Original Research

Histomorphological Evaluation of Medial Patellar Ligament Affected with Upward Luxation of Patella in Cattle

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Abstract

Clinical study was carried on twenty cattle aged between 6-13 years (6.25±0.59 years) presented with chief complaint of upward luxation of patella to investigate this musculoskeletal malady in dairy animals. Affected medial patellar ligament was harvested via open desmotomy whereas normal ligament was also collected from animals that died due to automobile accident for comparative purpose and preserved in 2.5% glutaraldehyde solution. Histopathological examination of affected ligament showed degenerative changes characterized by unevenly distributed and broken collagen fibers, atrophy and hypertrophied collagen fiber with loss of nuclei indicative cellular alterations. Scanning electron microscopy of affected ligament showed derangement in collagen fiber bundles arranged irregularly, wavy, loose and remarkable gap between two collagen fiber bundles was noted. Transmission electron microscopy showed thin and disorganized collagen fiber bundles with heterogenecity of size and diameter in collagen fiber bundles. To concludes, affected medial patellar ligament undergoes several degenerative changes in collagen fibrils that lead to changes in structure – function relationship causes luxation of patella in cattle.

Key words: Collagen, Cattle, Electron microscopy, Heterogenecity, Luxation and Medial Patellar Ligament

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Introduction

In Indian context, poor to marginal farmers adopts free grazing system in dairy sector to reduce the cost on feed and management especially in western part of Maharashtra. Under free range grazing system, dairy animals are highly prone to various occupational traumas to the ligaments (overstretching ligaments) during climbing or jumping. Continuous tear and wear of stifle ligaments, leads to laxity or flaccidity; results into luxation of patella. 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, C. B. 2004). The cause of upward fixation of patella is still debatable but possibly scientist have considered it to be a multifactorial etiology like nutritional deficiency, over exploitation, external trauma, intense contraction of quadriceps muscle and morphological changes in the stifle and associated ligaments (Chandrapuria *et al.*, 2012, and Mondal *et al.*, 2013). The varied incidence of upward fixation of patella (UFP) among bovine species has been reported by different authors (Hifny *et al.*, 2013 and Da Silva *et al.*, 2014).

Degenerative changes in collagen fibers and exudative changes are common in medial patellar ligament affected with upward luxation of patella bone in cattle (Naveen *et al.*, 2014 and Gali *et al.*, 2017). Nimni 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. Bayat *et al.* (2003) examined ruptured anterior cruciate ligament in dog and found that, wavy interlacing collagen fibres and fascicles with irregular network of collagen material. In India, there is paucity of available literature on histomorphological changes in medial patellar ligament in cattle encouraged us to investigate the cellular changes of affected ligament and its impact on tensile strength of ligament.

Materials and Methods

The present research work was conducted on the twenty clinical cases of cattle presented to teaching veterinary clinical complex of the institute with history of limping due to luxation of medial patellar ligament. All animals were randomly subjected to thorough anamnesis (age, breed, sex and physiological status); physical evaluation (swelling, trauma, arthritis, etc.); radiographic examination (position of luxated patella, femur tracheolar ridge, and joint pathology); ultrasonographic appearance (thickness) patellar ligaments and clinical examination to rule out the concurrent infectious causes of lameness in all animals.

Harvestment of Medial Patellar Ligament

Affected animals were restrained in lateral recumbency with affected limb in downward direction. In violent animals, Injection Xylazine HCl was given @ 0.01mg/kg in ear vein prior to surgery. Affected stifle joint at medial site was prepared aseptically with povidone iodine solution I.P.10%. After full extension of stifle,

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local anaesthetics were infiltrated beneath/around medial patellar ligament and about 3-4 cm straight incision were taken on skin parallel to medial patellar ligament to expose medial patellar ligament. Exposed full thickness ligament (2-3cm) resected (Fig.1&2) and preserved in 10% formalin and 2.5% glutaraldehyde for histopathological and histomorphological examination, respectively. For comparative study, normal medial patellar ligament from six cattle were harvested which were died due to automobile accident during the course of treatment at TVCC.

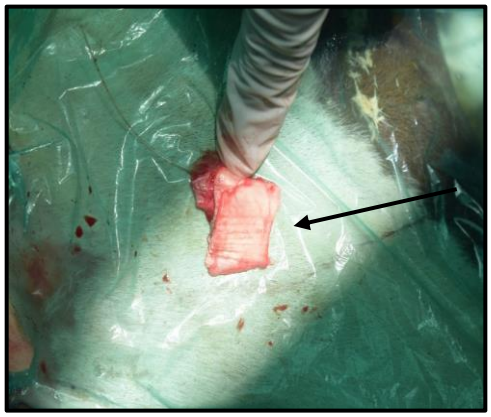

Fig. 1: Appearance of harvested medial patellar ligament during open method of medial patellar desmotomy.

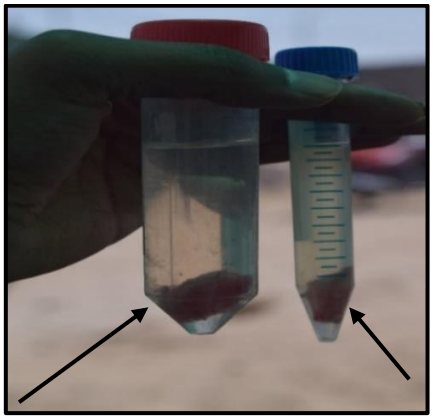

Fig. 2: Preservation of harvested pieces of medial patellar ligament for histomorphological examination.

Histopathological Examination

The collected tissue samples were fixed and preserved in 10% neutral buffer formalin. After fixation, the tissue pieces were processed as per the standard procedure and embedded in paraffin (60^0C with ceresin, Qualigens). Paraffin sections were cut at 3-5 µ thickness on spencer type microtone and stained by routine Haematoxylin and Eosin method (Culling, 1974).

Scanning Electron Microscopy

Tissue samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 hrs at 4^0C and post fixed 1% in aqueous osmium tetroxide for 4 h. 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. 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.

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Table 1: Scanning electron microscopic study of collagen fibril bundle thickness (µm) of medial patellar ligament in cattle

No. of field view	Normal medial patellar ligament	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

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 24hr at 4^0C and washed with PBS for four times each 1h, then post fixed in aqueous osmium tetroxide for 3h 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 80^0C for 72h for complete polymerisation. Ultrathin (60nm) 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 sections were then examined by TEM (JEM-2100 Electron Microscope JEOL Ltd., Tokyo, Japan) as described by Kuantzel (1933) and Schmitt et al, (1942) who concluded that, collagen is vital material in connective tissue of tendon and ligaments. The present clinical data was analysed with help of Web Agri Stat Package (WASP), ICAR-CCARI, Goa.

Results and Discussion

Incidence of Upward Luxation of Patella in Cattle

Among the 20 cattle, 6 (33.00%) were khillar and 14 (77.00%) were Holstein Fressian affected with upward patellar luxation.

Incidence of patellar luxation was higher in cattle than buffaloes and on contrary, Shivaprakash and Usturge (2004) who also reported that, maximum incidence was noted in bullock (48.58%) followed by buffalo (40%) and cow (8.00%). The average age of affected animals was between 3 – 12 years (6.25±0.59 years) and out of 20 animals, 17 (85.00%) were females and remaining three (15.00%) were male indicates that, the incidence of was higher in females as compared to male according with Da Silvaa *et al.* (2004) and Shivaprakash and Usturge (2004) findings. Duration of hindlimb lameness due to upward luxation patella in cattle was between 3-12 months (7.35±0.56months) indicates that, all animals were suffering with in present study showed chronic lameness due the negligence of owner to report. Similar study was conducted

by Dass *et al.* (1983) and Da Silvaa (2004) stated that, prevalence of upper fixation of patella in herd was variable and depending upon geographical area. Out of 20, 13 cases (65.00%) were reported in winter could be due to laxity of ligament as reported by Shivaprakash and Usturge (2004) and Salisha *et al.* (2015).

Radiographic and Ultrasonographic Findings

Latero-medial radiographic view of stifle in three animals showed patellar fragmentation and roughening of lateral condyle of femoro-patellar joint (Fig. 3). Similar findings were reported by Jeffcott *et al.* (1982) and Marino *et al.* (2010) in their study and conclude that, later medial view of stifle gives more information about joint pathology. 7.5 MHz, linear probe found to be more suitable for scanning of stifle joint in standing position in all animals controlled in standing position for better visualization of stifle ligaments (Kramer *et al.*, 1999). In present study, out of three patellar ligaments, middle and lateral ligaments give thin, flattened ribbon like appearance throughout investigation might be due to its superficial and palpable structure gives easy images as reported by Kassab and Badawy (2011) in his study. Thickness of medial, middle and lateral patellar ligament of patella showed varied thickness in both normal as well as in affected cases (Fig. 4, 5 & 6), indicates non-significant changes in ligaments accordance with Pennink *et al.* (1990) and Thrall (2019) evidenced that, ultrasonography allows visualization of three ligaments but due to their normal variation in shape of each ligament restricts accuracy of disease diagnosis.

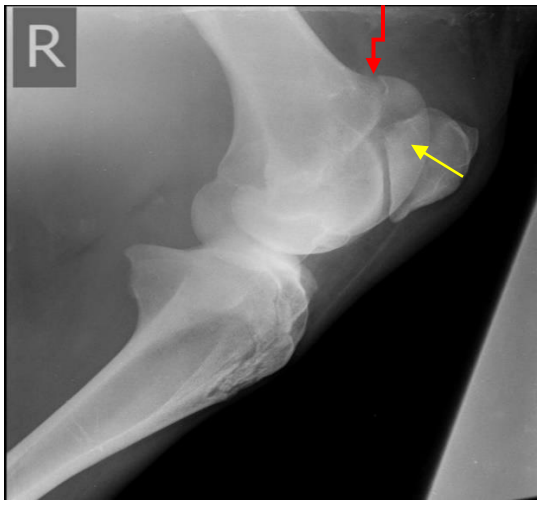

Fig. 3: Latero-medial view of stifle joint showing fragmentation of patellar surface (yellow arrow) and roughening of lateral condyle of femoro-patellar joint (red arrow) in Khillar cattle.

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Fig. 4: Transverse ultrasonographic image of medial patellar ligament affected with upward luxation of patella in cattle.

Fig. 5: Transverse ultrasonographic image of middle patellar ligament affected with upward luxation of patella in cattle.

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Fig. 6: Transverse ultrasonographic image of lateral patellar ligament affected with upward luxation of patella in cattle.

Histopathological Evaluation

Normal section of medial patellar ligament showed dense, regular and uniform length of collagen fibers with homogenous and uniform length of collagen fibers and elongated spindle shaped nuclei (Fig. 7). The collagen fibers were observed with uniform orientation in the collagen bundles without inflammatory or pathological changes with respect to length and diameter of the fibers. Affected ligament showed degenerative changes in the unevenly distributed collagen fibers (Fig. 8).

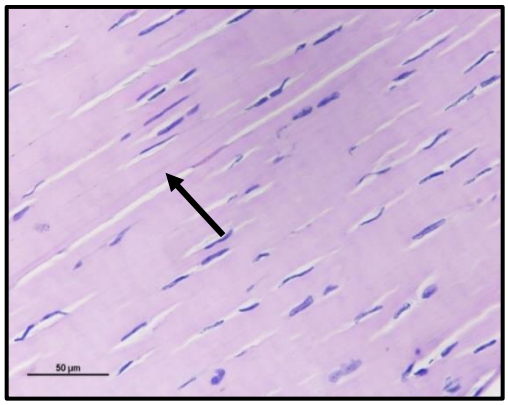

Fig. 7: Histopathological appearance of normal medial patellar ligament showed normal uniform length and compact dense pattern of normal collagen fibers (arrow) with intact cellular morphology of elongated nucleus (HE, X 400).

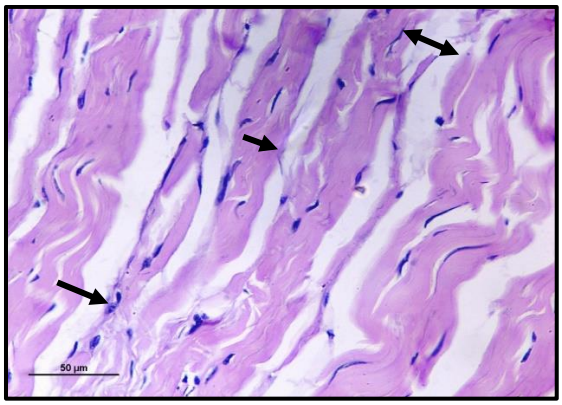

Fig. 8: Histopathological appearance of affected medial patellar ligament showed degenerative changes of collagen fibers with uneven appearance (short arrow) and break in continuity of fibers (long arrow). Note widened gap between collagen fibers (Double head arrow) (HE, X 400).

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The uniform length and homogenous dense compact arrangement of the collagen fibers was broken at multiple foci with presence of broken segments of the fibers. The degenerative and ruptured fibers showed variable cellular changes with atrophy and hypertrophy of fibers, loss of nuclei (Fig. 9) occasionally and enlarged oval to round nuclei in few of the fibers. Similarly, Naveen *et al.* (2014) revealed that, hypertrophied and oval nuclei of fibroblast were thickened in collagen bundles. Degenerative changes of the collagen fibers were observed in the all the affected animals. The intensity of microscopic cellular lesions in the collagen fibers was variable ranging from minimal changes to moderate in the affected cases observed for histopathology (Naveen *et al.,* 2014 and Gali *et al.*, 2017) stated that, degenerative changes in collagen fibers and exudative changes are common in medial patellar ligament affected with upward luxation of patella bone.

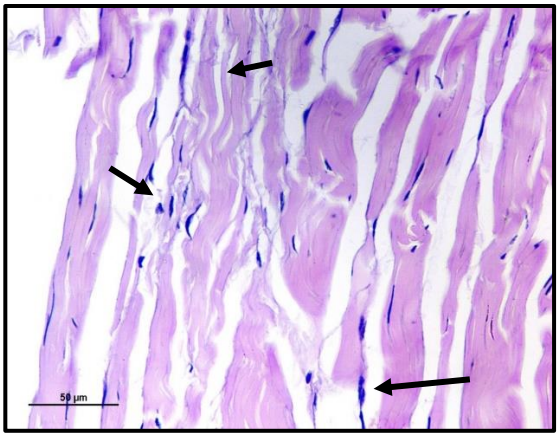

Fig. 9: Histopathological appearance of affected medial patellar ligament showed degenerative changes of collagen fibers with loss of nucleus (short arrow) with atrophy of collagen fibres and nuclear enlargement of collagen fibers (long arrow) (HE, X 400).

Few of the sections of ligament showed marked degenerative changes with presence of significant space between the fibers with hypertrophy. Few of the areas showed proliferation of fibrous tissue in the whole ligament sections interstitially with formation of few blood capillaries. The fibroblast tissue showed focal hypertrophy with large oval nuclei of fibroblasts. Ueda *et al.* (2018) also recorded unevenly distributed collagen fibers in skin and affected cruciate ligament in dogs characterized by marked gap between individual fibers. There was an excessive proliferation of fibrous connective tissue in 5 tissue sections of ligament from affected animals in present study (Naveen *et al.,* 2014). The inflammatory changes in the affected ligaments were observed in 4 cases from affected animals with infiltration of mononuclear cells (Fig. 10) principally comprised of lymphocytes. The inflammatory cells were observed in the areas of ligament with degenerated collagen fibers. One tissue section of affected ligament revealed uneven arrangement of collagen fibres and distorted fibers along the length (Fig. 11). Similarly, Deng *et al.* (2019)

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studied histology posterior cruciate ligament rupture in rabbit and concluded that, collagen fibrils were thick, loosely and disorderly arranges fibres indicative of stifle joint pathology.

Scanning Electron Microscopic Findings

Scanning Electron Microscopy (SEM) of medial patellar ligament was performed in both control and affected animals. Longitudinal sections of medial patellar ligament from two normal animals and four affected animals were carried out to observe the morphological and cellular changes in the ligament and collagen fibers as described by Laxman (2017) and Bozzola and Russell (1998). Section of normal ligament showed collagen fiber bundles with dense and compact arrangement. The collagen fibers were well organised lengthwise. The morphometric analysis for thickness (diameter) was performed and mean value was reported to be 3.66±0.58µm (Fig. 10). 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 manner. Inflammatory cellular changes were not observed in the

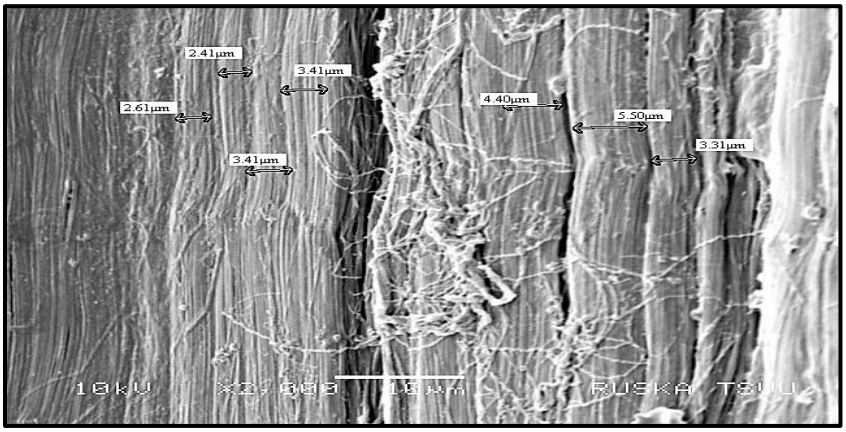

Fig. 10: Morphometric analysis for thickness of collagen fiber bundle and thickness in normal medial patellar ligament in cattle (SEM, X2000).

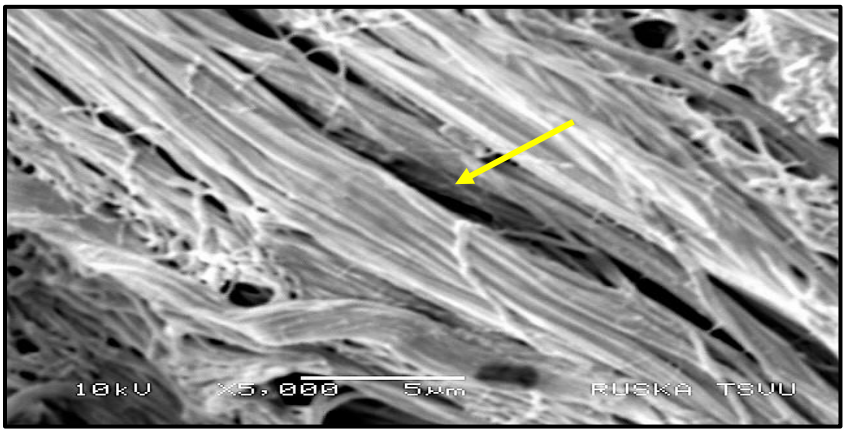

Fig. 11: Surface of collagen fibers appeared uniform and compact (Yellow arrow) (SEM, X5000).

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ligament sections from normal group. With help 5000x of SEM, the surface of collagen fibers appeared uniform, compact and uniform lengthwise (Fig. 11). The present findings in collaboration with Nimni 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. Copper Jr. *et al.* (2006) studied 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. Affected ligament showed derangement in collagen fiber bundles characterized by irregular and wavy pattern. Collagen fiber bundles were not well organized and loosely attached to each other (Fig. 12a and 12b).

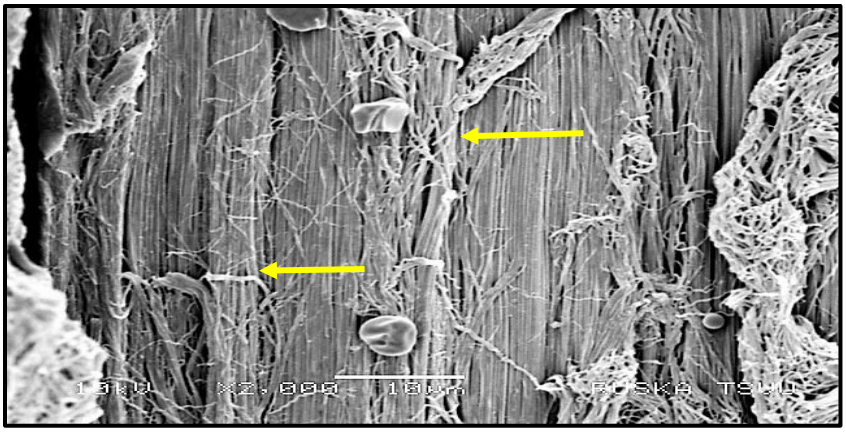

Fig. 12a: Collagen fiber bundles of medial patellar ligament are not very compact but loosely attached to each other in cattle affected with upward luxation of patella (Yellow arrow) (SEM, X2000).

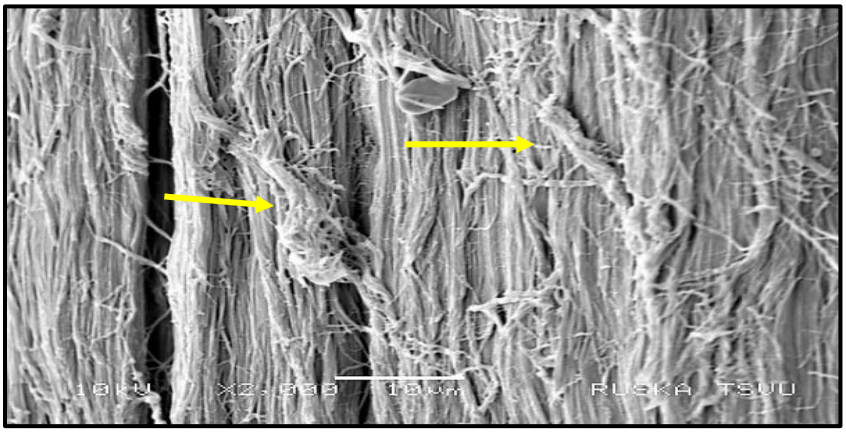

Fig. 12b: Collagen fibers of medial patellar ligament are irregular and arranged in loose and wavy manner from each other (Yellow arrow) (SEM, X2000).

The mean thickness of collagen fiber bundle was recorded to be 2.58±0.03µm (Fig. 13) and 3.06±0.07µm (Fig. 14). The gap between two collagen fibers was remarkable (Fig. 15) as compared with findings of

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normal ligament and fibers appeared as divided, broken and overlapping on each other. Collagen fibers were arranged interwoven and interlacing with each other. In certain sites under observation of SEM, presence of erythrocytes and leucocytes (Fig. 16) were seen in the collagen fibers. Under 5000x of SEM, the surface of collagen fibers appeared irregular, rough, and course 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, rough with disorganized fashion as compared with normal medial patellar ligament and depicted in Table 1.

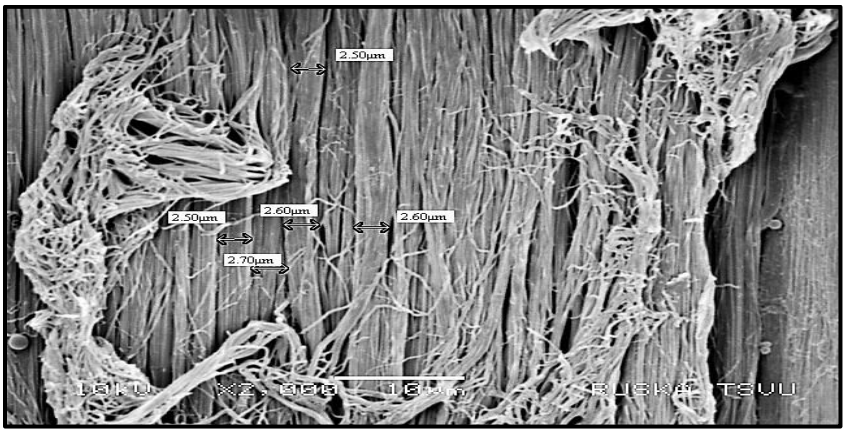

Fig. 13: Morphometric analysis for thickness of collagen fiber bundle and thickness in affected medial patellar ligament of cattle (SEM, X2000).

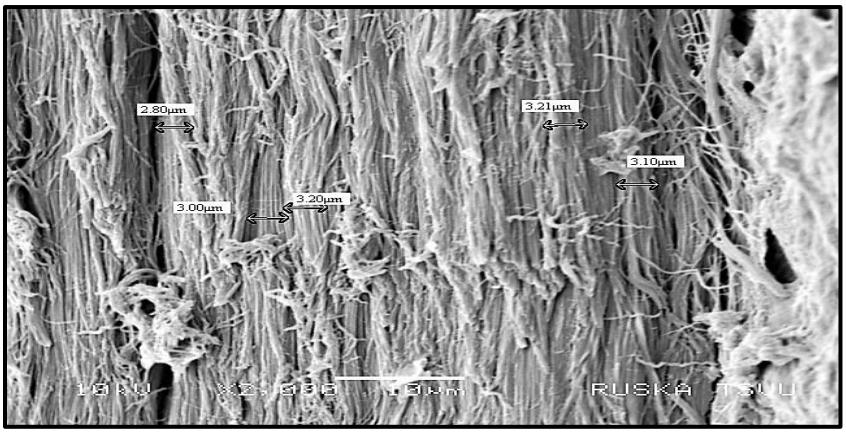

Fig. 14: Morphometric analysis for thickness of collagen fiber bundle and thickness of medial patellar ligament in cattle (SEM, X2000)

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) examined ruptured anterior cruciate ligament in dog and found that, wavy interlacing collagen fibres and fascicles with irregular network of collagen material as recorded in present clinical study. In present

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

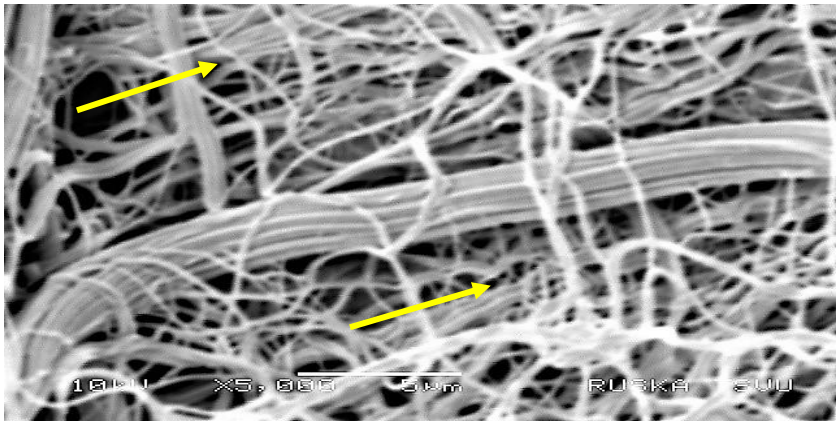

Fig. 15: The gap between two collagen fiber was remarkable in medial patellar ligament in cattle (Yellow arrow) (SEM, X5000)

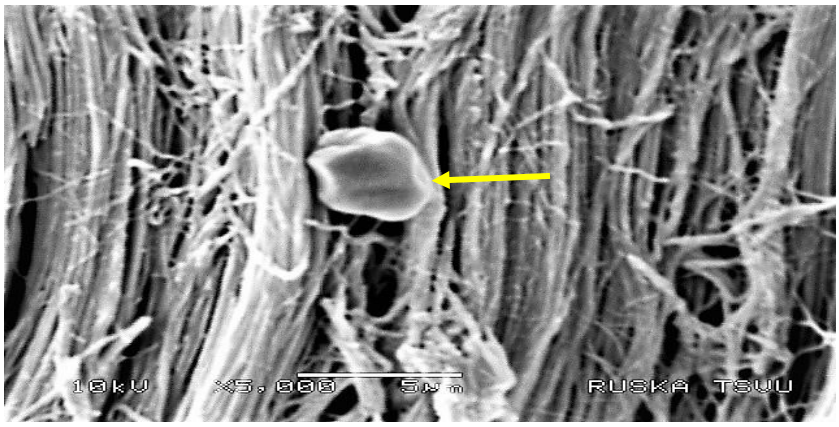

Fig. 16: Presence of leucocyte seen in the collagen fibers in medial patellar ligament of cattle (Yellow arrow) (SEM, X5000)

Transmission Electron Microscopic Findings

The electron microscopic observations of the ligament sections from normal animals showed presence of thick collagen fiber bundles packed in dense and compact arrangement (Fig. 17). Collagen fiber bundles were uniform, homogenous and well organised (Fig. 18). Surface area of collagen fibers was uniform and smooth in nature and there was no remarkable gap between the collagen fiber bundles with diameter 0.22±0.003 µm (Plate 4.26). Affected ligament showed degenerative changes of the fibers.

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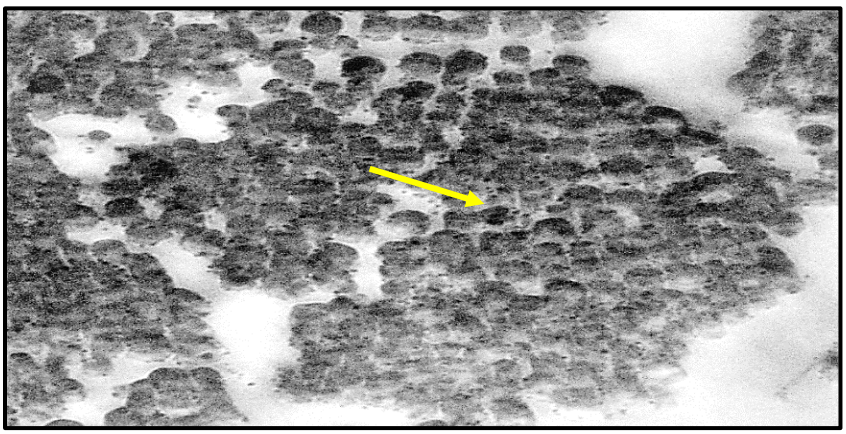

Fig. 17: Micrograph cross section of normal medial patellar ligament in cattle showing presence of thick collagen fiber with dense arrangement and uniform morphology of bundles (Yellow arrow) (TEM, X 5000).

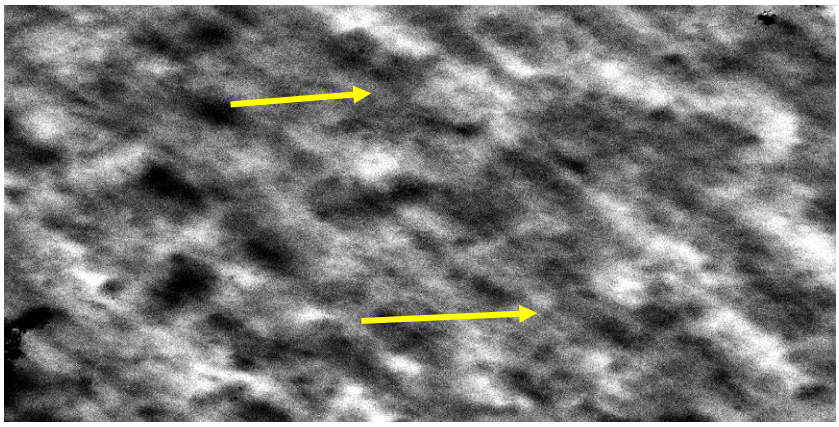

Fig. 18: Micrograph longitudinal section of normal medial patellar ligament in cattle showing uniform and homogenous collagen fiber bundle (Yellow arrow) (TEM, X 3000).

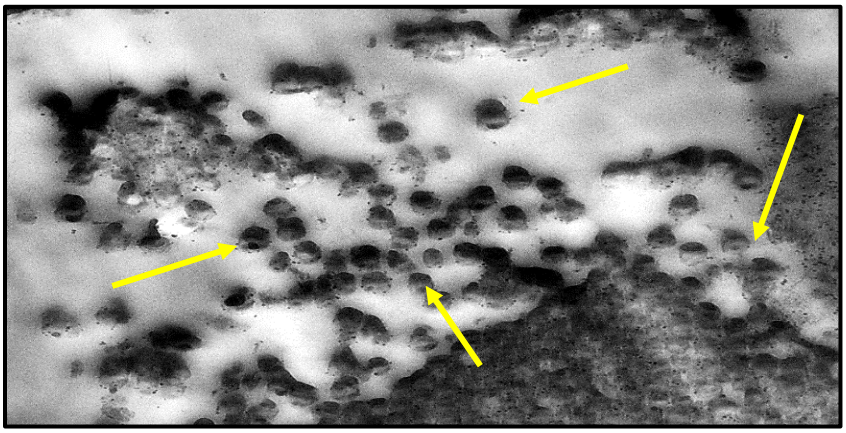

Fig. 19: Micrograph cross section of affected medial patellar ligament in cattle showing disorganized fibrils with uneven and loose arrangement (Yellow arrow) (TEM, X 5000).

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There was presence of thin collagen fiber bundles which were loosely arranged. The ligament fiber bundle showed disorganized fibrils (Fig. 19) and heterogenecity 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. 20). The diameter of collagen fiber was measured and was found to be 0.13±0.007 µm (Fig. 21) and compared with normal medial patellar ligament and depicted in Table 2. In present study, the affected medial patellar ligament of cattle showed degenerative changes, remarkable gap between collagen fibril and rough collagen surface. Similar conclusions were drawn by Ueda *et al.* (2018) in his study of patellar ligament with patellar luxation in dog and reported that, affected ligament showed uneven distribution of collagen fibril (103.67±15.58nm) with marked gap between collagen fibril indicates cellar damage.

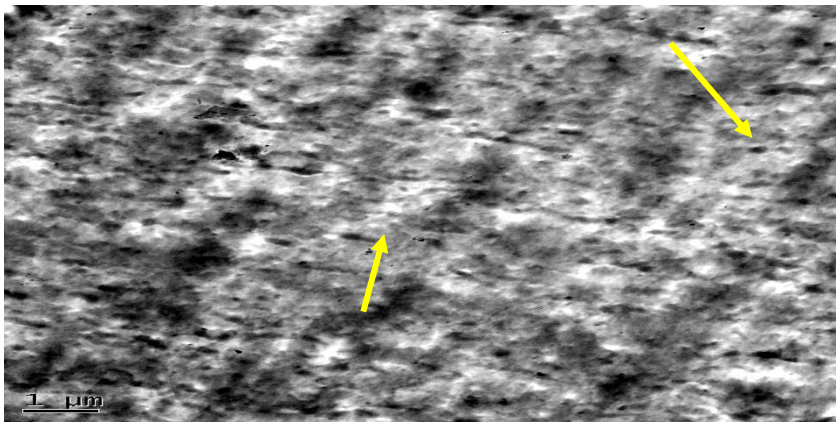

Fig. 20: Micrograph longitudinal section of affected medial patellar ligament in cattle showing surface area of collagen fiber bundles are rough, coarse in nature (Yellow arrow) (TEM, X 3000).

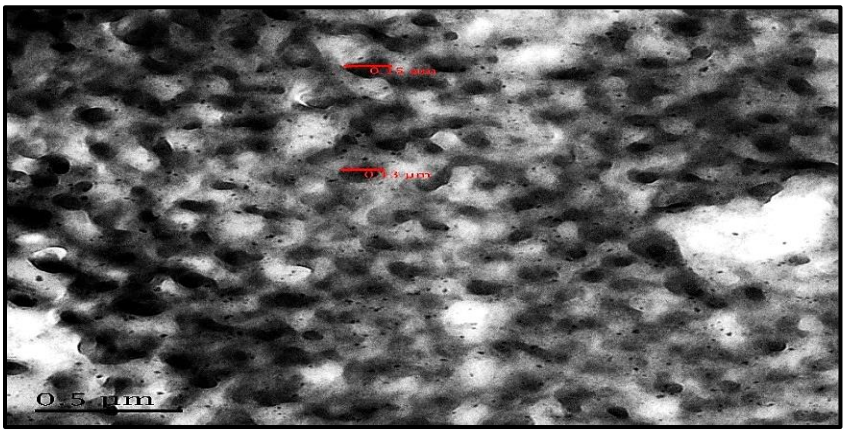

Fig. 21: Micrograph cross section of affected medial patellar ligament in cattle showing diameter of collagen fiber (TEM, X 5000).

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Table 2: Transmission electron microscopic study of collagen fibril diameter (µm) of medial patellar ligament in cattle

No. of field view	Normal medial patellar ligament	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

The present clinical study concludes that, histopathologically affected medial patellar ligament with upward luxation of patella in cattle showed several degenerative changes characterized by unevenly distributed collagen fibers with loss of compactness and homogenous properties of collagen fibers indicates cellular damage. Scanning electron microscopic revealed, derangement in collagen fiber bundles arranged in irregular and wavy with significant reduction in collagen fibers thickness and increased gap between two collagen fibers was remarkable. Transmission electron microscopic showed thinning of collagen fiber bundles which were loosely arranged, disorganized fibrils, heterogenecity of size and diameter in collagen fiber bundles. On basis of histomorphological alteration in medial patellar ligament i.e. loss of normal fibril architecture, broken fibrils and heterogenecity of collagen fibril bundles causes weakening and laxity of ligament leads to upward luxation of patella in cattle which affects the productivity of dairy animals.

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