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PAPAIN INDUCED PROGRESSIVE DEGENERATIVE CHANGES IN ARTICULAR CARTILAGE OF RAT FEMOROTIBIAL JOINT AND ITS HISTOPATHOLOGICAL GRADING

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ABSTRACT

Osteoarthritis (OA) is a chronic debilitating disease of articular joints in human beings and animals. Severity of this drastic malady relate directly with extent of changes at molecular level in affected joint. Progressive structural changes in knee joint cartilage during development of OA were graded using modified Mankin scoring system. OA was induced in male Wister rats (n=25) by injecting papain (10mg/joint) and compared with control group of rats (n=5) receiving sterile saline solution. Papain injected rats were divided into five groups (n=5) and sacrificed under high dose of anesthetic ether after 1st, 7th, 14th, 21st and 28th days. OA and control knee joints were removed and extent of histological changes in cartilage structure was assessed. Histological features of rat cartilage included were surface layer, population of chondrocytes, orientation of chondrocyte columns, morphology of cells and matrix. Score was allocated based on the severity of changes occurred in cartilage micro-texture in relation to time. Highest histological lesion score (12.82±1.64) was observed in papain induced OA cartilages on 28th day followed by 10.09±2.02 (21st day), 9.92±2.76 (14th day), 7.48±1.15 (7th day) and 3.09±.81 (1st day), respectively. Severity of lesions progressed with passage of time and maximum lesions were observed on day 28. Findings recorded after 28th day of papain injection seem to be consistent and resemble with early phase of OA in human articular cartilage. This method offers a rapid and minimally invasive method to produce OA like lesions in rodent model. Grading system will provide insight to chalk out therapeutic strategies for early OA.

Key words: Osteoarthritis, Papain, Knee joints, histological lesions and cartilage.

INTRODUCTION

Osteoarthritis (OA) is a chronic debilitating inflammation of joints characterized by destruction of articular cartilage which becomes soft and frayed with passage of time. Thickness of cartilage is reduced; subchondral bone eburnation and clustering of osteophytes result in immobilization of joints and pain. Rate of occurrence is higher in weight bearing joints at old age in human beings and animals (Goldring and Goldring, 2007).

There are many ways to develop OA in experimental animals like trauma, surgery, use of chemicals and enzymes that contribute towards robust histological changes of cartilage. Papain is a proteolytic enzyme that causes collagen degradation disrupting cartilage micro-architecture and integrity of affected joints. It produces OA like conditions in short time at low dose and is preferred over other methods of induction. There are many histological studies narrating use of papain for successful induction of OA in short time interval. It produces histological lesions resembling with those in early phase of natural OA in human beings.

Histological studies are more important to detect the accurate severity of OA at specific sites in humans and other species. For this purpose cartilage biopsy samples can be taken from joint of animals and human beings *in vivo* and histopathology analyzed. There are many limitations to collect whole tissue for standardization and grading of micro-molecular changes occurred in normal histological architecture. Experimental OA animal models are used for better understanding of disease progression and efficacy of treatments. Moreover, simple and reproducible histopathology grading of experimentally induced OA in animals provide necessary information about structural changes in joints, environmental or biological risk factors. In case of small animals, biopsy of joint tissue is very hard; hence whole joint can be examined (Collings and McElligott, 1960). OA model and type of animal species are used to play a critical role in deciding location within joint to be analyzed.

In present study OA was induced in Wister rats using papain under experimental conditions. Progressive changes in histological architecture of knee joint cartilage were graded in relation to time. Severity of histological

lesions was quantified by allocating histopathological score to each lesion. Thus, the aim of study was to quantify and compare histopathological changes on different days in papain induced experimental OA model till the consistent changes were observed that served as starting point of therapy.

MATERIALS AND METHODS

Experiment was carried out in accord with guidelines of committee for Research and Ethical Issues of the International Association for the Study of Pain ® (IASP) and institutional guidelines. Male Wister rats (n=30) weighing 150-200g selected from animal house of University of Veterinary and Animal Sciences, Lahore. Animals were housed (5 rats/cage) under standard management conditions.

Experimental plan: Out of thirty selected rats five were kept as control and twenty five were used to develop OA. Papain enzyme (Sigma, Cat # P 3125) at dose rate of 10mg in 0.05M sodium acetate (pH 4.5) with enzymatic activity of 31 I.U/mg was injected intra-articulary in each right knee joint of twenty five Wister rats as described by Murat *et al*. (2007). Five rats (n=5) were injected with 0.5cc of sterile saline solution (0.9%) in right knee joint that served as control group. Twenty five rats were divided into five groups (n=5) for development and assessment of osteoarthritis model also called as osteoarthritis (OA) groups. Rats were sacrificed under high dose of ether on 0 day (control group), 1st, 7th, 14th, 21st and 28th days post papain injection. Femorotibial joints of rats were separated with sharp knife on each sampling day. Ligaments and tendons were removed with sharp razor and then preserved in 10 per cent formalin solution (pH 7.4). Joints were decalcified in 5 per cent formic acid for 1 week, processed for paraffin embedding; frontal parts were sectioned at 5µm thickness and subsequently stained with haematoxylin-eosin (H&E) as described by Schmitz *et al*. (2010).

Staining protocol: All the slides were dipped three times in Xylene solution for 4 minutes, dipped two times in 95 percent alcohol for time interval of 1 minute each. Slides were passed once through 70 % alcohol solution for 1 minute and then rinsed with tape water for 1 minute. Afterwards slides were dipped in Mayer Haematoxylin solution (50 g alum, 1g haematoxylin, 0.2g sodium iodide, 1g citric acid were first dissolved in 250 mL distilled water then made up volume to 1000mL) for 6 minutes and then washed with tape water for 8 minutes. Slides were dipped 8 times in 95% alcohol and then placed in 0.25% Eosin Y solution for 1 minute prepared freshly by diluting 250 mL of Eosin Y stock solution (10g Eosin in 200 mL distilled water and 800 mL 95% ethanol) with 750 mL 80 % ethanol and 5 mL concentrated glacial acetic acid. Slides were dipped three times in 100% alcohol for 5 minutes each, dipped in xylene for 5 minutes and mounted with xylene based media.

Semi quantitative histopathological grading was performed in accordance with histological scoring system of Mankin *et al*. (1971). Histological features of normal rat knee cartilage were intact surface layer, normal population of chondrocytes, normal orientation of chondrocyte columns, normal morphology of cells and continuous matrix and allocated as grade zero.

Histological lesion scores calculated on different days were expressed as mean ± SD. Difference between all groups (OA and control) was analyzed statistically using one way analysis of variance followed by Duncan's Multiple Range post hoc test using SPSS software, version 13.0 (SPSS Chicago III, USA) at 95% probability.

RESULTS

Histopathological scoring system was used to estimate the effective induction of OA in experimental rats by papain. Control group injected with sterile saline solution did not caused any change in histological morphology of rat knee joint cartilage. Mean histological lesion score calculated was zero. All of the morphological features included in the criteria were normal in this control group. The histological findings for normal (control) group were comprised of intact surface layer, normal population of chondrocytes, normal orientation of chondrocyte columns, normal morphology of cells and continuous matrix (Fig. 01). Representative histopathological slides for allocated grades 1, 2, 3, 4 and 5 are shown as figures 01, 02, 03, 04, 05 and 06, respectively. Description of histological lesions along with individual allocated sub-scores for grades 1-5 are presented at table (01).

Histological picture having rough surface was graded one (01). This was sub graded as irregular superficial layer, condensation of superficial layer, proliferation of chondrocytes and initiation of chondrocyte clustering. Grade two (02) was allocated to surface discontinuity comprising of all pathological lesions of grade one plus discontinuous superficial layer, cell death till mid zone, hypercellularity, disorientation of chondrocyte columns and completion of chondrocyte cluster formation. Cleft formation in cartilage was included in grade three which was sub graded as large patches of cell death, sloughing of layers till mid zone and mild loss of matrix, hypertrophy of chondrocytes. The erosion of cartilage surface was graded as four (04) and additional lesions included were sloughing of cellular layers, maximum loss of matrix, excessive clustering of chondrocytes, hypocellularity and multiple patches of cell death. Grade five (05) was allocated denudation and sub

graded as denudation of bone, maximum loss of cellular layers and matrix, micro fracture of bone and initiation of fibrosis in addition to histological features of previous grades.

Development of experimentally induced OA was determined by euthanizing five rats on day zero (control) and days 01, 07, 14, 21 and 28 post papain injection. Descriptive histological lesions with scores and their statistical means are presented at table (02). Highest histopathological score (12.82±1.64) was observed on 28th day of post papain injection followed by 10.09±2.02 (21st day), 9.92±2.76 (14th day), 7.48±1.15 (7th day) and 3.09±.81 (1st day). Histopathological scores were compared statistically by DMR test at probability level of 0.05 using SPSS version 13.0. All the groups differed significantly from normal. The difference was not significant between histopathological scores on days 14 and 21 post papain injection. Representative histopathological slides of OA rat model on different days with descriptive lesions are shown as figures 07 (zero day), 08(1st day), 09 (7th day), 10 (14th day), 11 (21st day) and 12 (28th day), respectively. Histological lesions progressed till 28th day post papain injection and remained consistent later on.

Table 01: Main histological and associated lesions classified under different grades

Grade	Histopathological Features	Associated Lesions
0	Normal cartilage	Intact surface layer, normal population of chondrocytes , normal orientation of chondrocyte columns, normal morphology of cells and continuous matrix.
1	Rough surface	Irregular superficial layer (0.25), condensation of superficial layer (0.25), proliferation of chondrocytes (0.25) and initiation of cluster formation (0.25).
2	Surface discontinuity	All above and discontinuous superficial layer (1.20), cell death till mid zone (1.20), hypercellularity (1.20), disorientation of chondrocyte columns (1.20) and cluster formation (1.20).
3	Cleft formation	All above and large patches of cell death (2.25), sloughing of layers till mid zone (2.25), mild loss of matrix (2.25) and hypertrophy (2.25).
4	Erosion	Sloughing of cellular layers (3.20), maximum loss of matrix (3.20), clusters of chondrocytes (3.20), hypocellularity (3.20) and multiple patches of cell death (3.20).
5	Denudation	Denudation of bone (4.25), maximum loss of cellular layers and matrix (4.25), micro fracture (4.25) and initiation of fibrosis (4.25).

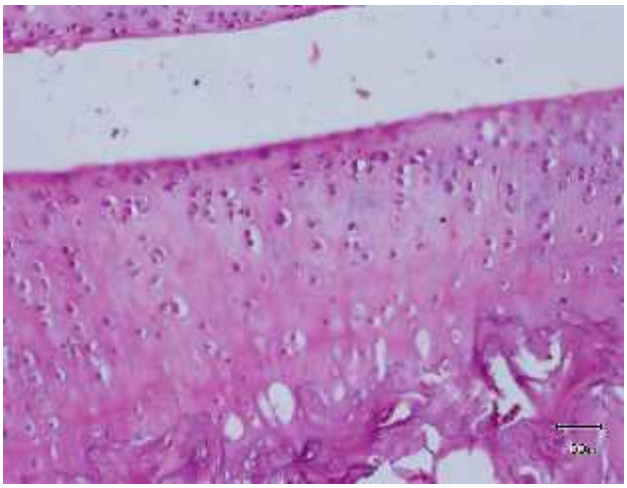

Fig. 01: Histological slide of rat cartilage (normal) allocated Grade-0 (100X) Intact surface layer, Normal population of chondrocytes, Normal orientation of chondrocyte columns, normal morphology of cells and continuous matrix.

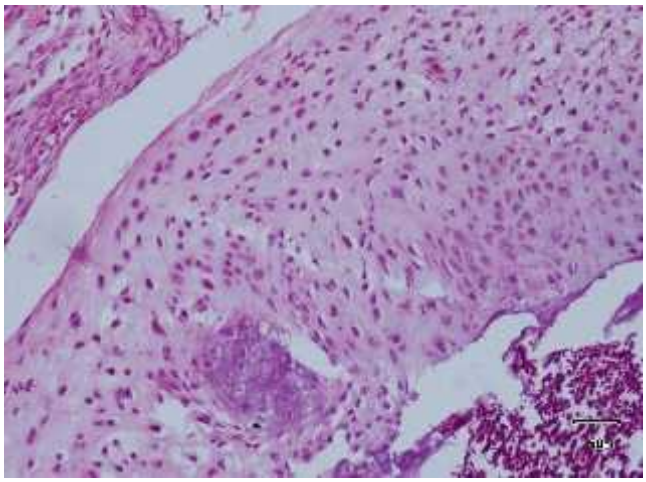

Fig. 02: Histopathological slide of rat cartilage allocated Grade-1 (200X) Fibrillation of superficial layer, condensation of superficial layer, proliferation of chondrocytes and initiation of chondrocyte cluster formation

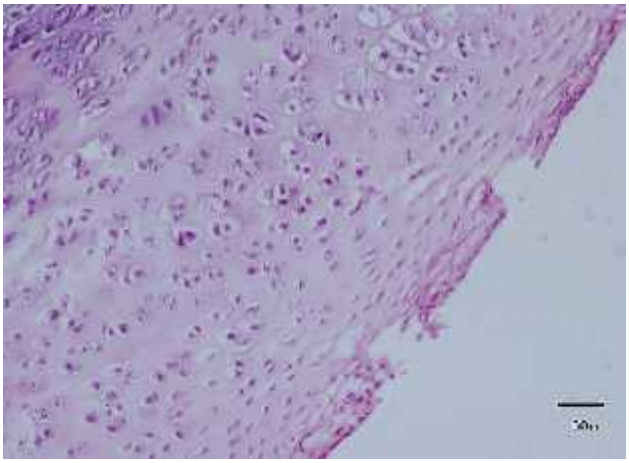

Fig. 03: Histopathological lesions of rat cartilage allocated Grade-2 (100X) Discontinuity of superficial layer, cell death in mid zone, hypercellularity, Disorientation of chondrocyte columns and cluster formation

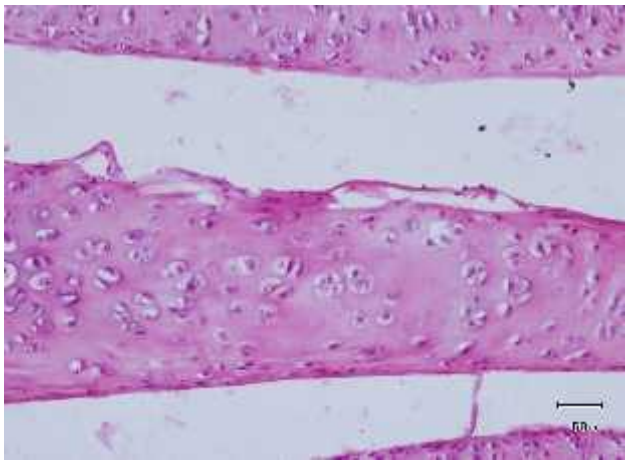

Fig. 05: Histopathological lesions of rat cartilage allocated Grade-4 (200X) Sloughing of superficial layer, desquamation of superficial layer from the middle zone, mild loss of matrix, Clusters of chondrocytes, hypocellularity and cell death

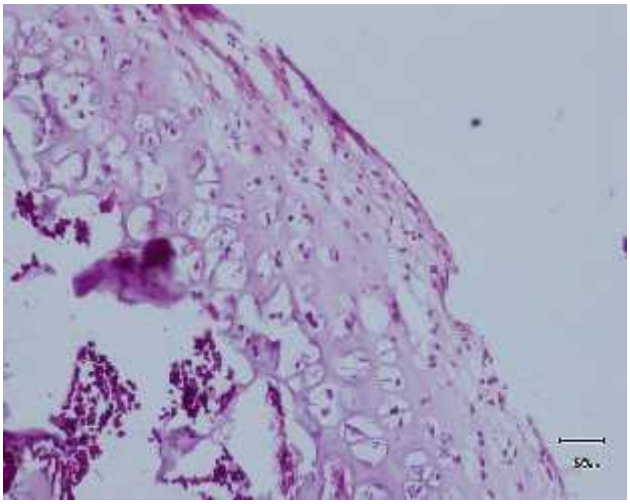

Fig. 04: Histopathological lesions of rat cartilage allocated Grade-3 (100X) Discontinuity of superficial layer, patches of cell death, Cluster formation of chondrocytes, sloughing of layer till mid zone and disoriented chondrocyte columns

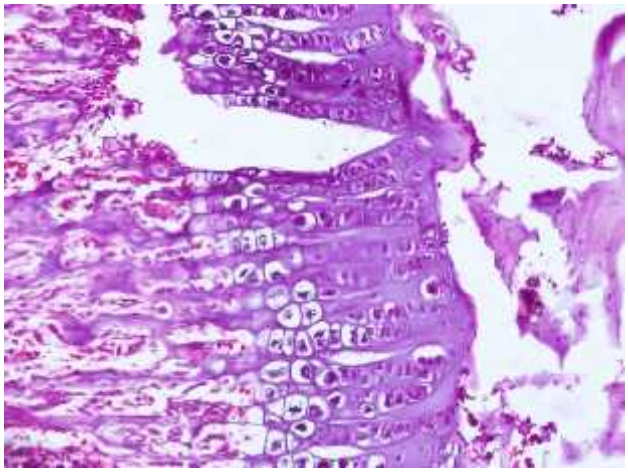

Fig. 06: Histopathological lesions of rat cartilage allocated Grade-5 (200X) Denudation of bone, maximum loss of cellular layers and matrix, micro fracture and initiation of fibrosis

Table 02: Mean histological lesion scores of control (Day 0) and experimentally induced OA groups on different days

Days	Replicates	Histological lesions	Histological Score	Mean±S.D.
00	01	Intact surface, normal population of chondrocytes, normal morphology of cells and continuous matrix.	0	.00±.00[a]
	02	Intact surface, normal population of chondrocytes, normal morphology of cells, continuous matrix, normal architecture of cells and matrix.	0	
	03	Intact surface, normal population of	0	

		chondrocytes, normal morphology of cells, continouse matrix, normal architecture		
	04	Intact surface, normal population of chondrocytes, continuous matrix,	0	
	05	Intact surface, normal population of chondrocytes, continuous matrix, normal orientation of chondrocytes	0	
	01	Surface irregularity (0.25), mild cluster formation (0.25), moderate cell death(1.20), little condensation of cells in superficial layer (0.25)	1.95	
	02	Discontinuous superficial layer (1.20), hypercellularity (1.20), cluster formation (1.20), condensation in superficial layer (0.25).	3.85	
	03	Hypercellularity (1.20), cluster formation (1.20),	2.65	
01		condensation of cells in superficial layer (0.25).		3.09±.81[b]
	04	Rough surface (0.25), condensation of cells (0.25), disturbed morphology of chondrocytes (0.25), cluster formation (1.20) and severe cell death (1.20).	3.15	
	05	Hypercellularity (1.20), Condensation of chondrocytes (0.25), mild sloughing of superficial layer (1.20) and cluster formation (1.20)	3.85	
	01	Roughness of layer (1.20), moderate cluster formation (1.20), loss of matrix (2.25) hypocellularity (3.20).	7.85	
	02	Hypocellularity (3.20), maximum cluster formation(1.20), disturbed architecture of chondrocytes (0.25), sloughing of superficial layer (2.25).	6.90	
	03	Hypocellularity (3.20), disorientation of	8.90	
07		chondrons (1.20), mild loss of matrix (2.25) and sloughing of superficial layer (2.25).		7.48±1.15[c]
	04	Sloughing of superficial layer (2.25), disorientation of chondrons (1.20), hypocellularity (3.20) and condensation of cells in superficial layer (1.25)	7.90	
	05	Hypercellularity (1.20), disorientation of columns (1.20), irregularity of superficial layer (1.20) and mild loss of matrix (2.25).	5.85	
14	01	Sloughing of superficial layer till mid zone (3.20), cluster formation (1.20), disoriented chondrocyte columns (0.25), hypercellularity (1.20) and condensation in superficial layer (0.25).	6.10	
	02	Loss of superficial layer (1.20), hypercellularity (1.20), hypertrophy (2.25), loss of matrix (2.25) and condensation of chondrons (0.25).	8.15	
	03	Sloughing of superficial layer till mid zone (2.25), hypertrophy (2.25), cluster formation (1.20), loss of matrix (2.25) and cleft formation (3.20).	11.15	9.92±2.76[d]
	04	Loss of superficial layer till mid zone (3.20), cleft formation (3.20), hypocellularity (3.20), hypertrophy (2.25) and cell death (1.20).	13.05	
	05	Loss of superficial layer, loss of matrix (3.20), hypocellularity (3.20), hypertrophy (2.25), disorientation of chondrons columns (0.25) loss of matrix (2.25).	11.15	

	01	Sloughing of superficial layer (3.20), maximum hypocellularity (3.20), chondrones columns disturbed (0.25) and cell death in deep zone (2.25).	8.90	
	02	Sloughing of superficial layer (3.20), disturbed chondrones columns (0.25), condensation of cells (0.25), cell death in patches (2.25) and cluster formation (1.20).	7.15	
21	03	Sloughing of layer till mid and deep zone (3.20), maximum hypocellularity (3.20), disturbed chondrones columns (0.25), cell death in deep zone (2.25) and hypertrophy (2.25).	11.15	10.09 ± 2.02^{d}
	04	Loss of superficial layer till mid zone (3.20), loss of matrix (2.25), patches of cell death (2.25), hypercellularity (1.20) and cleft formation (3.20).	12.10	
	05	Sloughing of superficial layer (3.20), hypertrophy (2.25), matrix loss (2.25), condensation of cell in superficial layer (0.25) and erosion (3.20).	11.15	
	01	Loss of superficial layer till deep zone (3.20), matrix loss (2.25), hypocellularity (3.20), cleft formation (3.20) and cell death in patches (3.20).	15.05	
	02	Loss of layer till mid zone (3.20), cleft formation (3.20), cell death in patches (3.20), hypercellularity (1.20) and disturbed columns of chondrons (0.25).	11.05	
	03	Loss of superficial layer till mid zone (3.20), cleft formation (3.20), multiple patches of cell death (2.25), hypercellularity (1.20) and matrix loss (2.25).	12.10	
28				12.82 ± 1.64^{e}
	04	Loss of superficial layer till mid zone (3.20), cleft formation (3.20), multiple patches of cell death (3.20), hypercellularity (1.20) and matrix loss (3.20).	14.00	
	05	Sloughing of superficial layer till mid zone (3.20), Hypocellularity (3.20), loss of matrix (2.25) and hypertrophy (2.25).	11.90	

Means±S.D with different superscripts vary significantly while same superscripts have non- significant difference

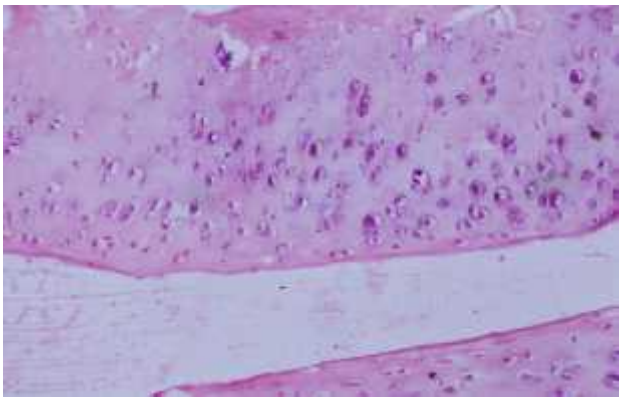

Fig. 07: Histopathological slide of rat cartilage at Day zero (100X) Intact surface, normal population of chondrocytes, normal morphology of cells, continuous matrix, normal architecture of cells and matrix

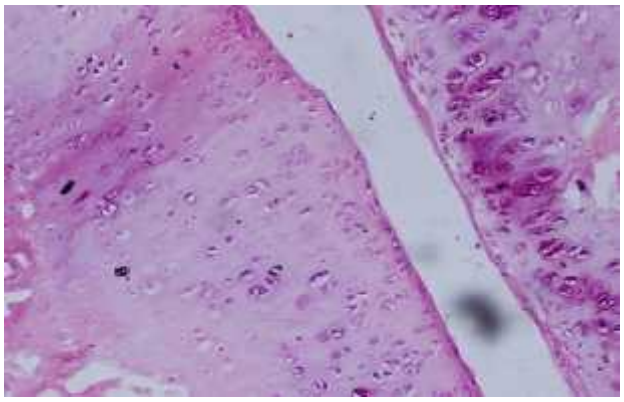

Fig. 08: Histopathological representation of rat cartilage at Day (01) post papain injection (100X) Hypercellularity, disorientation of columns, irregularity of superficial layer, initiation of cluster formation

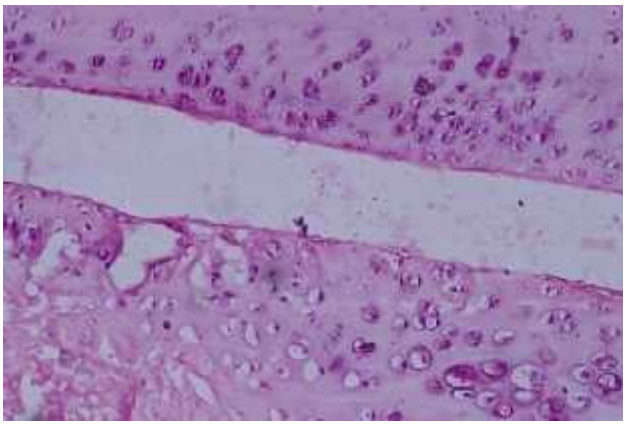

Fig. 09: Histopathological representation of rat cartilage at Day (07) post papain injection (100X) Sloughing of superficial layer, disorientation of chondrons, condensation of cells in superficial layer

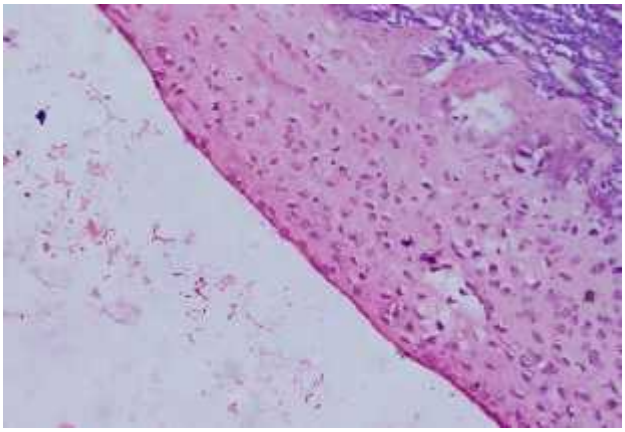

Fig. 10: Histopathological representation of rat cartilage at Day (14) post papain injection (100X) Loss of superficial layer till mid zone, cleft formation, hypocellularity, hypertrophy, cell death, disturbed architecture of cells

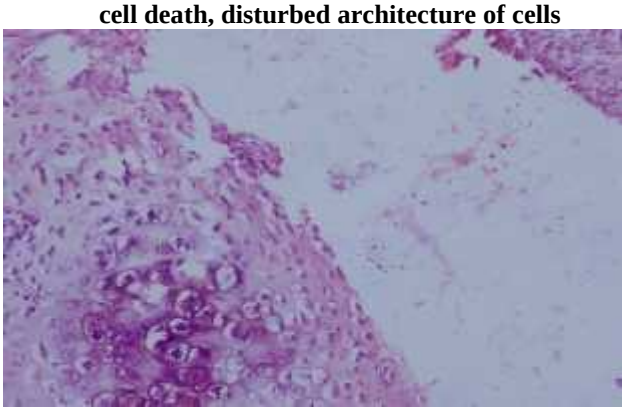

Fig. 11: Histopathological representation of rat cartilage at Day (21) post papain injection (100X) Sloughing of superficial layer, disturbed chondrones columns, hypertrophy, cell death in patches, cluster formation

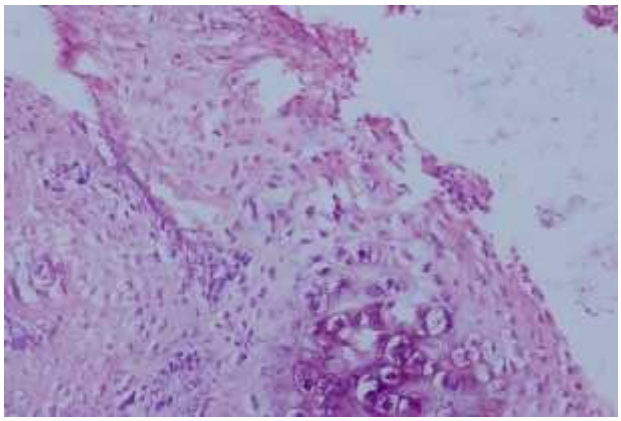

Fig. 12: Histopathological representation of rat cartilage at Day (28) post papain injection (100X) Loss of superficial layer till mid zone, cleft formation, multiple patches of cell death, morphology disturbed, hypercellularity, matrix loss

DISCUSSION

Osteoarthritis (OA) models were developed by surgical, mechanical or chemicals means in experimental animals. Specific knock out strains of mouse and guinea pig had been used for development of spontaneous OA (Glasson 2007). Papain (proteolytic enzyme) or monosodium iodoacetate (MIA) were used to alter histological morphology of articular joints (Guzman *et al.*, 2003). Enzyme collagenase caused injury of ligaments and tendons. Joint instability was induced in surgical models by partial meniscectomy combined with transaction of collateral and cruciate ligaments (Bendele 2001; Pmonis *et al*., 2005; Fernihough *et al*., 2004).

The Dunkin-Hartley guinea pig model was used mostly in experiments to develop OA of the medial joint compartment. In this model, mild focal changes in the area of medial tibial plateau and femoral condyle were observed in about 50% of animals at age of 3 months with weight of 700grams (Bendele 2001). Due to unavailability of various transgenic and knock out models naturally occurring models of mice were used in number of trials. Spontaneous OA had been evaluated in several mouse strains, including SRT/Ort (Mason *et al*., 2001), SRT/ IN (Schunke *et al*., 1988), and C57/ B16 (Glasson 2007).

Compression/immobilization technique was also used to produce degenerative OA in experimental animals in which joints were immobilized in forced fixed positions for a long period of time (Palmoski and Bean 1988). However, this method took much time for development of experimental model.

In present study chemical agent used to develop OA was an enzyme papain in accord with Kopp *et al*. (1983). Tanaka *et al.* (1992) injected papain intra-

articularly to induce experimental OA. Murat *et al.* (2007) quantified papain induced OA using Mankin scoring system. Cartilage changes were observed in papain induced OA in rats by Grevenstein *et al*. (1991).

Quantification of papain induced OA in experimental rat model was developed in relation to progression of lesions with days. In present study, histopathological scoring system was developed with minor modification following Mankin scores as described by Murat *et al*. (2007). This scoring system was used to estimate the level of clinical OA in Wister rats after papain injection intra-articularly. Lesions were divided into five grades starting from normal histology to maximum damaged cartilage. Division of histological lesions in grades was comparable with model of Pritzker *et al*. (2006) with the exception of an additional grade (6) denudation (micro fracture of bony tissue) which was not observed in present study. Grading systems used in experimentally induced OA using knee joint had minor variations. Hluchy *et al*. (2008) used description for histopathological evaluation of degenerative changes in cartilage and scored them on the basis of severity with progression of OA in relation to time. Descriptive histological lesions used were Loss of superficial layer, erosion of cartilage, fibrillation or fissures, loss of proteoglycan, disorganization of chondrocytes, loss of chondrocytes, exposure of subchondral bone and cluster formation. This description differs from that used in present study. Van der Kraan *et al*. (1989) induced OA in mice using papain, iodoactate and collaginase. Quantifcation of histological lesions was on the basis of cartilage fibrillation, erosion of noncalcified cartilage, erosion of calcified cartilage, chondrocyte death, chondrocyte clusters and loss of safranin O. Kleemann *et al*. (2005) quantified progression of OA following the Mankin *et al.* (1971) which was in accord to observations of present study.

Singh *et al.* (2012) developed cartilaginous defective model by using surgical means and measured its healing capacity by using cartilage repair assessment chart. This system was based on International Cartilage Repair System (ICRS) that closely resembles with Mankin scoring system used in this study to measure the progression of disease with passage of time.

Van der Kraan *et al*. (1989) observed distinct depletion of stain and cytolysis post papain and iodoacetate injection which was comparable with bovine serum albumin induced OA (Van den Berg *et al*., 1982). Schalkwijk *et al*. (1985) observed death of chondrocytes in patches which was due to variable sensitivity of chondrocytes in different areas of cartilage. These findings are comparable with present study. Chondrocytes clusters at area close to bony tissue in post papain injected knees were similar to observations reported by Bendele *et al*. (1987) and Walton *et al*. (1977) in spontaneous OA of guinea pigs. Similarly, localization of chondrocytes in OA had been reported by many researchers at multiple points in cartilage (Moskowitz *et al*., 1973; Schwartz and Levielle 1981; Colombo *et al*., 1983; Bendele *et al*., 1987). Areas of hypocellularity and hypercellularity observed with progression of OA post papain injection in relation to time in present work were comparable with results of Rostand *et al*. (1986), Havdrup *et al*. (1977), Moriizumi *et al*. (1986) and Coulais *et al*. (1983). Proliferation of chondrocytes response was due to depletion of proteoglycans initially and later on lowered number was observed which may be due to lowered metabolic activity of chondrocytes. Similar findings had been reported by Van der Kraan *et al*. (1989). In OA rat model, loss of matrix, chondrocyte clustering and perichondrocyte hollow areas were observed in accord with observations of Walton *et al*. (1977) and Schunke *et al*. (1988) in different mouse strains.

In present study highest histological lesion score (12.82±1.64) was observed in cartilages on 28^{th} day post papain injection. So, it was concluded that severity of lesions progressed with passage of time and maximum lesions were observed on day 28^{th} that seem to be consistent with early OA changes in human. The findings may provide therapeutic strategy to clinicians for treatment of early OA changes in human. This model offers a rapid and minimally invasive method to produce OA like lesions in rodent model.

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