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Glutaraldehyde cross linked bubaline acellular periosteum scaffolds augment healing of fractured long bones in dogs

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In the present study, glutaraldehyde-cross linked bubaline acellular periosteum (GBAP) scaffolds were used to augment long bone fracture healing in dogs. Twelve client-owned male dogs, aged 6-9 yr, with fracture of long bones, were divided randomly into groups A (control) and B (treatment). The fracture was stabilized by intramedullary pinning in both groups. Further, in group B animals, GBAP scaffold was placed circumferentially covering the fractured site. The functional limb usage, callus index, serum calcium, phosphorus, alkaline phosphatase, oxidant and antioxidant status of the patients were evaluated. By day 45, group B animals showed full function at standing and walking, and mineralization of bone at the fracture site. The serum calcium, inorganic phosphorus and alkaline phosphatase levels were higher in treatment group. Levels of MDA, GSH and catalase increased significantly in control group. It was concluded that GBAP scaffolds helped to augment fracture healing and reduce the healing time.

Key words: *Acellular periosteum, Bone scaffold, Dog, Fracture healing*

Fracture healing is a specialized type of healing process in which the regeneration of bone leads to reestablishment of skeletal integrity. Though, this process is considered biologically most appropriate, delayed or diminished healing may be noted in 5-10% cases of fracture (Fong *et al*., 2013) and requires biological enhancement. Limited availability and donor site morbidity restrict the use of autografts. Allografts and xenografts hold the cell surface antigen molecules, which evoke host immune response. These antigen molecules are removed by the process of decellularization to reduce their immunogenicity. The decellularized ECM should contain less than 50 ng dsDNA per mg ECM (Crapo *et al*., 2011). The success rate of any decellularized scaffold depends on certain physical properties like mechanical strength, pore size etc. The scaffold should have interconnected pores and a pore size of 200–350 ìm is optimal for regeneration of bone (Oryan *et al*., 2014). Gluteraldehyde treatment of the scaffolds has been shown to decrease immunological recognition of the tissue (Nimni *et al*., 1987), through crosslinking of collagen which leads to resistance against proteolytic degradation and masking of antigenic sites (O'Brien *et al*., 1984).

The xenogenic acellular periosteum has not been evaluated for augmentation of fracture healing. The present study is therefore designed to develop a biomimetic cross linked xenogenic acellular periosteal scaffold and to evaluate its potential for augmentation of fracture healing process in the long bone fractures in dogs.

Materials and Methods

Harvest and preparation of acellular periosteum

The native periosteum was collected from the long bones of dead buffalo foetus delivered after caesarean section and stored in chilled sterile phosphate buffered saline (PBS, pH 7.4) admixed with amikacin (0.1%) and ethylene diamine tetraacetic acid (EDTA) (0.2025%), a proteolytic inhibitor. The periosteum was thoroughly cleaned with sterile phosphate buffer saline, cut into 20 x 20 mm^2 pieces, transferred in a sterile plastic container having 100 mL solution of 1% SDS and subsequently agitated on magnetic stirrer for 24 hr at room temperature. Processed tissue samples were collected in phosphate buffered formalin saline and 5% glutaraldehyde at 12hr, 18hr and 24 hr intervals for confirmation of decellularization by H&E staining, DAPI staining and scanning electron microscopy.

Verification of cell removal

Native and decellularized periosteum tissues were fixed in 10% phosphate buffered formalin saline. These tissues were dehydrated in graded ethanol, cleared in xylene and embedded in paraffin to cut 5 micron thick paraffin sections, which were then stained with H&E, and with DAPI (4, 6-diamidino-2-phenylindole 2HCl) staining solution. The scanning electron microscopic (SEM) examination of the native and decellularised periosteum scaffolds was performed using SEM model Jeol JSM-840.

Animals and implantation

In this study 12 client-owned male dogs, weighing 18-21 kg and 6-9 yr of age with tibial fractures were divided randomly in to two groups having 6 animals each. After preoperative

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preparations, surgical anaesthesia was achieved using atropine (0.04 mg/kg body wt, i.m.)-diazepam (0.5 mg/kg body wt, i.v.)-thiopentone sodium (15 mg/kg body wt, i.v.) protocol. The fracture fragments were stabilized by Steinmann IM pins in both groups. In group B animals, GBAP was also applied circumferentially covering the fracture site (Fig. 1). Muscles and skin were sutured in standard manner. Injection of ceftriaxone (10 mg/kg body wt) and meloxicam (0.25 mg/kg body wt) were given intramuscularly for 5 and 3 days, respectively. Antiseptic dressing of suture line was done with 5% povidone iodine, and the skin sutures were removed on 10th day postoperatively. All the cases were studied at least up to 45 days or till complete fracture healing on the basis of following parameters.

Fig. 1: Intraoperative image showing implantation of glutaraldehyde treated acellular periosteum at the fracture site.

Observations

The functional limb usage of the affected limb in both groups was evaluated on postoperative days 0, 15, 30, 45 and 60 (Braden and Brinker, 1973).

The radiographs were taken preoperatively and on postoperative days 15, 30, 45 and 60. Callus Index (callus diameter at the fracture site divided by the diameter of the normal bone) was measured.

Blood was collected preoperatively and on postoperative days 7, 15, 30, 45 and 60. Serum was harvested for estimation of calcium, phosphorus and alkaline phosphatase using commercial test kits (Span Diagnostics Ltd). Blood collected in the heparinized vials preoperatively and on days 3, 7, 15, 30, 45 and 60 was used to prepare hemolysate to estimate lipid peroxides (LPO) (Placer *et al.*, 1966), reduced glutathione (GSH) (Prins and Loos, 1969), superoxide dismutase (SOD) (Masayasu and Hiroshi, 1979) and catalase (Cohen *et al.*, 1970), soon after collection of blood. Oxidative stress factor (OSF) was assessed preoperatively and on postoperative days 3, 7 and 15 (Bisla *et al.*, 2002).

Statistical analysis

The results were presented as mean±SE. Data were analyzed and measurements between groups were compared using an independent sample t-test. Biochemical values were analysed by one way analysis of variance (ANOVA) and means were compared by Duncan's multiple range test (DMRT) for multiple comparisons.

Results and Discussion

Evaluation and characterization of processed periosteum

Grossly the osteogenic layer of periosteum was smooth (Fig. 2a) and fibrous layer was rough in nature (Fig. 2b). Microscopically, the osteogenic layer of native periosteum showed progenitor cells that would develop into osteoblasts or chondroblasts at places, and fibrous layer was composed of compactly arranged thick collagen fibres (Fig. 3a). However, the fibrous layer of periosteum processed with 1% SDS showed intact thin and compact collagen fibres with slight porosity. Nucleus and cellular debris were absent in both osteogenic and fibrous layers of processed periosteum (Fig. 3b). Treatment of native periosteum with 1% SDS for 18 hr resulted in complete decellularization of periosteum without disturbing extracellular matrix (ECM) morphology. Sodium dodecyl sulphate was more advantageous for eradication of cellular components and debris from dense tissues as compared to Triton X-100 (Gilbert *et al.*, 2006). DAPI stained native periosteum tissue samples showed cold blue fluorescence, depicting the nuclear DNA (Fig. 3c). Decellularized samples of periosteum did not show any nuclear components (Fig. 3d). DAPI staining of processed samples confirmed the results of histological examination. SEM of native periosteum showed ultrastructure of fibrous layer of periosteum with cellular structures (Fig. 3e). However, decellularized periosteum showed fibrillar structure and cells were absent (Fig. 3f). SEM examination also confirmed effective decellularization of the periosteum and the preservation of collagen structure and integrity within GBAP.

Evaluation of fracture healing

By day 15, the animals of group B showed partial weight bearing with functional use of treated limb and set the limb down at a stand or a walk and carried the limb while running. However, the animals of

Fig. 2: Image of acellular periosteum showing (A) smooth cambium layer, and (B) rough fibrous layer.

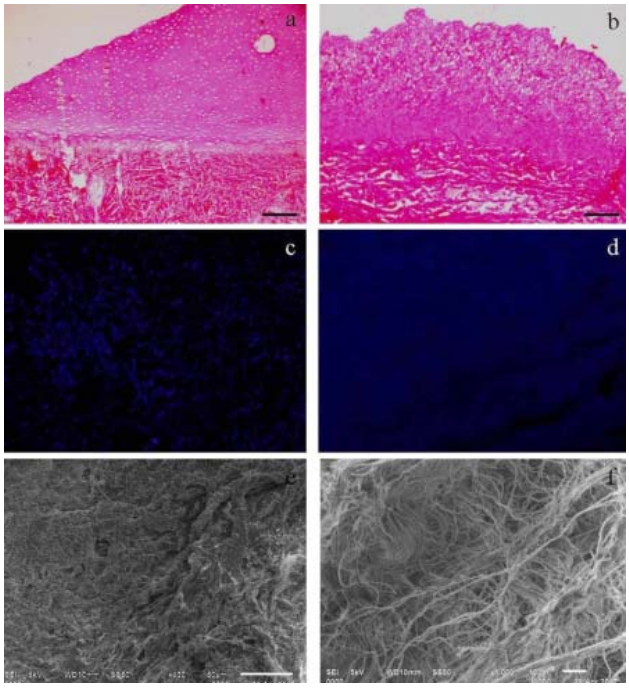

Fig. 3: Micrograph of (**a)** native periosteum (H&E stain, 100X) and **(b)** decellularized periosteum after treatment with 1% SDS for 18 hr (H&E stain, 100X); 4, 6-diamidino-2-phynylindole (DAPI) staining of **(c)** native (normal) periosteum, and (**d)** decellularized bubaline periosteal matrix (18 hr); SEM image of **(e)** native periosteum showing fibroplasia over the collagen fibers, and (**f)** decellularized periosteal matrix showing fibrillar meshwork without cells (18 hr).

group A showed no functional use of the limb and carried the limb most of the time. By day 30 and 45, the animals of group B showed functional use of limb and the animals set the limb down at a stand or a walk but limping was present during running. However, the animals of group A used the affected limb only slightly and showed partial weight bearing with some functional use of the treated limb. The group A animals showed only slight use of the affected limb even after 60 days. Mild inflammatory swelling was noticed in early postoperative days, which might be due to surgical trauma (Gangwar *et al.*, 2006). Early weight bearing in the animals of group B might be due to less pain and better fracture healing as compared to group A animals.

Radiographic changes in the tibia of both group A and B animals at different time intervals are presented in figure 4 and 5, respectively. In control group A, no periosteal reaction was visible on day 15. By day 30, the callus index was 1.39±0.042. By day 45, callus was observed at both the fracture ends but fracture line was clearly visible. By day 60, the fracture line was slightly radiodense. The fracture fixation with IM pinning alone tends to inhibit the large amount of callus (Eastaugh-Waring *et al.*, 2009). In treatment group B, by day 15, periosteal reaction with slight amount of callus was present at the transplanted site. By day 30 radiographic changes were consistent with a more mature stage of fracture healing. The callus index was 1.55±0.078. By day 45, the callus completely bridged the fracture ends. High callus index in group B animals might be due to more deposition of minerals within the GBAP scaffold.

Biochemical studies

Serum calcium (Ca), inorganic phosphorus (P) and alkaline phosphatase (ALP) values in animals of both groups at different time intervals are shown in table 1. Serum Ca values significantly ($P<0.05$) increased on postoperative days 7, 15, 30, 45 and 60 in both groups; however, maximum levels were noted in group B (13.042±0.36). Serum P level increased significantly ($P<0.05$) and ALP value decreased significantly ($P<0.05$) on day 60 in both groups. The serum Ca level on day 60 was significantly ($P<0.05$) higher. These results were in accordance with the findings of Hegde *et al.* (2010) who have reported increased Ca level in fracture union. Early rise in

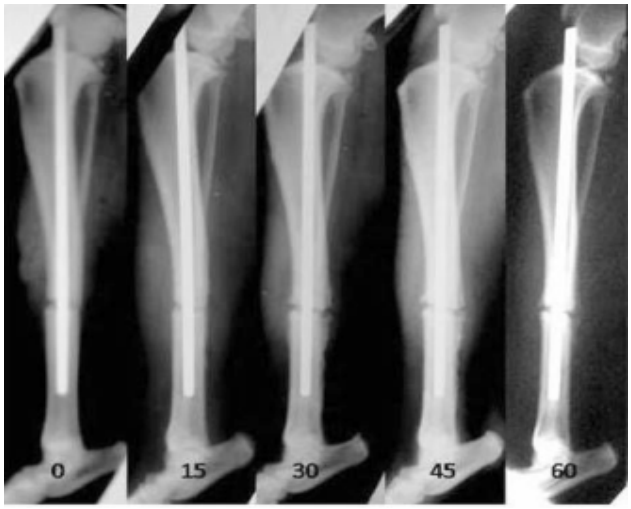

Fig. 4: Radiographs of the tibia of control group animals at different time intervals (medio-lateral view).

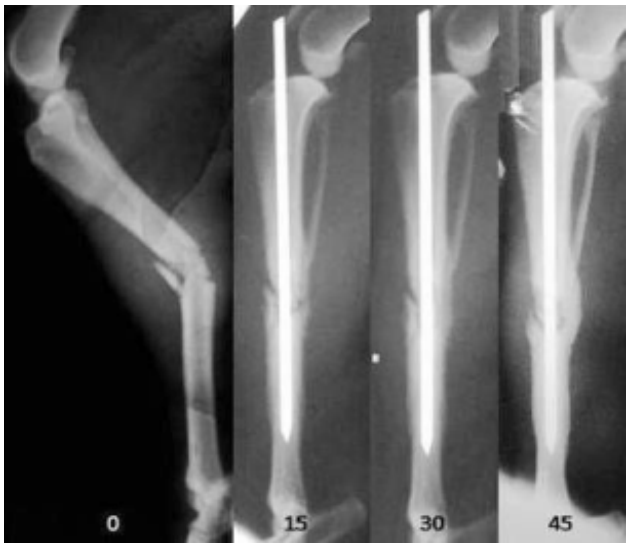

Fig. 5: Radiographs of the tibia of animals with GA (0.05%) treated acellular periosteum implant at different time intervals (medio-lateral view).

serum P in group B might be due to necrotic disintegration of the cells at the fracture site and ultimate liberation of calcium and phosphorus in the circulation (Saraswathy *et al.*, 2004). The highest serum ALP level in group B could be attributed to early proliferation and penetration of osteogenic cells within the collagen of GBAP scaffold (Kruger *et al.*, 2013).

Oxidant and antioxidant status

A relatively large output of radicals is produced after bone fractures. Lipid peroxidation, which produces MDA as a by-product, is one of the most harmful consequences of reactive oxygen species (ROS). Apart from its use as a lipid peroxidation marker, MDA has also been employed as an index for osteoclastic activity. Reduced levels of MDA in the serum and decreased activity of the antioxidant enzymes SOD may suggest that the body's defence may not be able to counteract the enhanced generation of superoxide by the osteoclasts. In group B, on day 7 the level of MDA significantly (P<0.05) decreased, whereas it significantly (P<0.05) increased in group A (Table 2). The level of MDA increased (P<0.05) on day 60 in group B, the level of MDA slightly decreased on day 7, which could be attributed to interruption of blood flow to the severed bone at the time of fracture. In later period, the increase of MDA might be due to more penetration of osteoblasts, fibroblasts and new capillary vessels within the GBAP matrices. Early remodeling phase is characterized by enhanced osteoclastic activity and increased production of ROS (Sontakke and Tare, 2002). The level of GSH increased slightly on day 3, thereafter decreased significantly (P<0.05) on day 60 in group B. However, the level of GSH significantly (P<0.05) increased on day 60 in group A. Lower level of GSH in group B might be due to more utilization for detoxification of free radicals (Reilly *et al.*, 1991). The level of SOD significantly (P<0.05) decreased on day 15 thereafter the values significantly (P<0.05) increased on day 60 in both groups. SOD decreased up to day 30 in groupB. This pattern may be contributed by the high production of superoxide radicals during the early phase of fracture healing. Depressed activities of the antioxidant enzymes, SOD illustrated a defence mechanism that may have been overwhelmed in mitigating the increased superoxide production by the osteoclasts (Chen *et al.*, 1997). The level of catalase increased (P<0.05) on days 3 and 60

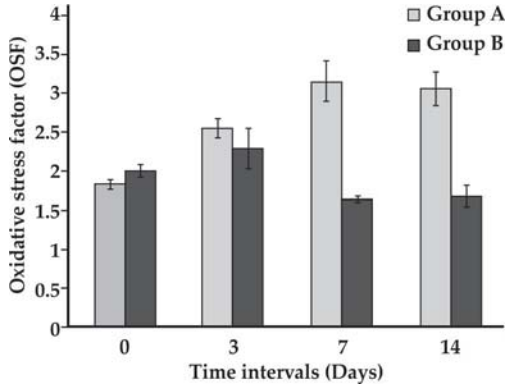

Fig. 6: Oxidative stress factor (OSF- mean±SE) in animals of two groups at different time intervals.

Table 1: Mean±SE of serum calcium (mg/dL), inorganic phosphorus (mg/dL), alkaline phosphatise (KA units) of animals of different groups at different time intervals.

Parameter	Group	Time intervals (days)					
		0	7	15	30	45	60
Calcium (mg/dL)	A	3.29±0.12	6.13±0.19*[a]	7.67±0.18*[a]	9.08±0.18*[a]	10.87±0.26*	12.33±0.18*
	B	3.79±0.12	6.92±0.11*[b]	8.54±0.28*[b]	10.25±0.39*[b]	11.58±0.37*	13.04±0.36*
Inorganic Phosphorus (mg/dL)	A	0.47±0.26	0.63±0.03[a]	0.83±0.02[a]	1.09±0.02	1.33±0.02*	1.42±0.03*
	B	0.32±0.01	0.78±0.03*[b]	0.92±0.03*[b]	1.14±0.05*	1.38±0.04*	1.66±0.05*
Alkaline Phosphatase (KA units)	A	4.46±0.08[a]	3.62±0.12*[a]	3.32±0.03*[a]	2.47±0.19*[a]	2.08±0.13*[a]	1.67±0.13*[a]
	B	7.90±0.14[b]	7.24±0.19[b]	6.70±0.26*[b]	6.10±0.16*[b]	5.20±0.13*[b]	4.60±0.09*[b]

[ab]Values with different alphabets differ significantly (P<0.05) between the groups at particular time interval; * Values differ significantly (P<0.05) from day 0 value

Table 2: Mean±SE of LPO (nmole eq. MDA/mL haemolysate), GSH (nmole/mL of haemolysate), SOD (units/mL of haemolysate) and catalase (units/mL of haemolysate) in the blood of animals of two groups at different time intervals.

Parameter	Group	Time intervals (days)						
		0	3	7	15	30	45	60
LPO	**A**	80.93±2.15[a]	91.02±1.58*[a]	88.35±0.70* [a]	69.15±2.75*	81.55±3.05	84.43±3.04	86.95±2.96
	B	72.38±2.05[b]	70.40±1.17[b]	58.84±4.96*[b]	70.50±1.71	73.10±1.53	77.36±1.83*	82.39±1.65*
GSH	**A**	0.86±0.03[a]	0.94±0.03*[a]	1.19±0.12*	1.19±0.05*[a]	1.24±0.02*[a]	1.17±0.03*[a]	1.25±0.02*[a]
	B	1.08±0.03[b]	1.15±0.08[b]	0.96±0.07	0.60±0.05*[b]	0.70±0.03*[b]	0.65±0.02*[b]	0.92±0.02*[b]
SOD	**A**	12.48±0.25 [a]	11.92±0.74*[a]	2.60±0.69*[a]	5.26±0.96*	30.07±0.56*[a]	23.13±0.64*	30.62±0.35*[a]
	B	27.68±0.50[b]	17.57±1.26*[b]	4.03±0.29*[b]	2.93±0.73*	32.84±0.52*[b]	29.52±1.05*	29.97±1.21*[b]
Catalase	**A**	48.85±6.07	89.33±11.58*	55.25±1.34	51.81±6.22[a]	74.04±0.55[a]	64.58±2.34[a]	61.17±2.33[a]
	B	60.25±2.63	83.98±11.43	86.78±15.48	97.92±2.02*[b]	93.15±5.82*[b]	83.51±3.67*[b]	78.08±3.54*[b]

in both groups. The scavenged superoxide radicals convert into hydrogen peroxide, which stimulate the expression of catalase (Bae *et al.*, 1999). Oxidative stress factor (OSF) was significantly ($P<0.05$) higher on day 15 in group A. However in group B, a significant ($P<0.05$) decrease in OSF was observed on day 15 (Fig. 6). Bisla *et al*. (2002) reported higher OSF in buffaloes suffering from diaphragmatic hernia as compared to that in healthy buffaloes.

Based on the results of the present study, it was concluded that the use of GBAP as an osteoconductive scaffold resulted in early and better healing of long bone fracture in dogs without any complication.

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