



Application of *ex-vivo* expanded autologous bone marrow derived mesenchymal stem cells for repair of transected tendon in caprine

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Received: 4 December 2013; Accepted: 3 February 2014

ABSTRACT

Clinically healthy non-descript adult goats (18) of either sex, were divided equally in 2 groups (group A and B) to study the application of *ex-vivo* expanded autologous bone marrow derived mesenchymal stem cells for repair of transected tendon in caprine. The bone marrow aspirate was collected from iliac crest and mesenchymal stem cells were cultured. When the cells reached 70–80% confluency, they were harvested using 0.25% trypsin-EDTA. In both the groups, the superficial digital flexor tendon (SDFT) was transected. In group B (test group), bone marrow derived mesenchymal stem cells (BM-MSCs) were implanted at the site of injury while in control group (group A), the operated limb was allowed to heal without BM-MSCs treatment. Post-operative care was similar in both the groups. The efficiency of treatment was evaluated by observing various clinical parameters on day 3, 7, 10, 15, 30, 45 and 60 postoperatively. The air-tendograms were performed on day 15, 45 and 60. There was early reduction in swelling, exudation, warmth and pain at the repaired site in group B. The early restoration of tendon gliding movement and weight bearing capacity were also observed in the test group as compared to the control groups. Air-tendograms revealed comparatively more adhesions in control group than in test group. Result concluded that autologous BM-MSCs enhance the tendon healing and thus can be used for early and better healing in cases of tendon injuries.

Key words: Air-tendograms, Bone marrow derived mesenchymal stem cells, Goats, Superficial digital flexor tendon

Tendons are dense connective tissues responsible for passively transferring forces generated by muscles to the opposite side of the joint, and support normal movements and the stability of the animal (Kannus 2000). When subjected to high physiological loads, these tissues are commonly injured and fail to heal optimally due to their low cellularity and vascularity (Strauss *et al.* 2007). Tendon injuries produce substantial morbidity, and at present there are only a limited number of scientifically proven management modalities (Sharma and Maffulli 2005). Bone marrow derived mesenchymal stem cells (BM-MSCs) were chosen for this study as they are the most investigated and characterized postnatally derived progenitor cells and they appear to perform superiorly to MSCs recovered from other tissues in terms of their ability to undergo differentiation under specific conditions (Toupadakis *et al.* 2010). In addition, BM-MSCs are thought to have profound anti-inflammatory effects via their inhibition of T cell mediated

response (Muller *et al.* 2008). The present study was designed using BM-MSCs in tendon injury model.

MATERIALS AND METHODS

Clinically healthy non-descript adult goats (18; 2 to 5 year-old) of either sex divided randomly into equal group A (control) and group B (treatment). All the animals were kept off feed for 12 h and water was withdrawn for 6 h before the start of experiment.

Bone marrow aspiration: The goats were placed in lateral recumbency and the surface above the one or both iliac wings was prepared in aseptic manner. The animals were preanaesthetized by intramuscular injection of atropine sulphate @ 0.02 mg/kg body weight followed by the xylazine @ 0.1 mg/kg body weight intramuscularly. Then 1–2 ml of 2% lignocaine was infiltrated locally at the site of collection of bone marrow aspirate. The bone marrow was aspirated according to Jian-wei *et al.* (2011).

MSCs isolation and cultivation: The bone marrow mononuclear cells were isolated from the marrow aspirate by volume reduction centrifuge “buffy coat” protocol as per Kasten *et al.* (2007) with some modifications. After dilution 1:1 with DPBS (Dulbecco’s phosphate buffered saline) bone marrow was carefully layered over Ficoll and centrifuged at 3,000 rpm for 30 min. Mononuclear cells

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were collected from the interface, diluted with triple volume of DPBS and again centrifuged at 2,500 rpm for 20 min. After discarding the supernatant, the cells were washed with DPBS and centrifuged at 2,000 rpm for 10 min. Again the supernatant was discarded and the cell pellet was resuspended in 5 ml. of DMEM-LG (Dulbecco's modified eagle medium-low glucose), counted and plated at 2×10^5 cells/cm² in T-25 flasks. The cells were maintained in DMEM-LG containing 10% FBS (fetal bovine serum) and antibiotics [mixture of 100 IU/ml penicillin and 100 µg/ml streptomycin and antimycotics nystatin 12.5 U/ml] at 37°C in a humidified atmosphere of 5% CO₂ and 95% air in CO₂ incubator. After 5 days of primary culture, the non adherent cells were removed by changing the medium. The medium was changed every 3 days thereafter until the cultures become confluent. After achieving 70–80% confluency cells were removed from the flasks using 0.25% trypsin/EDTA digestion and centrifuged at 2,000 rpm for 5 min to pellet the cells. The supernatant was removed and cells were suspended in 1 ml of DMEM-LG without serum and counted in a haemocytometer. After adjusting the cell count to 5×10^6 /ml, the cells were used for implantation.

Creation of defect and implantation of BMMSCs: The animals were preanaesthetized by intramuscular injection of atropine sulphate @ 0.02 mg/kg body weight followed by xylazine @ 0.1 mg/kg body weight intramuscularly. Subsequently 2–3 ml. of 2% lignocaine hydrochloride was injected epidurally in lumbosacral space aseptically. The animal was restrained in right lateral recumbency and the metatarsal region of right hind limb was prepared for aseptic surgery. A 3–4 cm long skin incision was made on the medial planter aspect of metatarsal and the superficial digital flexor tendon was exposed and transected (Fig. 1).

In the animals of both groups, the transected tendon ends were immediately repaired with 3–0 monofilament polypropylene using locking loop suture pattern. In the animals of group B, the BM-MSCs were injected on the pretensioned sutures. The skin incision was sutured with 2–0 silk using interrupted horizontal mattress sutures. Immediately after the operation, a PVC pipe splint was applied in all the animals to immobilize the operated limb for 10 days (Fig. 2). All the animals were kept free and allowed free access to feed and water. All the animals were administered with broad spectrum antibiotic ampicillin plus

cloxacillin @ 10 mg/kg body weight IM BID for 5 consecutive days. Daily dressing of the surgical wound with framycetin was performed till the complete healing of the incision and removal of the sutures.

The efficacy of the treatment was assessed by recording the different clinical parameters viz. wound condition, extent of pain, tendon gliding movement, weight bearing capacity in standing position and during locomotion on 3rd, 7th, 10th, 15th, 30th, 45th and 60th day postoperatively.

The condition of the surgical wound was evaluated on 3rd, 7th, 10th, 15th, 30th, 45th and 60th day postoperatively on the basis of the following parameters.

1. Swelling: The swelling at the operated site was graded on a 1–4 scale: 1 - no swelling, 2 - mild swelling, 3 - moderate swelling, 4 - severe swelling.
2. Exudation: The degree of exudation was assessed on 3rd, 7th, 10th, 15th, 30th, 45th and 60th day postoperatively as given below: 1 - no exudate, 2 - mild exudate, 3 - moderate exudate, 4 - severe exudates.
3. Warmth: The warmth at the operated site was graded as follows on a 1–3 scale: 1 - normal, 2 - mild warm, 3 - moderate warm.
4. Pain: The pain was evaluated by applying gentle pressure with finger on the operated site. The degree of pain was graded on a scale of 1–3 as per the details given below: 1 - no pain, 2 - mild pain, 3 - severe pain.

The presence or absence of the tendon gliding movements was determined manually before surgery and on 3rd, 7th, 10th, 15th, 30th, 45th and 60th day postoperatively and graded as follows (Ramesh *et al.* 2003): 0 - no gliding, 1 - mild gliding, 2 - moderate gliding, 3 - full gliding.

The weight bearing capacity on the operated limb was observed in standing and while walking on 3rd, 7th, 10th, 15th, 30th, 45th and 60th day postoperatively (Ramesh *et al.* 2003).

1. Weight bearing in a standing position: Weight bearing capacity in a standing position was graded on a scale of 0–3: 0 - no weight bearing, 1 - toe touching the ground, 2 - moderate weight bearing, 3 - full weight bearing
2. Weight bearing in motion: Weight bearing capacity during locomotion was assessed as per the details



Figs 1–3. 1. SDF tendon. 2. Goat with full limb PVC pipe splint. 3. Spindle shaped morphology of MSCs at day 15 (x400).

given below: 0 - Supports the limb on the ground and less weight bearing, 1 -Moderate weight bearing, 2 - Full weight bearing with visible lameness, 3 - Full weight bearing with no lameness.

Air-tendograms was taken from the animals on day 15, 45 and 60 postoperatively to evaluate the healing and adhesions at the site of tendon repaired (Kumar *et al.* 2002). Air provided adequate contrast (negative) for demonstration of the soft tissue changes and also valuable information regarding continuity of tendon and healing process after surgery. For this purpose lateral air-tendograms were taken using 45 kVp and 4.8 mAs.

The statistical analysis of the data was done using the analysis of variance as per the method described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

The superficial digital flexor tendon plays an important role during locomotion in animals thus it is more likely to injure (Sharma *et al.* 2005). Owing to its relatively easy surgical access and ease in postoperative immobilization of the limb of animal, superficial digital flexor tendon was selected for this study. In the present study, the lateral crest of iliac was suitable for collection of marrow aspirate due to the less post surgical complications. The density gradient centrifugation method allows the differential migration of

cells during centrifugation that result in the formation of layers containing different cell types. On day 0, the nucleated cells were round in shape representing a mixture of heterogeneous cell population. Many cells of the heterogeneous cell population started to adhere to tissue culture flask surface on third day. On day 5, the non-adhered floating cells were removed in first media change, leaving the potential MSCs adhered to the flask bottom. The plastic adherence property of mesenchymal stem cells is one of the criteria of identification of MSCs. The confluency was increased day by day as the cells multiplied and started to form colonies. On day 7–8, few of the adhered cells started to change their morphology from round to spindle shaped cells and formed small colonies in the culture flasks. These cells were grown further and on day 10–14 have covered almost 70–80% area of the tissue culture flask. The primary cultures of the bone marrow cells contained mainly fibroblastic cells that were seen as spindle shaped under inverted microscope on higher magnification (Fig. 3). On day 15, after reaching 70–80% confluency, as assessed by visual inspection under inverted microscope, the cells were harvested for implantation using the 0.25% trypsin-EDTA. The mononucleated cells represent only a tiny fraction i.e. 0.01–0.001% of the total marrow population (Pittenger *et al.* 1999). Thus these cells were needed to grow in culture to be used for treatment.

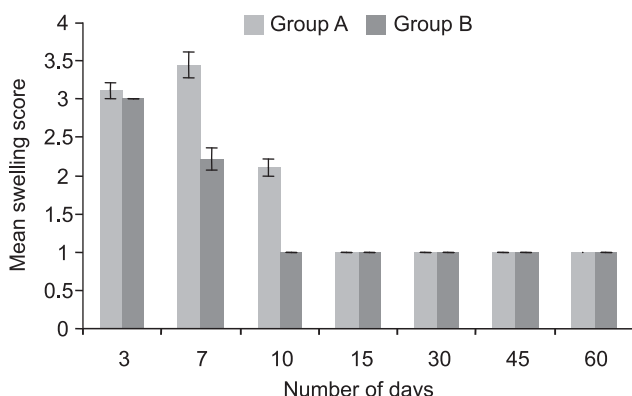


Fig. 4. Mean $\pm$ SE of swelling score in various groups at different time intervals.

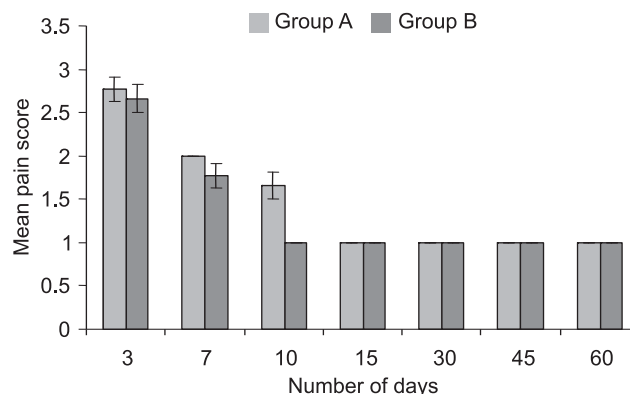


Fig. 6. Mean $\pm$ SE of pain score in various groups at different time intervals.

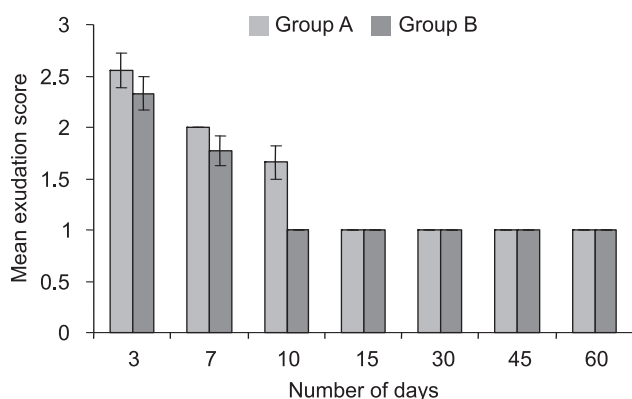


Fig. 5. Mean $\pm$ SE of exudation score in various groups at different time intervals.

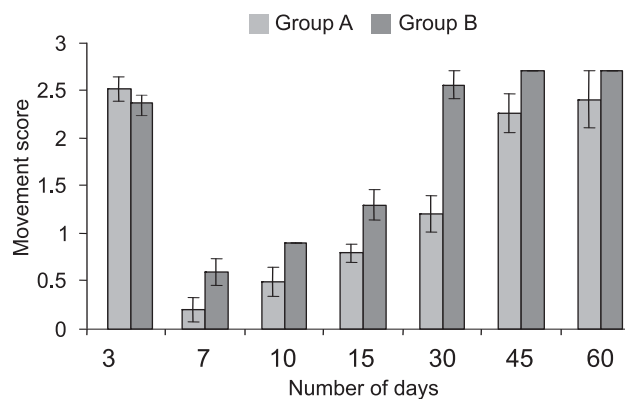


Fig. 7. Mean $\pm$ SE of tendon gliding movement score in various groups at different time intervals.

Surgical wound appeared healthy throughout the period of observation in all animals. For exposure of superficial digital flexor tendon, a medial planter approach was found satisfactory. This approach was also found quite convenient for the repair of SDF tendon by different workers (Maiti *et al.* 2006). Frequent use of 0.9% normal saline solution at the operated site helped to minimize bleeding and prevented peritendinous adhesions. Similar findings were also reported by Kumar *et al.* (2002) in bovines.

The transected superficial digital flexor tendon ends were repaired with locking loop technique using non-absorbable suture material in both the groups of animals. This technique was found satisfactory, easy to apply and secured the tendon ends in apposition (Maiti *et al.* 2006). The use of non-absorbable suture material monofilament polypropylene (3–0) was satisfactory as this provided easy handling, secured knot and adequate tensile strength. Application of PVC splint for 10 days prevented the undesired extension and flexion of the operated limb. It has helped in reducing the tension over the repaired site. It also helped in early revascularization of the repair site. Various workers like Suchak *et al.* (2008) and Saini *et al.* (2010) also reported that the early active mobilization shows benefits of increased healing rate, tensile strength, decreased adhesion formation and rupture of tendon.

Moderate swelling was observed at the site of injury on day 3 in all the animals of both groups. The mean swelling scores (Fig.4) increased significantly ($P<0.05$) on day 7 in group A. Reduction in inflammatory swelling was observed on day 10 in the group A and on day 7 in the animals of group B. Comparison between the 2 groups revealed faster resolution of inflammatory swelling in the test group than the control group. However, no inflammatory swelling was observed in the animals of group A and B on day 15 onwards. The presence of swelling at the operated site in all the animals after tendon surgery was related to the acute inflammatory reaction due to surgical trauma to the soft tissue (Saini 1995). The remodelling and organization of fibrous connective tissue in later stages caused reduction in inflammatory swelling at the site of injury in both the groups. The resolution of inflammatory swelling was faster in the MSCs treated group might be due to the anti-inflammatory effect of mesenchymal stem cells. MSCs secrete anti-inflammatory mediators such as prostaglandins and interleukin-10 (Ryan *et al.* 2005) that reduces the inflammation.

The mild to moderate exudation was observed at the operated site on day 3 in all the animals. The inflammatory exudate was reduced progressively on day 7 in both the groups. There was no exudation on day 10 and day 15 onwards in the animals of group B and A respectively (Fig. 5). The reduction in inflammatory exudation was faster in group B as compared to group A. The inflammation gradually subsided as healing progressed and thus no exudate was observed later on. The early reduction in the inflammatory exudates in the group B, was because of the anti-inflammatory properties of MSCs (Singer and Caplan

2011) that resulted in regression in inflammation and thus reduced the inflammatory exudates.

Mean warmth score (score=2) was observed in all the animals on day 3 postoperatively. There was significant ($P<0.05$) decrease in the mean warmth score on day 10 and day 15 in both the groups. In the remaining periods, the warmth of the operated site did not differ from that of the contra lateral limb. A mild increase in warmth at the operated site was due to the hyperaemia in response to soft tissue injury that is likely to occur during surgery. Acute inflammation at the site leads to vasodilatation of the local blood vessels and increase in the temperature of the reconstructive site (Guyton 1998). The MSCs treated group showed normal warmth on day 10 while animals of control group showed normal warmth on day 15. This indicated the early reduction of the inflammation and thus warmth at the injured site and enhancing the tissue healing. MSCs decrease the amount of IL-10 and TNF- α secreted by dendritic cells, and increase the amount of the anti-inflammatory IL-4 produced by T cells (Singer and Caplan 2011). Thus due to their anti-inflammatory action, MSCs reduced the inflammation as well as the warmth or heat (that is one of the cardinal sign of inflammation) at the site of injury.

Severity of pain was assessed by applying gentle pressure at the operated site. On day 3, all animals showed moderate pain (Fig. 6) which gradually decreased in due course of time. None of the animals showed pain at the operated site on day 10 and 15 in the animals of group A and B respectively. Thus the severity of pain reduced faster in the animals, treated with MSCs as compared to the animals of control group. Moderate pain on day 3 in all the animals might be due to acute inflammatory reaction after surgery. Pain after tendon reconstruction was also reported by Kumar *et al.* (2002) in buffalo calves. Decrease in pain after day 3 indicated the reduction in the inflammatory response. Early reduction in pain in the group B as compared to group A indicated early reduction of inflammation in group B treated with the MSCs. The mesenchymal stem cells also reduce the pain perception in various chronic painful conditions. Guo *et al.* (2011) suggested that the infused MSCs stimulate release of endogenous opioid peptides from peripheral and central sites, leading to prolonged attenuation of pain and hypersensitivity.

The tendon gliding movement was absent on day 3 in all the animals (Fig. 7). The slight tendon gliding movement was observed on day 7 in both the groups. The tendon gliding movement improved faster in the test group and full gliding movement was observed on day 45. The tendon gliding movement was comparatively slower in the control group and full gliding movement was seen on day 60 except in 1 animal. Tendon gliding movement is important for normal functioning of the tendon. The absence of gliding movement on day 3 may be due to the inflammation and peritendinous adhesion formation with the surrounding tissues (Maiti *et al.* 2006). There was early restoration of tendon gliding movement in MSCs treated group as

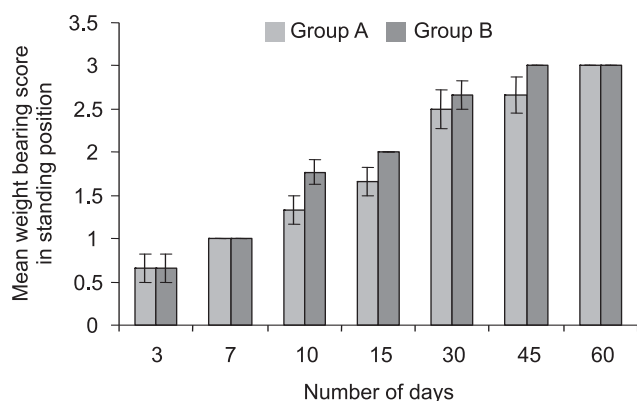


Fig. 8. Mean $\pm$ SE of weight bearing score in standing position in various groups at different time intervals.

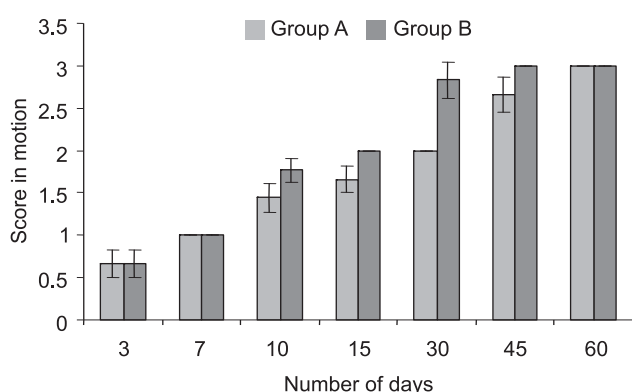


Fig. 9. Mean $\pm$ SE of weight bearing score in motion in various groups at different time intervals.

compared to control group, as the MSCs reduce the inflammation at the site of injury thus they prevent the excessive scar tissue formation and adhesions with the peritendinous structure, resulting in restoration of tendon gliding movement (Ryan *et al.* 2005).

The weight bearing scores in standing as well as during motion showed a gradual increase in both the groups (Figs 8, 9). There was no significant ($P > 0.05$) difference in mean weight bearing scores of group A and B on day 3 and 7. All the animals of group B showed moderate weight bearing on day 15 and full weight bearing was noticed on day 45. While the animals of group A showed full weight bearing on day 60. Lameness and reduced weight bearing capacity in standing as well as during locomotion of the operated limb, observed after the repair in all the animals was due to the acute inflammation and postoperative pain at the operated site. comparatively lower extent of lameness in the animals of group B as compared to the animals of group A may be due to the anti-inflammatory properties of mesenchymal stem cells and early healing process of injured tendon and improvement in tensile strength of healing tendon in stem cell treated groups (Chong *et al.* 2007).

Air-tendograms has helped in evaluating the continuity of the superficial digital flexor tendon and the density of the tissue at the reconstructed site. Air-tendograms also provided useful information regarding the type and intensity

of adhesion formation and status of reconstructive site of tendon. The use of tourniquet helped in providing good contrast with relatively less amount of air. On day 15, the air-tendograms (Fig. 10) of all the animals showed the skin, SDF and DDF tendons as homogenous thickened mass, which was indicative of presence of adhesions between them. Adhesions between suspensory ligament and SDF and DDF tendons were also seen at the site. Extensor tendons on the dorsal aspect of limb were clearly visible and separated with the skin. On day 45, there was a little regression of adhesions between skin and SDF tendon in group A. On day 60, the adhesions between the skin, DDF tendon and SDF tendon were markedly reduced and the thickening of SDF tendon at reconstructed site was also reduced. In group B, air-tendograms (Fig. 10) revealed marked reduction in swelling and adhesions between the skin and flexor tendons on day 45. On day 60, the SDF tendon, DDF tendon and suspensory ligaments were well separated by air density throughout their length. Tendon at the reconstructed site was further organized and near to normal thickness and density. Air - tendograms of metatarsal region were evaluated for the extent of adhesions between

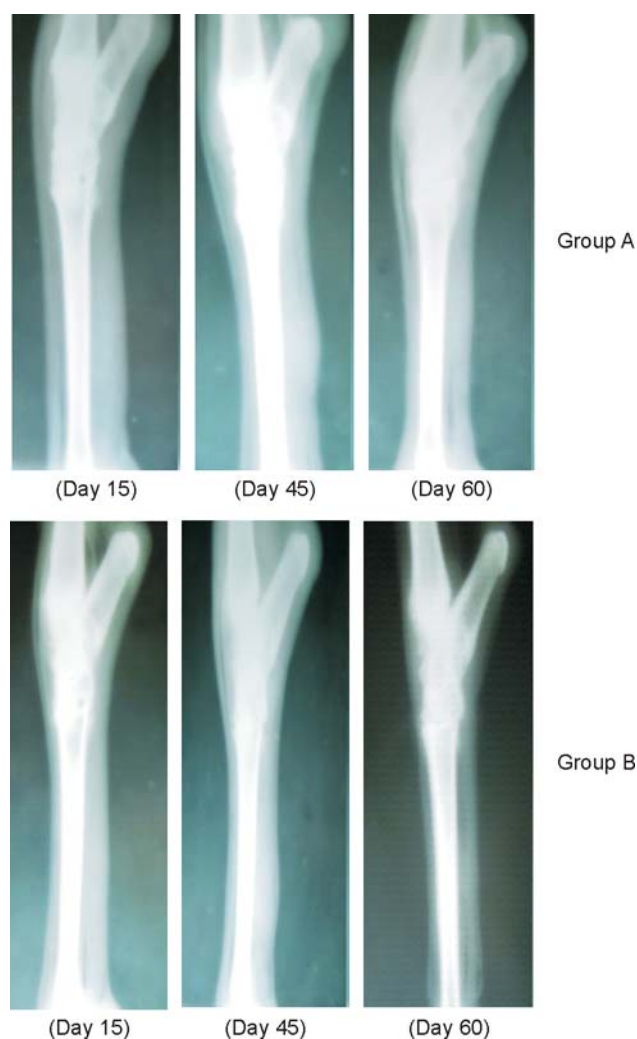


Fig. 10. Air-tendograms of group A and B on day 15, 45 and 60.

the healing tendons and surrounding structures. Air-tendograms clearly depicted the tendon outline, its thickness and peritendinous adhesions (Saini 1995). In both the groups, adhesions were more at initial stages and gradually decreased as healing advanced. The increased thickness of SDF tendon in the animals of group A indicated more fibroblastic activity as compared to the animals of group B. There was progressive organization and regression of adhesion with time in both the groups, but it was more pronounced in the MSCs treated group.

On the basis of the various parameters observed in the present study it was concluded that the *ex-vivo* expanded autologous bone marrow derived mesenchymal stem cells can be used for early and better result in tendon healing in cases of damaged tendons.

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