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The Efficacy of Cryopreserved Amniotic Membrane seeded with Mesenchymal Stem Cells for Management of Bone Defect in a Canine Model.

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ABSTRACT

Bone substitutes and scaffolds play continually progressing role in management of bone defects. The amniotic membrane (AM) has been used in surgical transplantation as a biomaterial and scaffold. Autologous mesenchymal stem cells (MSCs) represent an excellent source for tissue engineering for the low risk of immune complications. The present study was planned into 2 main stages. The first stage, AM bank was prepared from 10 full term pregnant female-dogs. Prepared AMs were preserved at -80°C and later were used as a scaffold. The second stage, experimental bone defect was carried out on 27 apparently healthy mongrel dogs. Dogs were divided into 3 main groups; Group A: control group; defect left without scaffold. Group B: received AM only and Group C: received AM seeded with MSCs. The animals in each group were divided into 3 sub-groups (n=3) according to follow up periods as 6, 12 and 24 weeks. Animals of group C were used for MSCs acquisition and preparation. Under complete aseptic condition; the prepared AM was co-cultured with MSCs. The animals were observed for clinical, radiological and morphohistological examinations. The results showed that the use of AM seeded with MSCs has good promising outcome in bone defect healing than AM alone.

Keywords: Cryopreserved, Amniotic membrane, Mesenchymal, Stem, Defect, Dog.

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Introduction

Schmitz and Hollinger [1], described the critical size defect (CSD) as the smallest interosseous wound that doesn't heal by bone formation during animal's life. Bone substitutes and scaffolds play an important continually progressing role in the management of CSD. The production and use of various types of bioengineered bone substitutes and scaffolds has led to dramatic improvements in the chances of survival for patients with great bone losses. Scaffolds are developed to support the host cells, promote bone differentiation and proliferation during bone healing. Therefore, the design and selection of the biomaterials used for scaffolding is critical in bone tissue healing [2].

A major criterion for choosing a scaffold is its biocompatibility [3], unchanged in inflammation and reaction to an appropriate host response [4]. In addition, their mechanical properties should include permeability, stability, elasticity, flexibility, plasticity, and resorbability at a rate congruent with tissue replacement [5]. Scaffolds should also allow cell adhesion and the potential delivery of biomodulatory agents such as growth factors and genetic materials [6].

Amniotic membrane (AM) has been used in surgical transplantation as a biomaterial 100-years ago. The presence of hyaluronic acid and collagen types I, III, IV, V and VI, fibronectin, elastin, laminin, nidogen and proteoglycans makes the AM act as a scaffold for proliferation/differentiation and delivery of stem cells. Amniotic cells also express both of the mesenchymal and epithelial stem cell markers. Fresh AM have a short life span compared to preserved membranes [7&8].

The use of autologous bone marrow derived mesenchymal stem cells (MSCs) is an intrinsic part of tissue engineering and plays a key role in the creation of implantable tissue [9] because of the low risk of immune complications. However, they are not cost-effective or batch-controlled for universal clinical use [10].

The present work aimed to study the efficiency of a cryopreserved AM scaffold loaded with autologous undifferentiated MSCs in the management of experimentally induced critical femoral bone defect in a canine model.

MATERIALS AND METHODS

The experimental work was approved by the ethical committee of Faculty of Veterinary medicine, Cairo University (EAURC) code (Cu F Vet/F/SUR/2013/15).

Study Design

Ten female mongrel dogs were used as AM donors. Freshly collected AMs through C-section were prepared and cryopreserved to be used as scaffolds [6].

Twenty-seven apparently healthy mongrel dogs weighing 15-20 kg body weight with average age 2-5 years were used for the experimental procedures. All dogs were vaccinated and dewormed. A femur defect of 2-cm in length was created in all animals.

Groups

Experimental animals were allocated randomly and equally (n=9) into three groups.

Group-A: the defect was treated conventionally without a scaffold (control group).

Group-B: the defect was treated with AM scaffold alone and in

Group-C: the defect was treated with cryopreserved AM scaffold seeded with autologous MSCs.

Each group was divided into 3 sub-groups (n=3) according to the follow-up period; 6, 12 and 24 weeks.

Procedures on donors

After the placentae were collected, the AMs were stripped off aseptically. The following procedures were conducted:

Amniotic Membrane Preparation

AMs were immersed in 1L sterile normal saline containing 100 U/ml penicillin and 0.2mg/ml streptomycin and 0.025mg/ml amphotericin. The epithelial covering was marked by external knot of silk stitch which was placed on the surface of the amniotic membrane. Ten times serial washing of the membranes in sterile petri-dishes contained 20ml normal saline with the former additives were applied. During washing, each individual membrane was finger rubbed and squeezed of the blood vessels gently. AMs were then kept in a container of normal saline with additives for a period of two hours in a refrigerator [6].

Amniotic Membrane Banking

AMs were spread uniformly without folds or tears on individually sterilized 0.22m nitrocellulose membranes of the required size (47mm, commercially available-Millipore or Sartorius, Biobasic Canada[®]) with the epithelial/basement layer surface up. The molded membrane sheet were cut to 4×5cm pieces, each piece was put in plastic sterile dish containing the preservative medium 1:1 vol/vol ratio sterile glycerol and Dulbecco Modified Eagles media (DMEM) with 3.3% L-glutamine, 25µg/ml gentamicin, 50units/ml penicillin-100µg/ml ciprofloxacin and 0.5mg/ml Amphotericin B. Then membranes were preserved in a temperature -80°C.

Amniotic Membrane Assessment

AMs were assessed bacteriologically and mycological through: direct microscopic examination (swapping and staining) with additional aerobic and anaerobic culturing immediately after collection. Samples of the cryopreserved AMs were tested, one, two and three weeks after preservation using the same previous procedures to detect any bacterial or fungal contamination during preservation.

Before using, the cryopreserved AMs were left for thawing at room temperature and washed with normal saline solution several times at the time of implantation.

Procedures on experimental animals

The procedures included 2 steps; **Step-1:** Bone marrow aspiration to obtain autologous MSCs. This was conducted only on animals in **Group-C**. The MSCs were loaded on the AM of donors to treat animals of this group. **Step-2:** The experimental bone defect was created identically on animals.

Step-1:

Autologous MSCs Acquisition and Preparation

The iliac crest region was prepared aseptically and the animals were generally anaesthetized. A Rothensal bone marrow biopsy needle (stylet and needle, 15G, 1.8") was used to obtain MSCs [7]. Bone marrow samples (20 ml/sample) were collected and transported to the laboratory at 26°C then processed within 4-hours after aspiration.

Culture and propagation of bone marrow cells:

Under complete aseptic conditions; the isolation of MSCs was performed as described by Mokbel [8]. The cell viability was tested with trypan blue stain (0.4%). Then the washed pellet cells were re-suspended in Roswell Park Memorial Institute medium (RPMI 1640 medium- Sigma[®]) supplemented with 10% fetal bovine serum (FBS; USDA, Gibco, Grand Island, NY, USA), antibiotics (penicillin 10000-U/ml, streptomycin 10000-U/ml) and Amphotericin-B 25-U/ml. This medium was also used as a control medium for the experiments. The nucleated cells were plated as primary culture in tissue culture flask at $2.5 \times 10^5/\text{cm}^2$ and incubated at 37°C in a humidified atmosphere containing 5% CO₂. On day 4 of culturing, the non-adherent cells were removed along

with the change of medium every 2-days. Undifferentiated MSCs were transplanted in upon reaching 70-80% confluence.

MSCs identification

MSCs were identified by their morphology; the adherent colonies of spindle fibroblast like-cells were trypsinized, and counted. MSCs phenotypes were confirmed by flow cytometry and analysis of cell surface molecules as detailed elsewhere [9] for CD34⁻ and CD29⁺. Cells were also characterized by their in vitro ability to differentiate into osteocytes and chondrocytes [10].

Loading of stem cells on amniotic membrane

Under complete aseptic condition; the previously prepared cryopreserved AMs were washed with phosphate buffer saline contained antibiotic several times till become free from preservative glycerol. The isolated, propagated and characterised MSCs for two weeks in culture were counted with haemocytometer loaded on AM as a feeder layer.

AM co-cultured with MSCs was maintained in RPMI-1640 media for one week and supplemented in with 10% fetal bovine serum (FBS; USDA, Gibco, Grand Island, NY, USA), antibiotics (penicillin 10000-U/ml, streptomycin 10000-U/ml) and Amphotericin-B 25-U/ml MSCs. Co-culture was incubated at 37°C in a humidified atmosphere containing 5% CO₂ [9,10].

Step-2

The pelvic limbs of all experimental dogs were radiographed to document normal femoral bone anatomy and size prior to surgery.

Femoral Bone Defect Induction

A Prophylactic antibiotic course of Cefipim (Maxipim[®] Smith-Kline Beecham Co., A.R.E) at a dose of 4.5-mg/kg.b.wt was administered [I.V] immediately preoperatively. The right pelvic limb was prepared for aseptic surgery. After complete general anaesthesia, the craniolateral approach for femur bone exposure was performed [11]. After exposure the femoral shaft modulation of the dynamic compression bone plate (DCP) was performed to simulate the contour of the femur. An artificial bone defect of 2-cm length in the mid-shaft of the femur was created.

Femoral Bone fixation

Fixation of the bone segments was performed primarily by insertion of intramedullary bone pin (Synthes, Wayne, Pa) either 3 or 4-mm Ø according to the medullary cavity diameter using pneumatic drill by retrograde method. After that the steps for fixation of the modulated DCP was performed the holes of the plate were loaded with screws except the hole above the defect. The implantation site was then flushed several times with normal saline solution and irrigated with Gentamycin solution (Gentamycin[®] 10%, Alexandria Co., ARE).

Femoral Bone Defect implanting:

Conventional bone segments fixation was achieved in all groups. Additionally implantation of cryopreserved AM and cryopreserved AM seeded with MSCs was added in **Group-B** and C respectively. The surgical wound was closed routinely.

Post-operatively (P.O); All dogs were individually housed along the study period in metal galvanized cages at the animal house of Surgery department, Faculty of veterinary Medicine, Cairo university. Dogs were given Maxipim[®] every twelve hours for five successive days. The skin wound was daily dressed and the sutures were removed 10 days P.O.

Post-operative assessment

Post-operatively, all animals went through Clinical, Radiological and Morpho-histological evaluation as follows:

Clinical; daily regular clinical examination included wound drainage and evidence of infection, limb function, popliteal lymph node size and weight bearing capacity in standing and motion positions.

Radiological; Mediolateral (ML) and Craniocaudal (CC) Sequential radiographs of the operated limbs were performed using mobile x-ray machine (Ficher Machine, Eureka X-ray tube/ Model E-Merald-125, 1985, U.S.A); with radiographic factors 50-54 kV/32 mAs. X-ray films were taken immediate P.O. and monthly till the end of 6th month observation period. The fracture gap was examined for radiographic visibility and its filling by new osteophytes. At the end of the respective experimental period, euthanasia was performed by [I.V] injection of overdose thiopental (30 mg/kg.b.wt). The operated femora were harvested and passed contact radiography before and after removal of the plate.

Morphohistologically; harvested operated femurs were examined before and after removal of the plate for periosteal/endosteal reactivity, stability of the bone segments and the nature of tissue formed within the defect. Tissue sections at host-defect interfaces were stained with hematoxyline and eosin and examined microscopically.

RESULTS

Step-1; In vitro study

MSCs were cultured, propagated at 0, 7 and 14 days as illustrated in figures 1. MSCs and were identified.

Step-2: The evaluations of the recipient animals showed;

Clinically; all dogs were lifting the operated limb for 2-days P.O. They bear their weight partially on the limb at the end of the first week P.O. Animals regained their full weight bearing at 4-6 weeks P.O. All animals used their limb successfully at 12-weeks P.O in walking and fast movement except two animals of **Group-A** showed partial weight bearing till the end of the study.

Radiologically (Table-1 and Figure 2); Immediate P.O. radiographs of all animals revealed adequate metal-implant stability. Bone alignment was very good and the defect was clear radiolucent area.

At 4-weeks there were no observable radiographic changes except some rounding of the fracture ends in all animals. Moderate osteoperiosteal reactivity at the proximal and distal segments was detected in Group-B and C while in Group-A it was slight and limited to the proximal side. There was no changes at the defect in all groups.

At 6-8 weeks there was an increase in the periosteal reactivity on both bone segments in all animals. Slight osteophytic formation in the gap was noticed in Group-C represented by increasing in gap radiodensity, while in Group-B the gap radiodensity increased but less than Group-C. In Group-A; the gap was clear radiolucent area.

Contact radiography of the harvested femur at 6-weeks showed noticeable osteoperiosteal reactivity at both fracture ends. The bone defect was covered with slight radiopaque sleeve while the defect was narrowed but still radiolucent in Group-B and C. In Group-A there was a slight amount of osteoperiosteal reactivity at the proximal fracture end appeared as radiopaque thickening of the cortex and scanty amount at the distal one as well as the defect.

At 12-weeks, in all dogs, the osteoperiosteal reactivity was still present in both proximal and distal bone segments. Concerning the fracture gap; there was a noticeable newly formed bridging callus formed

inside the gap in Group-C while in Group-B there was increasing in osteophytic formation. In Group-A there was slight increase in gap radiodensity.

The contact radiography showed decrease of the osteoperiosteal reactivity at both fracture segments. The defect was filled with radiopaque material (new bony tissue) and partially filled at the medial cortex in Group-C. The same results was noticed in Group-B the defect was still clearer than Group-C. Group-A showed no radiographic changes except some increase in the periosteal reactivity at the fracture segment while the defect was still radiolucent.

At 16-weeks there was noticeable decrease in the osteoperiosteal reactivity at the segments in all groups (remodeling) especially at the proximal bone segment. The fracture gap was almost filled with newly formed osteophyte that was represented by high radiodensity and decrease defect length all over that period in Group-C. In Group-B the gap was nearly filled with new osteophytes but less than Group-C; while in Group-A there were no observable radiographic changes except slight increased gap radiodensity.

At 20-24 weeks P.O The osteoperiosteal reactivity had almost disappeared at both proximal and distal bone segment in all groups. The animals of Group-C showed fracture defect filled with new callus and markedly decreased in length. In case of Group-B; there was radiolucent area in the defect with increasing callus radiodensity. However Group-A showed the defect increasing in radiopacity especially at the proximal part but still clear.

Contact radiography at 24-weeks, in Group-C, the osteoperiosteal reactivity had almost disappeared at the proximal segment while still present at the distal one. The radiodensity of the defect had significantly increased and the new bony tissue extended from both cortices while the defect had almost disappeared. In Group-B the defect was more radiopaque and to some extent filled with new bone from the medial cortex. On the other hand; Group-A showed osteoperiosteal reaction which was still visible and the defect was filled with low radiodense material (fibrous tissue) but still noticeable.

Morphohistologically; the gross examination of the harvested femora (**Table-2**) showed that;

At 6-weeks the entire bone and the defect was completely surrounded by a fibrous periosteal sleeve in all animals of Group-C. While in Group-B fibrous reactivity observed on the proximal and distal host segments while it was scanty on the defect under the plate. The bone showed some stability after plate removal in Group-C than Group-B. On the contrary, Group-A periosteal reactivity observed on the proximal more than distal host segments while it was absent on the defect. The bone was unfixed after plate removal.

At 12-weeks in Group-C, there was complete disappearance of the proximal bone-defect interface, while the distal one has not completely disappeared. The bone plate was covered with fair amount of hard callus. The osteotomy line was almost stable at the proximal and distal host-implant edges after plate removal. Group-B showed some cortical union at the proximal bone-defect interface while the distal one almost disappeared that result in somewhat bone instability after plate removal. The remodeling of the periosteal reaction at the proximal segment was observed. In Group-A the defect and fracture segments were completely covered with fibrous tissue sleeve (periosteal reactivity). The segments were still completely unstable after plate removal.

At 24-week the harvested femora of Group-C showed clear cortical union at the proximal bone-defect interface and incomplete union at the distal one. The bone plate was covered with fair amount of osseous tissue. The Periosteum were completely remodeled at the proximal host-graft interface. The bone segment was fixed after plate removal. The same observations were noticed in Group-B except the union was incomplete at both bone-defect interfaces especially the distal one but the bone segments were stable after plate removal in both groups. While in case of Group-A both the segments and the defect covered with large fibrous tissue sleeve. The defect was nearly visible and the bone showed instability of the bone segments after plate unloading.

The results of histological examination of tissue sections at different observation periods (Figure 3) are summarized as:

At 6-weeks in case of Group-C, there was some bony necrosis in the fracture segments edges with resorption cavities. The defect either proximally or distally was filled with vascularized fibrous connective tissue and there was some osteoplastic activity. While in case of Group-B, the gap showed numerous connective tissue reactivity more than noticed in Group-C and few aggregations of osteoblasts. On the other hand; the control Group-A showed sever bone necrosis at the segments edges and the defect showed abundant connective tissue with severe inflammatory reaction represented by mononuclear cells.

At 12-weeks the sections of Group-C showed new bony tissue formation at different stages and some vascular channels within the defect intermixed with fibrous connective tissue. In case of Group-B sections; they showed less osteogenesis process, few osteocytes intermixed with chondrocytes and resorption cavities at bone segments ends. Group-A showed sever fibrous connective tissue reactivity with some osteoblastic activity near the segments edges while the defect was free from osteogenic cells. Some inflammatory cell still be seen between the fibrous fibers.

At 24-weeks there was noticeable osteoblastic activity with numerous bony cells (osteoblasts and osteocytes) with more vascular cavities in case of sections of Group-C. Also there was primitive bone marrow channels formed at the bone-defect interfaces especially at proximal segment. Whereas Group-B sections showed little osteogenic activity than that of Group-B. Less fibrous tissue was observed with vascularized cavities. However; in case of Group-A the fibrous connective tissue was the dominant and observed in the defect with no osteogenic cells expect at the bone segment-defect interface with some resorption cavities.

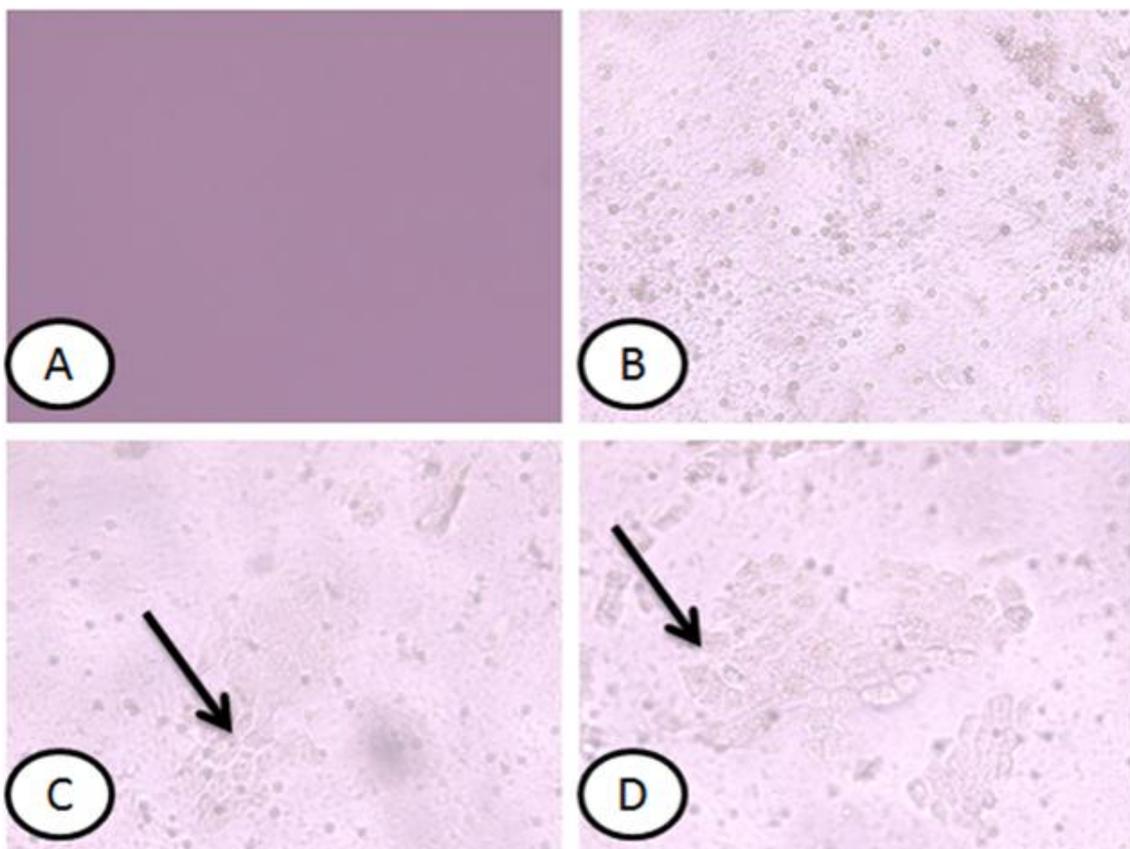


Figure (1): Showed in vitro culture of MSCs loaded on amniotic membrane:
A: Amniotic membrane free MSCs, **B:** Amniotic membrane loaded with MSCs at 0 day of culture (spherical rounded shaped cells). **C & D:** Amniotic membrane loaded with MSCs at 3 & 7 days of culture (arrows at fibroblast shaped cells).



Figure 2: In Sequential radiographs A; Craniocaudal and B; Mediolateral views of the operated limb of experimental groups at 24 weeks P.O showing the defect (red arrow) almost filled with new bony tissue. In contact radiographs of the harvested femurs a,b; before and c,d; after plate removal showing the defect occlusion in both group B and C with variable degrees while in group A still somewhat radiolucent.

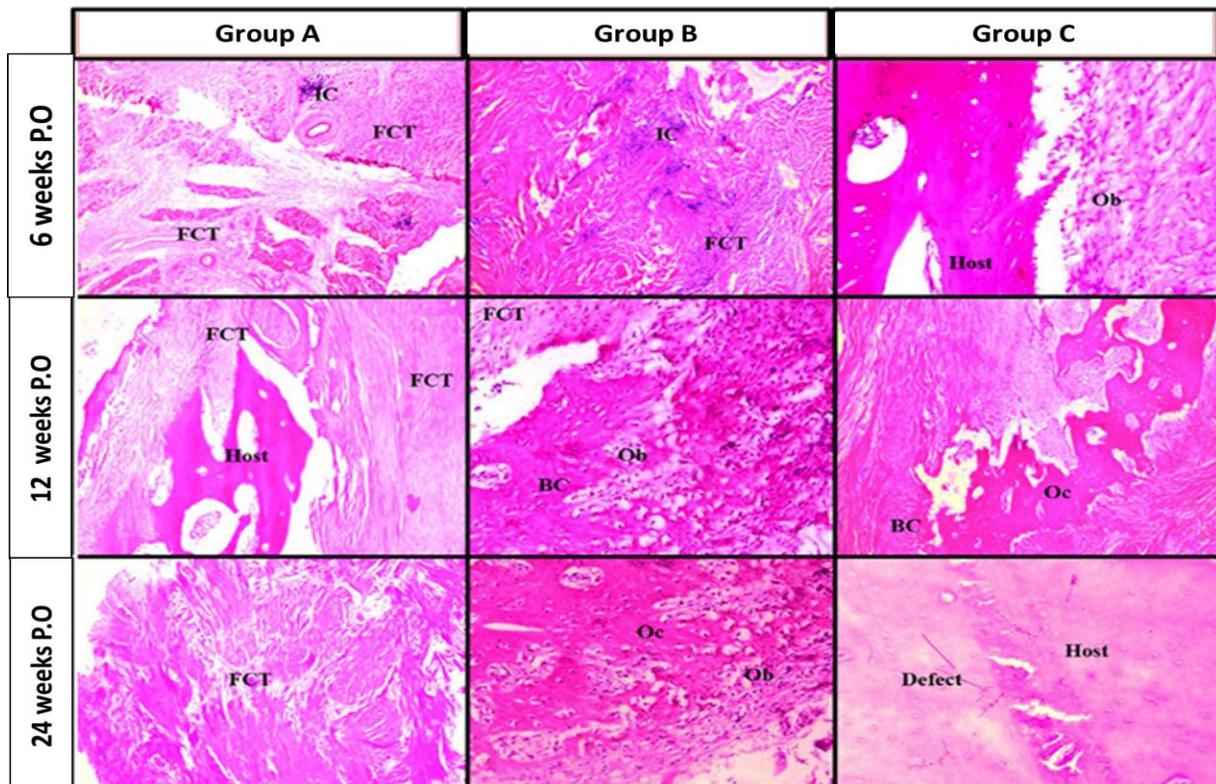


Figure 3: The histopathological examination of the tissue sections at different periods showing; at 6 weeks that fibrous tissue (FCT) was the main in the field with inflammatory cells infiltration (IC) in group A&B. In group C showing some osteoplastic (Ob) activity at the host-defect interface. At 12 weeks P.O vascularized FCT persist in group A while groups B&C showing some osteogenic activity (Ob&Oc). At 24 weeks P.O organized FCT present in group A while in group B increasing the osteogenic activity. Group C showing bony fusion at the host-defect interface.

Table 1: The osteoperiosteal reactivity as a radiographic measure in different experimental groups:

	Osteoperiosteal Reaction								
	Group-A			Group-B			Group-C		
	PBS	DBS	G	PBS	DBS	G	PBS	DBS	G
4W	+	n	n	++	++	n	++	++	n
8W	++	++	n	+++	+++	+	+++	+++	+
12W	++	++	-	+++	+++	+	+++	+++	++
16W	++	++	-	++	++	++	++	++	+++

PDB= Proximal Bone Segment; DBS= Distal Bone Segment; G= Gap; n= no change

Table 2: the fibrous reactivity and bone segments stability at the morphological examination of the harvested femora:

	Connective Fibrous Tissue Reaction											
	Group-A				Group-B				Group-C			
	PBS	DBS	G	Stability	PBS	DBS	G	Stability	PBS	DBS	G	Stability
6W	+	+/-	+/-	-	++	++	+	-	+++	+++	++	-
12W	++	++	++	-	+++	+++	++	+/-	+++	+++	+	+
24W	++	+++	+++	+/-	-	-	+	++	+/-	+	+/-	+++

PBS=Proximal Bone Segment; DBS= Distal Bone Segment; G= Gap; stability; segments fixation after plate removal.

DISCUSSION

Amniotic membrane is a biodegradable material known to accelerate wound healing, provide healthy granulation tissue formation, avoid immunological reactions, and have an antibacterial and angiogenetic effect [12&13].

Stem cells undergo asymmetric division to produce cells that become more specialized, such as osteoblasts or chondroblasts, while maintaining the ability to self-perpetuate [14], if seeded on scaffolds of predefined dimensions and shape to fit in the defect [15].

In the present study the canine AM was used as scaffold both alone or seeded with mesenchymal stem cells in management of artificially induced critical bone defect in dogs.

In this study full term (58-63 days) AM was used according to previous studies [16-19] that reported that although the preterm AM has a greater mechanical integrity than Full-term AM, the stiffness of full-term AM is more reasonable for a majority of tissue engineering protocols. Although placenta obtained shortly after elective caesarean delivery is the preferred source of an AM, since placenta from normal birth delivery are known to be contaminated and therefore unsuitable for transplantation [6].

Since the fresh AM is not available regularly cryopreserved AM was used as a scaffold in the present study. AM preservation is essential to reach a ready-to-use source for clinical applications. Fresh AM have a short life span as compared to the preserved membranes. Also it was suggested that the AM processing and cryopreservation was decreasing the immunogenicity of its epithelial layer if present as a result of loss of some epithelial cells during the procedures and preservation period [20].

The size of the created defect (≥ 2 cm) is considered a critical defect, because the shorter defect may heal spontaneously with normal fracture repair according to previous studies [21-23]. In the present experiment the critical femoral bone defect was managed through implantation of MSCs loaded on AM scaffold in a Group-C, AM scaffold alone was used in Group-B and the defect left without cell management in Group-A.

According to Sinibaldi [24] and Stevenson [25] the success or failure of full bone grafting depends upon two factors: rigid fixation of the graft to the host bone by standard ASIF plating techniques and cortical contact between the graft and the host bone.

In the present study, the host bone was fixed by 6-8 cortical screws (3-4 proximal and distal). This was in augmentation with introduction of 3-4 mm \varnothing intramedullary bone pin occupying about 65% of the medullary canal that increases the construct stiffness and estimated number of cycles to fatigue failure and plate bending when compared with a plate only construct. Mechanically, the intramedullary pin acts as a replacement of the transcortical defect that was in contract with that reported by different authors [11, 26-29].

The animals were evaluated for bone healing through clinical, radiological and morphohistological examination. The clinical evaluation results of all groups were satisfactory. There was no clinical evidence of infection or tissue reaction. Most dogs in all groups 70-80% did extremely well. They were bearing weight on the operated limbs within 2-4 weeks post-operatively, while full limb function was obtained by the end of 12-weeks post-operatively. These observation correlate well with those reported by Sinibaldi [24] and El-Keiy et al [30].

The radiographic examination was considered as a basic technique for assessing fracture healing process [24, 31]. The radiographic changes showed promising bone defect healing. The formation of a progressive wall of periosteal new bone as an attempt to bridge each host-scaffold interface was seen in animals of both Group-B and Group-C. Also increasing the gap radiodensity indicated new bony tissue formation. The osteophytic formations were observed at the proximal segment prior to the distal one. This might be attributed to the insufficient nutrition at the level of the distal segment. The remodelling phase of fracture healing was noticed also proximally former to distal segment. These observations were in agreement with El-Keiy et al [30].

In Group-A these observations were noticed very slow and late. There was no tendency of defect healing appeared radiographically as the defect appeared radiolucent all over the observation periods. Slight periosteal reactivity and some osteophytic reaction at the host segments edges were observed lately at 24-weeks P.O. this is in contract with Giannoudis [15].

The beginning of osteoperiosteal reactivity and increasing the defect radiodensity were noticed in Group-C at 4-6 weeks P.O. while in Group-B they were seen late at 6-8 weeks. The defect was nearly stuffed with new bone about 70% in Group-C at 24-weeks P.O while it was filled lower in Group-B at the same period. These results indicated that MSCs may improve and accelerate defect healing process augmented with AM than using AM alone. The results were in contract with other studies [32-34].

The contact radiographic evaluation of the harvest femora at each successive observation period showed the same results in sequential radiography. The cortical union was almost achieved in both Group-C and Group-B at 24-weeks P.O while no union in Group-A.

The gross examination of the harvested femora showed that at 6-weeks the entire defect was completely surrounded by a fibrous tissue sleeve which was hard proximally and somewhat elastic distally communicated with osteoperiosteal reactivity at both bone segments. The host-implant interfaces showed some instability after plate removal at that period. These were observed in Group-C and Group-B while in Group-A the tissue amount was less and bone segments were completely unstable.

The gap was covered with a semi-hard callus indicating beginning of calcification at 12-weeks P.O. and complete hardening with cortical union were noticed at 24-weeks P.O in both Group-C and B with variable degrees. While in Group-A there was fibrous tissue organization without hardening. This may indicate the differentiation of MSCs seeded on AM scaffold and AM cells into osteogenic cells in both Group-C and B resulting in callus formation and hardening. These results in agreement with the previous studies [33-37].

The histological examinations of tissue sections from harvested femora at different observation periods proved that AM scaffold either seeded with MSCs or alone may have an osteoconductive and osteoinductive properties with some differentiations [38]. At 6-weeks P.O the defect was filled with vascularized fibrous connective tissue (FCT) with some osteoblastic activity at the proximal host implant interface in Group-A. While in Group-B all the defect was filled with fibrocellular matrix and few osteoblast aggregations. In contrary Group-C sections showed abundant fibrous tissue without evidence of osteogenesis.

The osteogenic reactivity increased by time that indicated through MSCs differentiation into fibroblasts, fibrocytes, osteoblast and osteocytes all over the histologic field in Group-A and Group-B while in case of Group-C the FCT was the dominant. These results coincided with previous published research work [39].

It was concluded that AM can be used as a scaffold in segmental bone defects either alone or seeded with MSCs.

The bone necrosis that was noticed at the segment edges at early periods may be attributed to heat liberated during osteotomy. This necrosis was replaced with new bone in Group-A and B while it was persist in Group-C.

It was noted that the defect healing process began with fibroblastic and osteoblastic activity. These results are in contrary to John [40] who stated that the incorporation of the cortical graft is initiated by osteoclastic rather than osteoblastic activity.

The new lamellar bone had been deposited within a few resorption cavities close to each host-scaffold interface. The preceding reparative processes were advanced and faster in Group-A than B. These results may be attributed to the presence of MSCs and their potential ability to differentiate into osteoblasts. These results were similar to those obtained by other authors [41-44].

In the present study neither osteomyelitis nor infection were recorded. The cryopreservation process has antimicrobial action beside the antimicrobial activity of AM [45-47].

AM showed good and ideal characters to be used as a scaffold for either implantation or stem cells seeding and cultivation. It give good feeding and attachment media for MSCs as a results of its extracellular matrix component of collagen, laminin and fibronectin. This in contract with other literatures [48, 49].

CONCLUSION

The results of the present study revealed that the use of cryopreserved AM scaffold alone or seeded with MSCs in reconstruction of femoral bone defects in canine model is successful as determined by the achievement of full limb function, fracture healing and scaffold incorporation. These results are promising for the clinical application of AM implantation procedures and may open a way in using this type of graft in extensive bone injuries, defects or bone neoplastic disorders. Further studies need to answer the questions concerning the immunogenicity, biomechanical properties and bone remodeling rates.

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