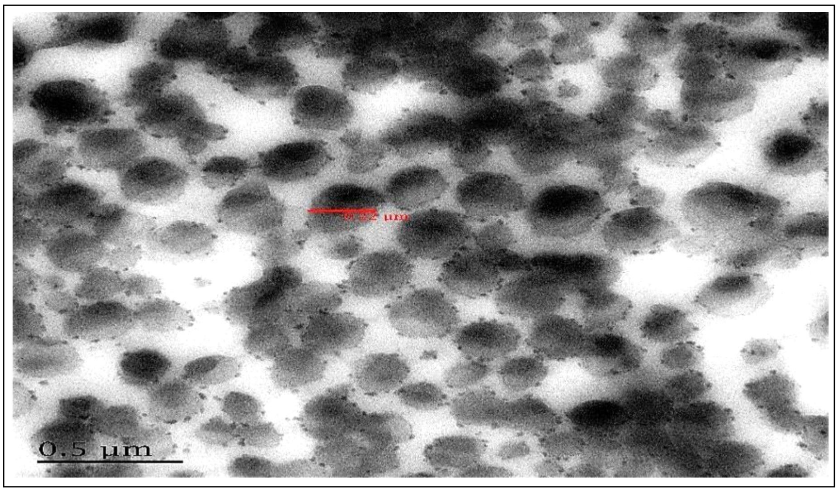

Fig 11: Micrograph cross section of normal medial patellar ligament in cattle showing diameter of collagen fiber (TEM, X 5000).

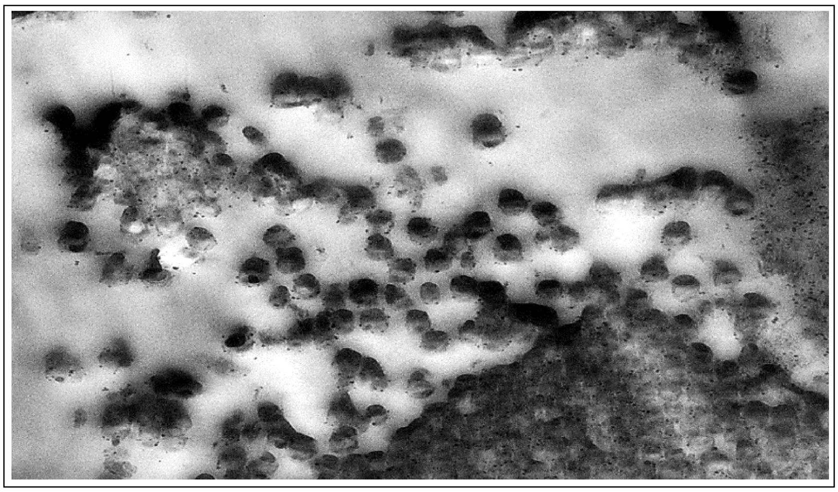

Fig 12: Micrograph cross section of affected medial patellar ligament in cattle showing disorganized fibrils wiht uneven and loose arrangement (TEM, X 5000).

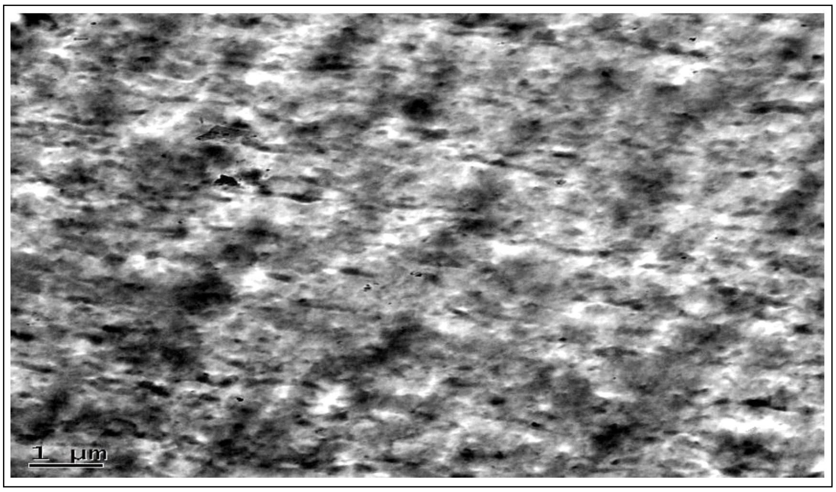

Fig 13: Micrograph longitudinal section of affected medial patellar ligament in cattle showing surface area of collagen fiber bundles are rough, coarse in nature (TEM, X 3000).

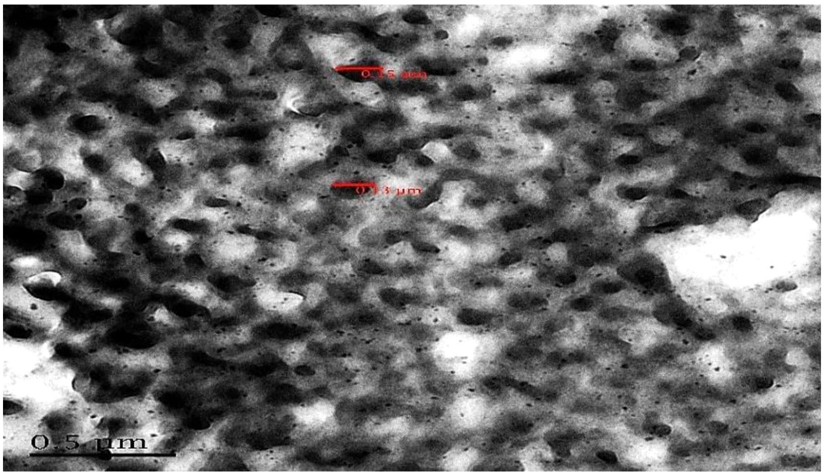

Fig 14: Micrograph cross section of affected medial patellar ligament in cattle showing diameter of collagen fiber (TEM, X 5000).

Table 2: Average mean (±SE) diameter of collagen fibrils recorded during transmission electron microscopic study of normal (control) and affected (luxated patella) medial patellar ligament in cattle.

No. of field view	Normal medial patellar ligament (control)	Affected medial patellar ligament (case 1)	Affected medial patellar ligament (case 2)
1	0.22 µm	0.13 µm	0.12 µm
2	0.22 µm	0.13 µm	0.14 µm
3	0.22 µm	0.15 µm	0.14 µm
Mean±SE	0.22±0.003 µm	0.13±0.007 µm	0.13±0.007 µm

CONCLUSION

It was sum up that, the scanning electron microscopy of affected medial patellar ligament in cattle suffering with patellar luxation showed derangement in collagen fiber bundles characterized by arranged in irregularly and wavy pattern with drastic reduction in fiber thickness and increased gap between two collagen fibers. Whereas, transmission electron microscopy revealed broken and overlapping fiber bundles, surface of collagen fibers appeared irregular, rough and coarse in nature with few fibers released outside from the bundles due to degenerative changes.

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Conflict of interest: None.

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