Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

TGF-β1 Improves Articular Cartilage Damage in Rabbit Knee

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ARTICLE HISTORY

Received: October 19, 2011
Revised: December 01, 2011
Accepted: January 05, 2012

Key words:
Cartilage
Neochondrogenesis
Mesenchymal stimulation
Osteochondral defect
TGF-β1

ABSTRACT

The present study was designed to assess the role of TGF-β1 in cartilage repair in an in *vivo* model of articular cartilage defect in rabbit. Twenty four New Zealand white rabbits of either sex, 6-7 months old (1-2 kgs) were divided into two groups i.e. A (Control) or B (TGF-β1). The articular cartilage defect of 3mm diameter and 2mm depth was created in the femoral groove of femoropatellar joint. Animals of both the groups were provided with collagen scaffolds at 10µg/cm2 in the articular defect. However, Group B animals were further provided with 20ng/20µl of TGF- β1 in the defect stuffed with collagen scaffold. Articular defect grossly appeared re-surfaced completely and healed with better joint movement in group B as compared to the animals of group A. Repaired articular surface maintained the transparency and sheen as hyaline cartilage. Synthesis of proteoglycans, enhanced filapodia activity, significant increase in the collagen type II and aggrecan in the repaired tissue further supports that TGF-β1 at the tested dosage improved the articular cartilage management and repair.

To Cite This Article: Singh NK, S Shiwani, GR Singh, DK Jeong, P Kinjavdekar, Amarpal, JD Lohakare and SJ Lee, 2012. TGF-β1 improves particular cartilage damage in rabbit knee. Pak Vet J, 32(3): 412-417.

INTRODUCTION

Cartilage exposure to any injurious agent can suppress proteoglycans synthesis and stimulate degeneration process of articular cartilage (Douglas *et al*., 2001). Proteoglycans and collagen are the major macromolecules which help in the maintenance of the cartilage proper function. These macromolecules are regulated by number of biological/ growth factors (Fortier *et al*., 2002) during chondrogenic development.

TGF- β1 has been demonstrated in the past to have role to play in cellular proliferation, differentiation, matrix production (Chai *et al*., 2003) and stimulates proliferation and inhibits terminal differentiation during chondrogenesis (Alvarez *et al*., 2001). Moreover, *in vitro* studies have also shown that TGF-β1 can inhibit catabolism, alter cell proliferation rate and counteracts interleukin-1 (Poole, 2003) and in turn it promotes proteoglycan synthesis and cartilage repair (Loveridge *et al*., 1993).

Use of locally delivered TGF- β1 in cartilage repair *in vivo* (Abe *et al*., 2003) has been reported experimentally however, its detailed analysis has not been so far elucidated in conjunction with collagen scaffoldings. Keeping into account paucity of literature on the application of external TGF-β1 along with scaffolds on cartilage repair, the present experiment was therefore, designed to study in detail the cartilage repair under the influence of transforming growth factor- β1.

MATERIALS AND METHODS

Experimental Animals: Twenty four clinically healthy New Zealand white 6-7 months old rabbits of either sex weighing 1-2 kg were chosen randomly in the study. Animal experimentation was approved by Institutional Animal Ethics Committee. The animals were provided with the standard diet and were acclimatized to approaching and handling for a minimum of one week prior to the study.

Experimental Design: The experimental animals were divided into two groups having twelve animals in each group i.e. group A (Control) and group B (TGF-β1). Total dose of 20 ng/20 µl of TGF-β1 per animal with collagen (10 µg/cm^2) was used in this study as per the

recommendation and description given earlier by Singh *et al*. (2007). Each group had 12 animals; four animals from each group were sacrificed on day 15, 30 and 45 of the experiment. All animals were kept off feed for 24 h and water was withheld for 12 h before the start of experiment to ease the handling of animals.

Operative procedure: Operative procedure and post-operative management was followed as described by Singh *et al*. (2007) (Fig. 1). Briefly, after xylazine and ketamine anesthesia, left knee was aseptically opened and patella was dislocated to expose the patellar groove of the femur. With the help of hand held trephine (modified from stainless steel reamer whose diameter was 3 mm, depth 2 mm and thickness of the trephine blade was 0.095 mm) osteochondral tissue was harvested from femoro-patellar groove and discarded (control).

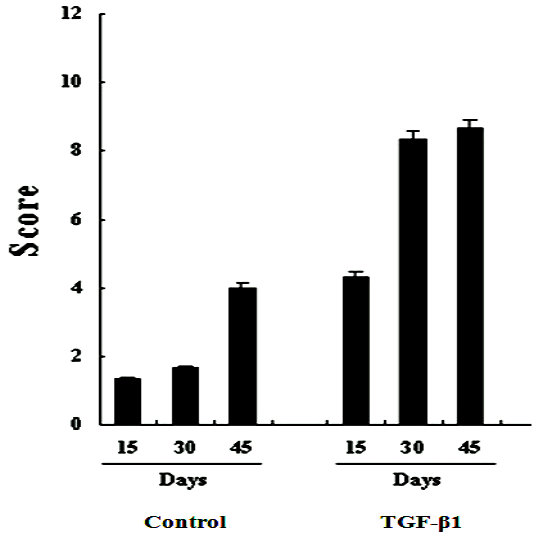

Fig. 1: Assessment of cartilage repair based on International Cartilage Repair Society (ICRS) assessment scale given in table. 1.

Evaluation of healing of cartilage was done on the basis of following parameters: Gross-observation: Cartilage repair assessment chart (ICRS) was taken into consideration for gross evaluation of cartilage healing (Table 1). The ICRS system of assessment of cartilage repair has been developed from the work of Brittberg and Peterson described by Peterson *et al*. (2000). This is a 3-point scoring system in which protocol 1 is made to assess the filling of the cartilage defect where as protocol 2 is made to assess the quality of the new tissue (such fibrillation, roughness, smoothness, coloration and congestion) which was formed after the creation of the defect.

Radiology: Lateral and antero-posterior views of the test joint for all the animals were taken in the flexion and extension before and on d 15, 30 and 45 respectively.

Histopathology and histochemistry: Entire femoral condyles were harvested and specimens were fixed in 10% neutral buffered formalin and decalcified in 25% formic acid for histopathological examination. Presence and or production of proteoglycans and collagen in the repaired cartilage were assessed qualitatively under polarized-light condition at different time intervals using toluidine blue (TB) and Masson's trichome (MST) respectively (Singh *et al*., 2007).

Surface electron microscopy: The samples for surface electron microscopical studies were fixed in 2% glutaral-dehyde. Hexamethyl disilazane (HMDS; Sigma Aldrich; Saint Louis; Missouri; USA) technique was used to dry the specimens (Singh *et al*., 2007). The specimens were observed at 5kv and the magnification range of the unit was calibrated using colloidal latex particles of 1μm size.

Table 1: Cartilage repair assessment chart (ICRS). ICRS scaling based description and score points

Protocol	Detail	Score
I: A	In level with surrounding cartilage	4
	75% repair of depth defect	3
	50% repair of depth defect	2
	25% repair of depth defect	1
	0% repair of depth defect	0
2: A	Macroscopic appearance	
	Intact smooth surface and glistening	4
	Fabrillated and rough surface	3
	Small, scattered, fissures or cracks	2
	Several, small or few but large fissures	1
	Total degeneration of grafted area	0
B	Macroscopic appearance in coloration	
	Transparency	4
	Transparency with whitish ring	3
	Whitish opaque	2
	Yellowish opaque and whitish ring	1
	Reddish and whitish ring	0
C	Macroscopic appearance in relation to congestion of the area	
	No congestion	4
	Slight congestion	3
	Mild congestion	2
	Moderate congestion	1
	Severe congestion	0
Overall repair assessment		
	Grade I normal	12
	Grade II near normal	11-8
	Grade III abnormal	7-4
	Grade IV severely abnormal	3-1

Quantitative real-time PCR for the chondrocyte specific cell type marker gene: Cartilaginous tissue samples were collected from the repaired area of the femoral condyles and the total RNA was extracted using Trizol reagent according to the manufacturer's instructions. The sequences of primers were i.e. aggrecan (L38480.1); forward: 5'-CCAGACCGGCTACCCCGAC CC-3'; Reverse: 5'-CCAAGGGCGGCTTCGTCAGCAA A -3', collagen type II (S83370.1); forward: 5'-GGGTGG ACATAGGGCCCGTCTG-3'; Reverse: 5'-CTTGCTTCT GGGCGGGGCGTTG-3', Probe; 18S rRNA gene (NR002170); forward: 5'-AGTCGGCATCGTTTATGGT C-3'; Reverse:5'-CCGCGGTTCTATTTTGTTG-3'. 18S rRNA gene was used as an internal control. Fold change in the relative gene expression of target were determined by calculating the 2-ΔΔCT (Pfaffl, 2001).

Statistical analysis: Differences between groups from different time interval were analyzed by one-way analysis of variance (ANOVA). Significant differences were detected ($P<0.05$) by Duncan's multiple range tests using a PC statistical package (SAS Institute Inc., 2002).

RESULTS

Assessment of cartilage repair (ICRS): Group B (TGF-β1) was scored grade II compared to group A (Control) which scored grade III on 45th day of observation based on ICRS protocols and scaling (Fig. 2). Grossly, on d 15, the defect appeared quite fresh in group A with reddish clot along with thin white circular ring at the margins of the defect and congested adjacent femoral ridges. On d 15 in group B (TGF-β1) defect created appeared 25% filled up as compared to healthy articular surface. Though the formed tissue was irregular and white but articular surface appeared quite smooth, glistening with no hemorrhagic signs. Later on, the tissue further organized as healthy cartilaginous tissue on d 45 (Fig. 3).

Radiological Evaluation: On d 15 in group A (Control), the lateral radiographs showed discontinuity in the articular surface. There was marked decrease in the size of circular defect and area appeared radiolucent but hazy as compared to the cortex and surrounding bone on d 45. Defect appeared radiolucent in group B initially but later on it became almost invisible on d 45 and articular surface appeared completely restored radiologically (Data not shown).

Histological Evaluation: Extensively hemorrhagic region in the deep epiphysis that was observed on d 15 (Control) covered with thick fibrous connective tissue. On day 45, the surface connective tissue had reduced in amount with slight proliferation of the mesenchymal layer over the connective tissue. Bony spicules were also evident in the repaired region of the control group (Fig. 4).

Group B (TGF-β1) on day 15 showed defects filled with fibrin and overlaid by new loosely arranged fibroblasts with lots of neutrophilic infiltration. Early attempt to cover the granulation tissue by synovial mesenchymal cells was quite evident. Increased fibroblastic activity was noticed in the deeper layers however, there was disappearance of matured megakaryocytes. Fibrocellular reaction and the production of proteoglycan became prominently evident when visualized by Masson's trichome (collagen specific) and toluidine blue (proteoglycan specific). Defect was filled with fibro-osseous tissue (evident by osteoblast like cells) (Fig. 4) on d 45.

Scanning electron microscopic (SEM) observation: On day 45, salient features of cartilage healing on SEM included surface contour, surface quality, presence of cells (chondroblast, chondrocyte, and lacunae), orientation of collagen fibers and blood vessels. Periphery of the defect showed a number of cells with filopodia (Fig. 5). These cells appeared to be in active multiplication phase as progressing towards the defect.

In group B (TGF-β1), the junction of defect at lower magnification appeared hillocky with hollows and prominences. At higher magnification the dense area showed collagenous projections and cells with filopodia showing its higher activity. At lower magnification the area appeared as trabeculated with the formation of dense trabeculae connected with each other leaving hollows in between giving an impression of unorganized osseous/ cartilaginous structure (Fig. 5).

Quantitative real-time PCR for he chondrocyte specific cell type marker gene: Fold change in the relative gene expression pattern of collagen type II and aggrecan during the healing process in the presence TGF-β1 was found to have increasing trend However, an increase in the expression pattern of aggrecan invariably followed expression pattern of collagen type II (Fig. 6). It was observed that the expression of both the chondrocyte specific gene showed significant increase ($P<0.05$) soon after d 15.

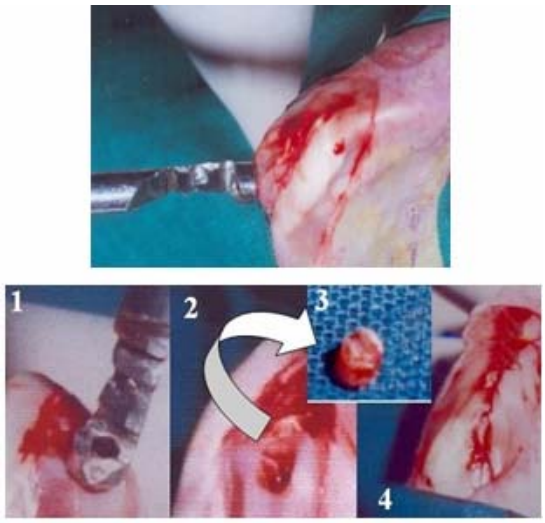

Fig. 2: Operative procedure depicting formation of defect in the femoro-patellar groove with the hand held trephine. 1) Harvesting osteochondral tissue, 2) Showing osteochondral defect, 3) Showing harvested osteochondral tissue and 4) Closing the knee joint after repositioning of the patella.

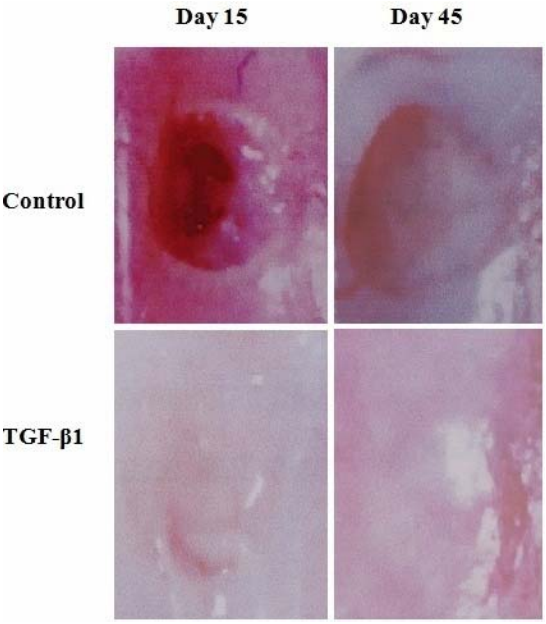

Fig. 3: Macroscopic appearance of articular cartilage defect during repair process. Control: d 15: Reddish clot along with thin white circular ring; d 45: Slight presence of blot clot towards one edge of the defect. TGF-β1: d 15: Gap appeared slightly below the normal articular cartilage; d 45: Gap filled up completely. Color of the defect appeared same as the healthy articular cartilage.

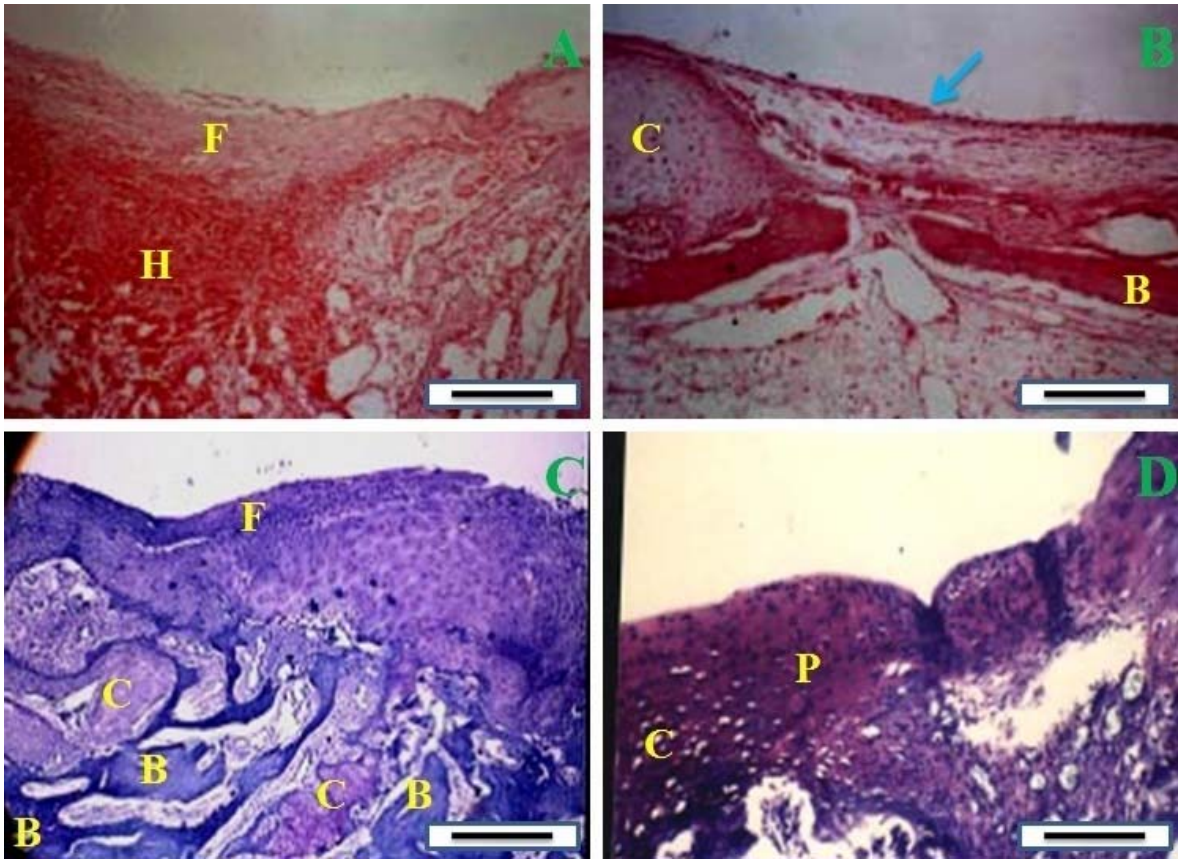

Fig. 4: Histological observations of articular cartilage during repair process. A. (Control - d 15): Hemorrhage (H) in the epiphysis of the defective region covered by fibrous tissue (F). B. (Control- d 45): Mesenchymal cells (arrow) lining the surface of the defect and indicating underlying bone (B) and peripheral chondrocytes (C) of the cut end of the defect. H&E X 80. C. (TGF-β1-d 15): Fibrinocellular reaction (F) seen prominent. MST X 100. D. (TGF-β1-d 45): Chondrogenesis (C) in the central portion of the gap showing intense proteoglycan (P) production: TB X 100.

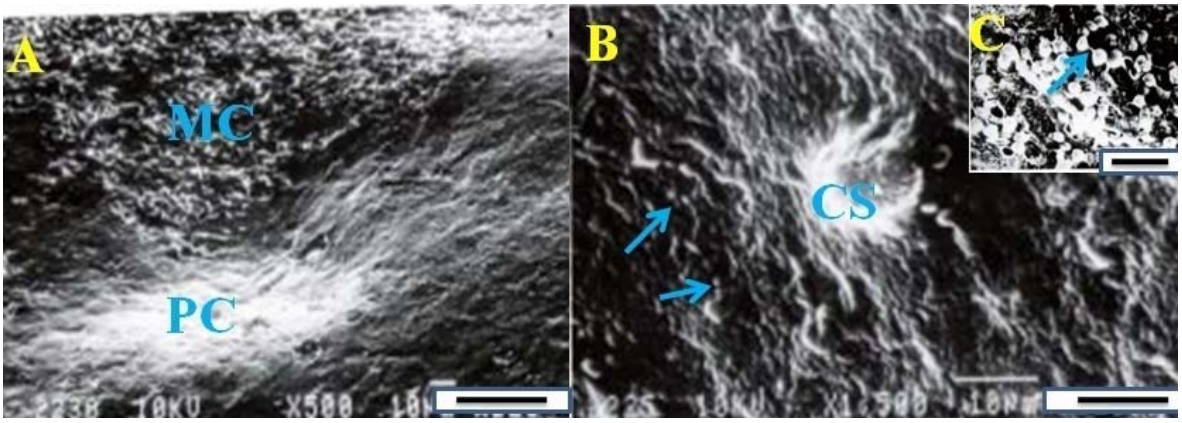

Fig. 5: Surface electron microscopic evaluation of articular cartilage repair on day 45. A. (Control): Proliferation of mesenchymal cells (MC) in the defect and the cell showing circular arrangements towards the defect indicating peripheral chondrogenesis (PC). X 500. B. (TGF-β1): Defect showing relatively dense prominent areas which had fine projections with newly formed chondrocytes forming filopodia's between themselves (arrows) indicating cartilage stimulation (CS) in the region of the defect. X 500. C. Developing filopodia's between the chondrocytes (arrows) shown in higher magnification. X 1000.

DISCUSSION

Osteochondral lesions such as Osteo-chondritis dessicans (OCD) in clinical patients develop over time and the responses of those tissues may be different compared to the experimental situation where the defect created was repaired immediately as in our study. We followed six weeks duration to study the healing capacity of cartilage, defect of appropriate size that normally does not heal without some form of treatment (Brittberg and Winalski, 2003). Moreover, we studied healing for six weeks duration as this has been demonstrated as sufficient time points for the detection of complete healing of cartilage defect (Brittberg and Winalski, 2003).

The fibrous materials developed along the cut edges, but it was not well recognized and organized only in the depths of the defect (Shapiro *et al*., 1993) in the control animals. Grossly, slight bluish white tinge was noted in

the repaired tissue without any signs of hemorrhage which might be attributed to the action of TGF-β1 on fibroblast. Significant increase (P<0.05) in the aggrecan and collagen type II gene in this study supports the hyaline production which was indicated as bluish white tinge grossly in the present study. TGF-β1 is known to cause increase in the cellular mRNA for connective tissue growth and stimulate connective tissue cell growth and extracellular matrix (Grotendorst, 1998) and increase the production of aggrecan and collagen type II factors to induce hyaline production. Role of fibroblast and other progenitors such as bone marrow elements have been documented to undergo differentiation either to chondroblast, osteoblast and adipoblast under the influence of certain growth factors (Shuler *et al*., 2000; Singh *et al*., 2007).

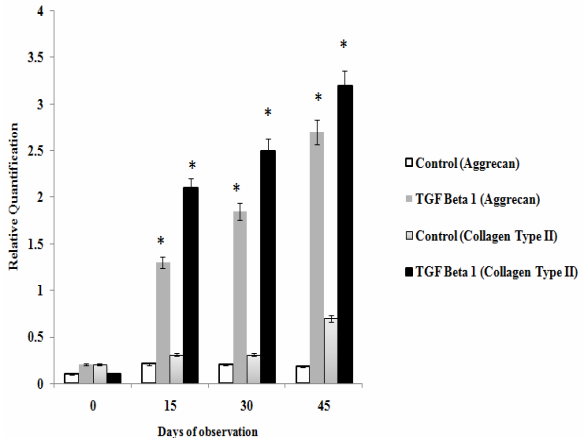

Fig. 6: Quantitative real-time PCR for the chondrocyte specific cell type marker gene during healing of full thickness defect at different point of time. *Indicates a significant difference (P<0.05) within an item compared with the undifferentiated (d 0) cells.

Neo-cartilagenous tissue was radiolucent radiographically and the continuity of the articular surface was in restoration and progression suggested progressive healing in general. Diameter of the defect, with the progress in the healing process, appeared to decrease significantly and the defect became hazy suggested the presence of osseous and chondroid cells (Shapiro *et al*., 1993) in the formed fibrous mass. Increased density at the periphery of the defect revealed the initiation of healing process however, slow and the defect could not be repaired completely in the control animals.

Lateral radiograph showed complete restoration of articular surface of the defect in group B (TGF-β1). Gradual decrease in the size of the defect was viewed in the lateral radiograph of group B animals which further suggested sequential healing process (Singh *et al*., 2007) that seems to hasten up under the influence of TGF-β1 (Lind, 1996). And the entire process in turn would have increased extracellular matrix in the process of chondrogenesis. However, the chondrogenic tissue appeared radiolucent probably because of the nascent and immature cluster of chondroblasts.

Articular defect gets filled up initially with blood clot (Shapiro *et al*., 1993) leading to fibrin network (Singh *et al*., 2007) formation and pooling of pleuripotent cells from marrow and cut margins of the synovial membrane (Dreher, 1982). Percent increase in the repair of the defect depth could be attributed to the proliferation and differentiation of mesenchymal cells to osseous tissue (Singh *et al*., 2007) and moreover it has also been documented earlier that the use of TGF-β1 for cartilaginous tissue development generally end up with forming osseous tissue or produce side effects which could end up in a bone production (Singh *et al*., 2007). Osseous formation and congested region in the defect was quite evident histologically in our study depending on the stage of the trauma or repair. Congested areas in the defect assumed a smooth and glistening surface over the period treatment in the animals (group B) which have been treated with TGF-β1 and such improvement might be attributed to the increase in the cellular mRNA of TGF-β1 (Grotendorst, 1998) and anti-inflammatory action TGF-β1 (Shuler *et al*., 2000). Significant increase in total aggrecan and Collagen type II genes as indicated in the real time quantification in the present study apparently indicated that there might have been higher cellular m RNA production of TGF-β1.

The periphery of the defect showed number of cells (chondroblast) in active multiplication phase progressing towards the defect center (Shapiro *et al*., 1993) in electron microscopy. Multiplying cells appeared to have cellular projections termed as filopodia at higher magnification. At lower magnification the area appeared as dense trabeculae with hollows in between indicating the presence of cartilaginous structure (Horas *et al*., 2003).

Hillocky surfaces as per the electron microscopy have been reported to be invariably rough or knobby. Fine projections, which were observed extending towards the hollows, would have been either filapodia or fine collagen, synthesized by the chondroblasts (Suso *et al*., 2004).

Expression of aggrecan and collagen type II on different days of healing of full thickness defect apparently shows the magnitude of TGF-β1 in mediating certain signaling pathway (Wnt) and instigating the chondrocyte differentiation for the repair process (Zhu *et al*., 1999). The expression patterns that indicated proper healing were in synergy with the macro and microscopic observations of the present study. Conclusively, TGF-β1 improved cartilage repair and yielded tissues equivalent to hyaline cartilage however, TGF-β1 needs to be further researched in clinical settings such as autografting and autologous chondrocytes transplantation.

Acknowledgement: The work was partly supported and facilitated with the grants from Indian Council of Agricultural Research, Department of Biotechnology (BT/PR10040/MED/31/12/2007), New Delhi, India and National Research Foundation of Korea (Grant No. 120110393).

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