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Using Of Chitosan Scaffold Seeded With Autologous Undifferentiated Mesenchymal Stem Cells For Femoral Bone Defect Management In Dogs.

Ahmed Syed Soliman[1], Mohammed Said Amer[1]*, Ashraf Ali Shamaa[1], Dina Sabry Abdelfatah[2], Gehan Gamil Shehab[3], Ayman Abdelmonem Mostafa[1], and Omaima Hagag Darwish[4].

[1]Department of Surgery, Anesthesiology & Radiology, Faculty of Veterinary Medicine, Cairo University, Cairo, Egypt
[2]Department of Biochemistry, Faculty of Medicine, Cairo University, Cairo, Egypt
[3]Department of Pathology, Animal Health Research institute, Ministry of Agriculture, Egypt.
[4]PhD Student, Veterinary Researcher.

ABSTRACT

Twenty seven apparently healthy mongrel dogs (2-5yeays old) weighing (15-20Kg) were divided into three groups (n=9). Group A; control group in which no implant was used to fill the bone defect in the transected femur of the operated limb. Group B; in which Chitosan without MSCs was used as a bone scaffold to fill the bone defect in the transected femur. Group C; in which Chitosan with MSCs was used to fill the bone defect of the transected femur. Each group was subdivided into three sub-groups (n=3) according to the time of postoperative observation (1½, 3 and 6 months). The dogs were checked clinically and radiologically till the end of the study designed. After euthanasia, the femurs of the operated limb were examined histologically. The results showed that, using Chitosan as a bone scaffold seeded with MSCs enhanced the process of bone healing than using of Chitosan alone.
Keywords: Bone, Chitosan, defect, Femoral, Mesenchymal, Stem.

*Corresponding author

INTRODUCTION

A great deal of interest has been recently focused on orthopedic surgery in small animals. Extensive or segmental defects of canine long bones are common orthopedic problems associated with severe trauma. The critical size defect (CSD) was known as the smallest interosseous gap that does not repair by normal fracture bone healing process during the life time of the animal [1]. Concerning the literatures several bone substitute materials either natural or synthetic were used to replace the loss of bone and initiate bone healing. The bone substitutes and scaffolds play a significant continually developing starring role in the management of critical bone defects which led to major advances in the chances of survival for patients with large bone losses [2, 3].

Scaffolds are developed to support the recipient cells during bone healing process. They stimulate cells differentiation and propagation throughout their formation into a new bone. So, the design and selection of the biomaterials used for scaffolding is a very important step in the process of bone healing [4]. The scaffold should be biocompatible [5], able to react to an appropriate host response [6]. In addition, their mechanical properties should include permeability, stability, elasticity, flexibility, plasticity, and resorbability at a rate congruent with tissue replacement [7]. Scaffolds should also allow cell adhesion and the potential for delivery of biomodulatory agents such as growth factors and genetic materials [8].

Chitosan, a natural polymer obtained from deacytilation of chitin [9], has a wide range of properties biodegradability, biocompatibility, antibacterial activity, wound healing properties, and bio-adhesive character that make it appropriate for tissue engineering [3,10,11].

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 [12] because of the low risk of immune complications. However, they are not cost-effective or batch controlled for universal clinical use [13].

The present study aimed to study the efficiency of using of Chitosan as a scaffold loaded with autologous undifferentiated bone marrow derived mesenchymal stem cells (BM-MSCs) in the management of artificially induced critical sized femoral bone defect in a canine experimental model.

MATERIALS AND METHODS

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

Study Design

The experimental work was performed on 27 apparently healthy mongrel dogs weighing 15-20 kg body weight and age range between 2-5 years. All animals were vaccinated and dewormed. A critical sized femoral bone defect of 2 cm in length was induced in all animals.

Groups

Experimental animals were allocated randomly and equally into three groups (n=9). **Group A**: the bone defect was managed conventionally through fixation only without a scaffold (control group), in **Group B**: the defect was managed with Chitosan (Ch) scaffold alone and **Group C**: the defect was managed with Ch-scaffold seeded with autologous undifferentiated BM-MSCs. Each group was divided into 3 sub-groups (n=3) according to the follow up period; 1.5, 3 and 6 months.

Preparation of Chitosan scaffold [14]:

The Ch-scaffolds were produced from natural polymers, Chitosan (ACROS Organics, USA) with 100.000-300.000 molecular weight and gelatine powder (Science Lab, USA). Chitosan: gelatine was added with a ratio 3:1and were cross linked with 25% glutraldehyde solution at 0.1% concentration. The mixture of Chitosan-gelatine and glutraldehyde were molded into three dimensional shape segment simulate the shape of mid-shaft of the femur bone of the dogs of **Group B & C**. The scaffold was dehydrated using Ethyl Alcohol

70%, then were put in the Lypholyzer for deprivation of Ethyl Alcohol. The scaffold took 7 days to be ready. The scaffold was then wrapped and kept in a sterile container till the time of use **(Fig.1)**.
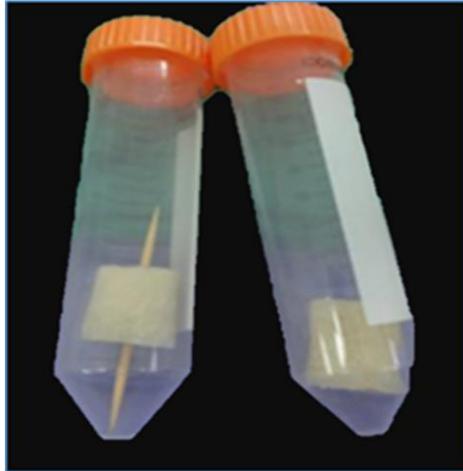

Figure 1: Chitosan-gelatin scaffold after preparation and packing in sterile containers, simulating bone segment shape and size.

Procedures on experimental animals

The experiment comprised 2 steps; **Step-I**: Bone marrow aspiration to obtain autologous MSCs. This was conducted only on animals in **Group C**. The MSCs were loaded on the Ch-scaffold to treat animals of this group. **Step-II**: The experimental bone defect, which was created identically in animals of all groups.

Step-I:

Autologous Bone Marrow derived MSCs Acquisition and Preparation

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

Culture and propagation of bone marrow cells

Under complete aseptic conditions; the isolation of MSCs was done as referred by Mokbel et al., [15]. Cells viability determination and the number of viable cells by trypan blue staining was performed, 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 $2.5 \times 10^5/cm^2$ and incubated at 37°C in a humidified atmosphere containing 5% CO_2. On day 4 of culture, the non-adherent cells were removed along with the change of medium every 2 days. Undifferentiated MSCs were transplanted upon reaching 70-80% confluence.

MSCs identification

Identification of MSCs was done 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 [16] for CD34$^-$ and CD29$^+$. The cell viability was tested with trypan blue stain (0.4%). They were also characterized by their in vitro ability to differentiate into osteocytes and chondrocytes [17].

Loading of stem cells on Ch-scaffold

Under complete aseptic condition; On the day of the operation, the propagated MSCs were checked for viability and its number, then poured on the Chitosan scaffold and spread between Chitosan pores, then was packed in ice box then transferred to the surgery room. The calculated cells to be seeded at the scaffold are 3000,000 cell/ml. Total No. of cells = 5 x 3000,000 = 1, 5000,000 cells.

Step-II

The right pelvic limb of all experimental dogs was radiographed to document the normal femoral bone anatomy and shaft size before surgical operation.

Femoral Bone Defect Induction

A prophylactic antibiotic course of Ceftriaxone sodium (Ceftriaxone® Sandoz, A.R.E) at a dose of 25 mg/kg body weight was administered i.v. immediately preoperatively. The right pelvic limb was prepared for aseptic surgery. The animals were generally anesthetized. Femoral shaft exposure was performed via the craniolateral surgical approach [18]. After bone shaft exposure, the dynamic compression bone plate (DCP) modulation was performed to simulate the contour of the femur shaft. An artificial bone defect with length 2 ± 0.2 cm in the mid-shaft of the femur was then induced [19].

Femoral segments fixation

The bone segments were fixed primarily by retrograde bone pinning by intramedullary bone pin (Synthes, Wayne, Pa) either 3 or 4 mm Ø according to the medullary cavity diameter. Then, the steps for fixation of the modulated DCP was performed. The holes of the plate were loaded with cortical screws (3-4 screws in each segment) except the hole above the defect. The implantation site was then flushed several times with normal saline solution and Gentamycin solution (Gentamycin® 10%, Alexandria Co., A.R.E.) [19].

Femoral Bone Defect Management

Conventional bone segments fixation was achieved in all groups. Additionally implantation of the Ch - scaffold and Ch-scaffold 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. The dogs were given Ceftriaxone® every 24 hours for five successive days. The skin wound was daily dressed and the sutures were removed 10 days P.O.

Postoperative assessment [19]**:**

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

Clinical: All animals were subjected to daily regular clinical examination, included wound drainage and infection evidence, 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 a 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 the observation period. The fracture gap was examined for its radiodensity and its filling by new osteophytes. At the end of the respective experimental period, euthanasia of the animals was performed through i.v. injection of 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 morphologically before and after removal of the plate for periosteal reactivity, the bone segments stability and the nature of tissue formed within the defect. Tissue sections at host-defect interfaces and within the defect were stained with hematoxyline and eosin and examined microscopically.

RESULTS

Step I; In vitro study

MSCs were cultured, propagated at 0, 7 and 14 days displayed characteristic spindle-shaped morphology. MSCs were identified by their ability to differentiate into osteoblasts and chondrocytes and by their specific surface markers.

Step II: The evaluations of the experimental animals showed;

Clinically: Generally; all experimental animals showed decreased appetite, seromal reaction in the operated limb and mild increase in rectal temperature (39± 0.5°C). The popliteal lymph node showed mild enlargement in both groups-A and B while in group-C, there was moderate enlargement, The previous recorded signs subsided spontaneously within 7 days P.O except two animals, one in group-A and another in group-B showed sever seromal reaction with limb swelling. These animals were treated with alpha-chymotrypsin injection once daily for 3 days. The swelling subsided in 10 days P.O.

Concerning the weight bearing capacity and limb function; all the animals showed no-weight bearing on the operated limb for 2 days P.O. In case of group-A, the animals landed the operated limb 3-5 days P.O. There was partial weight bearing in fast motion while the full limb function was achieved at 6-8 weeks P.O.

In case of group-B; all animals were ranged from partial to no-weight bearing until the end of the two weeks P.O. The animals' gait was ranged from observable moderate lameness in slow motion to severe lameness in fast motion during one month P.O. The full weight bearing was achieved within 4-6 weeks P.O.

While in group-C; there was partial weight bearing on the operated limb with mild degree of lameness in walking 15 days P.O. The animal's gait showed moderate-lameness in fast motion. The animals showed full weight bearing on the operated limb at 4-6 weeks P.O.

Radiologically (Fig.2): Immediate P.O. radiographs of all animals showed good metal-implant stability. Bone alignment was very good and defect appeared as a clear intersegmental radiolucent area.

At 2-4 weeks P.O: Generally, all radiographs showed no radiographic significance within the defect as it was still as intersegmental radiolucent area. The segments end showed some rounding with some osteophytic reaction. Slight osteoperiosteal reactivity at proximal bone segment was detected at 3-4 weeks P.O in groups-B & C while in group-A there were no observable radiographic changes.

At 6 weeks P.O: in case of group-A; the recorded radiographic changes was slight in the form of osteoperiosteal reaction on both fracture segments. The fracture gap was still relatively radiolucent. In case of group-B; the radiographs showed increased osteoperiosteal reaction proximally and distally with comparatively increased radiodensity at the defect area. While in group-C; there was an observable increase in osteoperiosteal reaction in both bone segments. The defect radiodensity was markedly increased with a slight osteophytic formation.

At 8-10 weeks P.O: in all groups there was increased osteoperiosteal reaction proximally and distally. The gap had a more osteophytic reaction with increased radiopacity in group-C than group-B while in the group A the gap was still clearly radiolucent.

At 12 weeks P.O: in all animals, the osteoperiosteal reaction was still present in both proximal and distal segments. Then, the proximal segment showed a smooth osteoperiosteal reaction which descends lower i.e. begin of remodeling. Concerning the fracture gap; the gap radiodensity was hardly increased in case

of group-A, while in group-B there was increasing in osteophytic formation inside the defect. But in case of group-C; there was a noticeable newly formed clear bridging callus formed inside the gap.
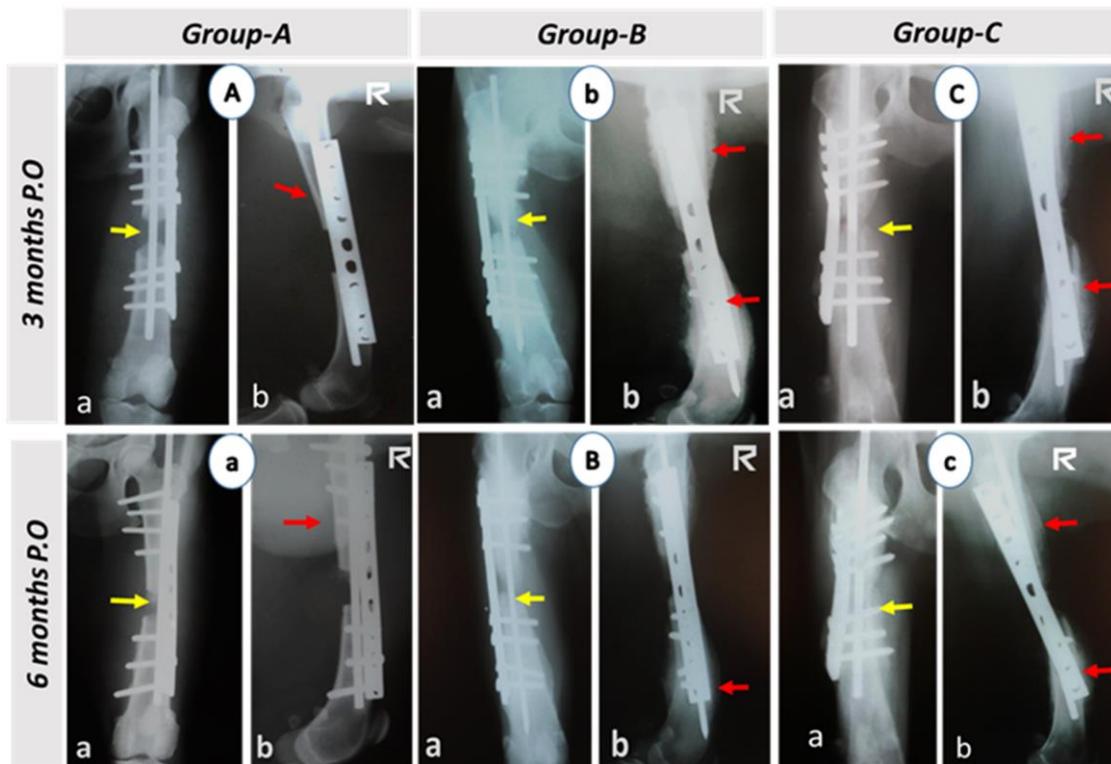

Figure 2: a; Craniocaudal and b; mediolateral radiographs of the operated femurs of groups A,B and C at 3 months P.O showing that the osteoperiosteal reactivity at both bone segments in case of groups B and C. the bone defect was clear radiolucent in group-A while in groups B and C it was more radiodense with variable degrees of closure with new osteophytes. At 6 months P.O the osteoperiosteal reactivity was decreased proximally and the intersegmental defect was almost disappeared in groups B and C. while in case of group-A the defect still obvious with increasing radiodensity at the proximal end.

At 16-22 weeks P.O: in all groups a reduction in the osteoperiosteal reaction (remodeling) especially at the proximal bone segment. In group-A there were non-observable radiographic changes except a little increase in gap radiodensity proximally. While the fracture gap in case of group-B was (about 40% filled with new osteophytes) but the gap was still detectable. But in case of group-C (50-60%) filled with newly formed callus that represented by high radiodensity and decreasing defect length all over that period.

At 24 weeks P.O: the osteoperiosteal reactivity was almost disappeared in proximal segment and still somewhat present at the distal one segment in all groups of animals. In case of group-A, the gap showed a relative increase in radiopacity especially at the proximal part. While in case of group-B; there was radiopaque area in the gap (filling about 50%). But in group-C the fracture gap was approximately filled with new callus (60-70%).

Morphohistologically: the examination of the harvested femora grossly **(Fig.3)** at the end of each respective study period revealed that:

At 1.5 months P.O: in group-A; there was connective fibrous tissue with osteoperiosteal reactivity covering the bone segments proximally and distally. The area of the intersegmental-defect was devoid from any reactivity except organized hematoma. There was no bone stability after plate removal. In group-B; the segments were covered with fibrous connective tissue extending and covering the defect. The segments were unstable after plate unloading. While in case of group-C; the segments with the defect were connected with fibrous connective tissue sleeve with some organization. The segments showed little stability after screws removal.

At 3 months P.O: the femora of group-A showed increasing of the fibrous reactivity which extending and covering the defect. The bone segments were unstable after removing of the plate. In group-B; the fibrous tissue sleeve was organized with osteoperiosteal reactivity. The defect was filled with semi-hard connective tissue, but the bone still somewhat unfixed. In case of group-C; the fibrous sleeve was decreased proximally. The defect was filled with semi-hard fibrous connective tissue. The bone was almost stable after plate removing.
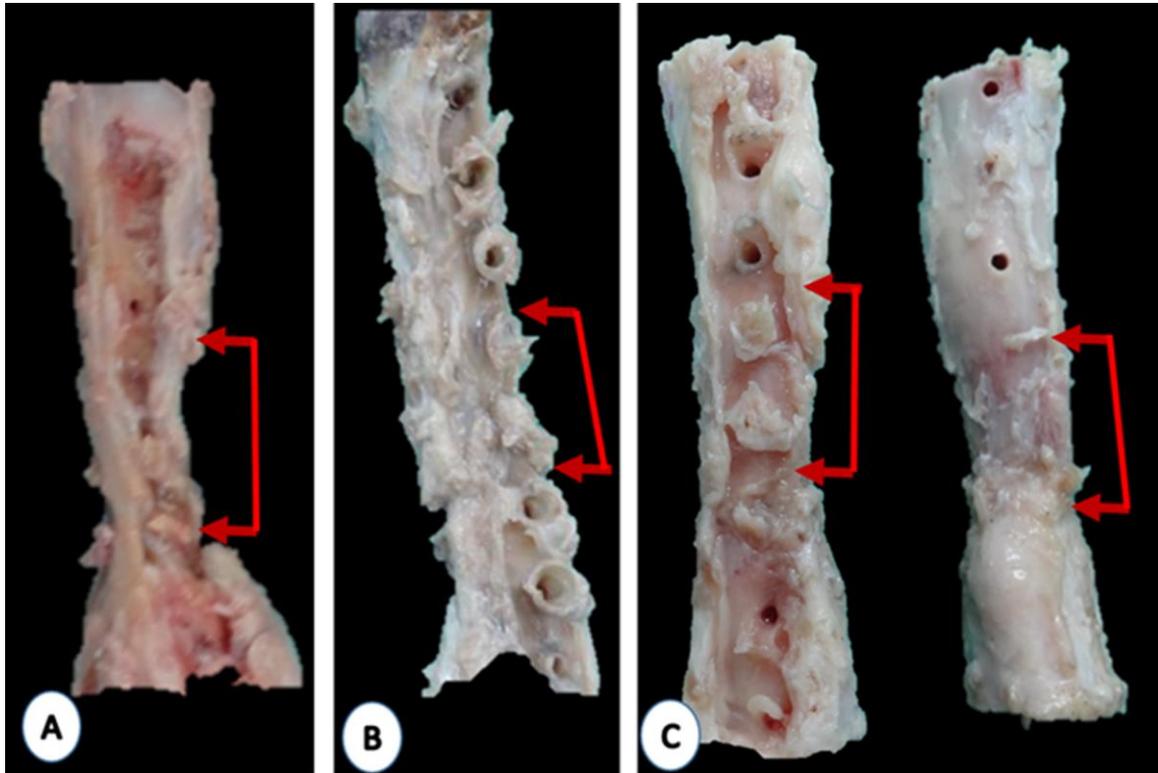

Figure 3: Morphological photographs of the harvested femora of A; group-A, B; group-B and C; group-C at 6 months P.O showing the intersegmental defect (arrows level). The defect is smaller than bone segments in group A while in group B and C the defect nearly the same size of the bone segments with osteoid tissue in group C.

At 6 months P.O: in case of group-A; the fibrous connective tissue was appeared covering the bone and the plate. The defect was filled with organized fibrous tissue. After plate removal; the bone showed elastic movement, but the segment was attached together. In group-B; the periosteal reactivity was covering the plate but decreased proximally. The segments were stable after plate unloading. While in group-C; the periosteal reactivity was almost disappeared proximally but still present at distal segment. The plate was clamped with fibrocartilagenous tissue. The bone-defect interfaces were not detected. The segments were stable with hard bony callus within the defect.

The microscopic examination of H&E stained tissue sections at the bone-defect interfaces **(Fig.4)** and at the defect area via different powers revealed that;

At 1.5 months or weeks P.O: in case of group-A; the microscopical field was filled with fibrous connective tissue (F.C.T) with some hemorrhages within the defect. Some inflammatory cells were found infiltrating the C.T fibers. In group-B; the fibrous tissue was organized with some inflammatory cells infiltration all over the field. While in case of group-C there were fibrocytes at different maturation stages and inflammatory cells. Few osteoblastic activity and vascularized F.C.T were found at the bone defect interfaces especially proximally.

At 3 months P.O: there were no changes in case of group-A except that, the F.C.T became more organized and vascularized. The inflammatory infiltration was decreased. In case of group-B, there was some osteoplastic reactivity at the proximal bone defect interface while organized C.T was filling the defect with the

presence of inflammatory cells. In group-C, the field showed more osteogenic reactivity at the bone-defect interface and within the defect. The F.C.T was still present and clear.
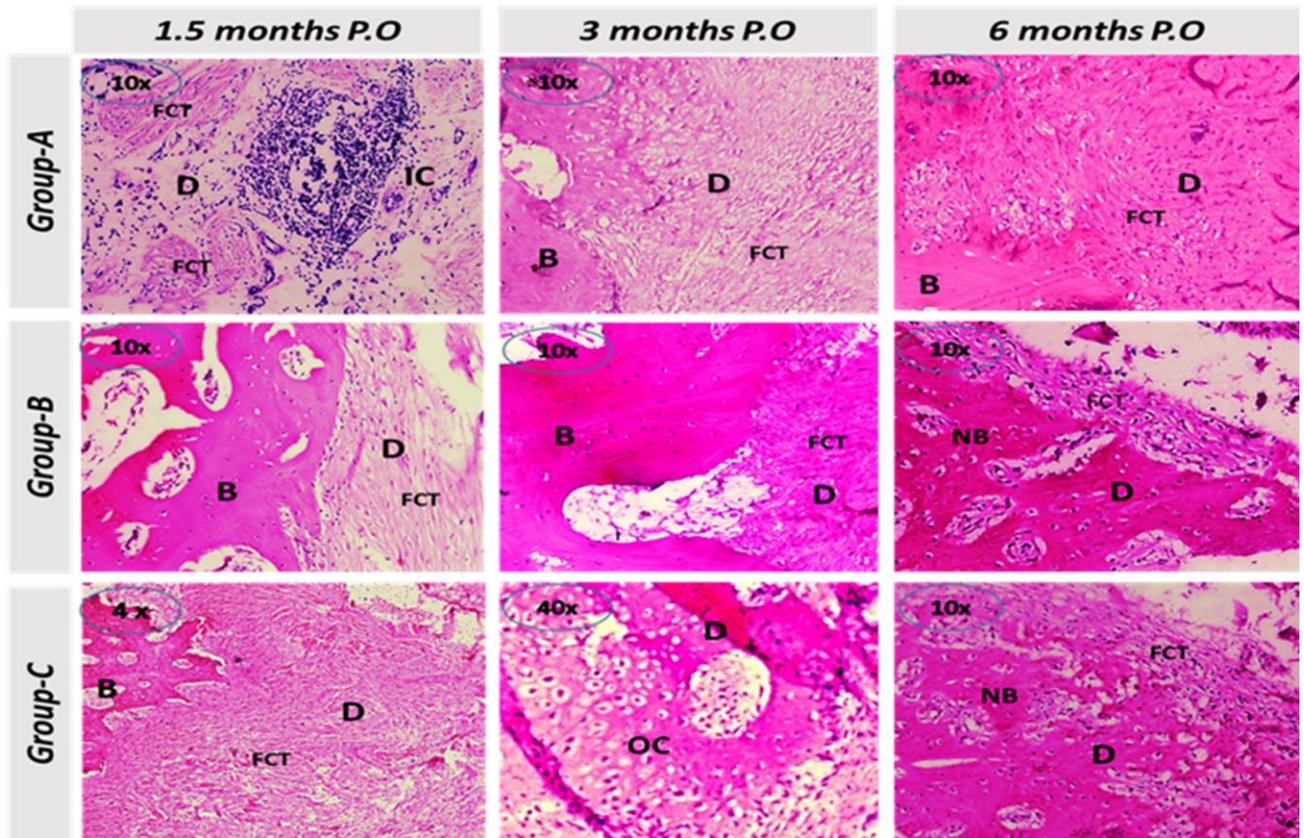

Figure 4: Microscopical photographs of tissue sections at the defect (D) level of groups A, B and C at different observation periods at 1.5 months the FCT and inflammatory cells were the dominant in group A. in group B and C the FCT become more organized and vascularized attached to host bone (B). At 3 months P.O group C showing osteogenic reactivity with some ossification centers (OC). At 6 months P.O the compact FCT is clear in group A, while in group B and C new bone tissue (NB) with increasing osteogenic reactivity is noticeable.

At 6 months P.O: in case of group-A; the examined slides showed compact organized fibrous C.T with some vascularization. No osteogenic reactivity was observed within the defect, it appeared at the end of the bone segments as small osteoblast aggregations. In group-B; the osteogenic activity was increased with the formation of trabecular bone at the proximal bone-defect interface. Different bone cells aggregations (ossification centers) were found inside the defect. Concerning the group-C; the plates showed mature bone cells with the defect. Bony tissue was observed at the bone-defect interfaces either proximally or distally. Few F.C.T was observed in between the newly formed bone tissue.

DISCUSSION

The rate of nonunion fracture is reported to be 3.4% of the total bone fractures in dogs. In nonunion fractures, the regeneration of bone is a major difficulty because the progression of fracture healing has ceased [20]. Recent developments in bone tissue engineering have been considerable and introduced as alternative solutions for bone regeneration instead of bone graft [21, 22]. Both cells and biomaterial components need to be optimized to produce a functional bone tissue-engineered therapy [23].

Chitosan has been extensively studied as a biomaterial for bone tissue engineering scaffolds, but in practice it is still used as a wound dressing and hemostatic agent in medicine. Several scaffold shapes and sizes can be successfully obtained by different processing techniques, which make Chitosan attractive for constructing scaffolds [3, 11].

Stem cells have shown to express their strong proliferative and regenerative potentials, which make it more important in tissue engineering based therapeutic approaches [24, 25]. Bone marrow contains not only mesenchymal fibroblast-like stem cells, but also a high amount of haematopoietic stem cells [26]. Yet, using cells alone has not solved the problem of nonunion fractures in which a wide gap exists. This requires the space to be filled with other space-filling-substance [19] (Scaffold) like Chitosan.

In the present study Chitosan and gelatin were conjugated producing Chitosan-gelatin mixture, which with glutraldehyde was molded into three dimensional, 2cm length scaffold simulating the femoral mid-shaft. The porous nature of the ch-gelatin, which favored its interaction with the seeded cells. In fact, the Chitosan-gelatin (Ch-gel) scaffold showed a capacity to the adherence and differentiation of mesenchymal stem cells, which was in accordance with previous in vitro and in vivo studies [9, 14, 27].

The BM-MSCs were preferably employed in this study; they were easily isolated from bone marrow, aspirated and expanded in vitro [15].

Some previous studies have demonstrated the effect of Chitosan on the bone healing and the formation process [28, 29]. Other studies have reported the biological enhancement of scaffolds with the addition of Chitosan and its influence over the osteogenic differentiation and bone regeneration; however, the mechanism of action remains unclear. This osteogenic character could be attributed to its porosity which traps the MSCs within to proliferate and exert its role in osteogenesis. Also, using Chitosan in combination with gelatin gives the adhesiveness to cells to be trapped inside the scaffold [3, 11].

In the present study a critical sized bone defect (≥ 2 cm) was created in the femur bone of the dogs, as at this length there is no osteogenesis or bone healing occur [30]. This defect was managed through either conventional fixation techniques only without scaffolding in Group-A (control) (n=9) or Ch-gel scaffold alone in Group-B (n=9), and implantation of MSCs loaded on Ch-gel scaffold in Group-C (n=9).

In our experiment, the femur bone was fixed as described earlier by the introduction of 3-4 mm Ø intramedullary bone pin occupying about 65% of the medullary canal. The fixation was augmented with 3.5 mm Ø DCP bone plate. The intramedullary pin increases the construct stiffness and estimated number of cycles to fatigue failure and plate bending when compared to a plate only construct. Mechanically, the intramedullary pin, replaces the transcortical defect that was in the contract with that was reported by of Hulse et al. [18, 31]; Reems et al. [32] in the treatment of femoral shaft fracture.

In the current study, the bone healing in animals was assessed via clinical, radiological and morphohistological evaluations. The clinical evaluation results of all animal groups were acceptable. The clinical evidence of infection or tissue reaction was not noticed as assessed by naked eye examination or palpation, monitoring the degree of swelling and surface temperature of the limb, popliteal lymph node size and absence of wound drainage. Except two animals, one in group-A and another in group-B showed sever seromal reaction with limb swelling that may be attributed to severe soft tissue dissection and improper dead space suturing, as they responded to alpha-chymotrypsin treatment. Most of the dogs in all groups 60-70% did tremendously well and bear weight on the operated limbs within 2-4 weeks post-operatively, while the full limb function was obtained by the end of 12 weeks post-operatively. These observations are in a good correlation with those reported by Sinibaldi [33] and El-Keiy et al. [34] for segmental cortical bone autograft and xenograft respectively and also Amer et al. [19] in case of amniotic membrane scaffold.

Assessment of bone healing is mainly depending on using conventional radiography in our experimental models to detect the degree of callus formation between the fractured ends, gap radiolucency and bone remodeling. Also, it provides a cheap and convenient technique for sequential assessment of the bone healing defect as reported by Sinibaldi, [33] and Lane and Harvinder, [35].

Increasing the gap radiodensity indicated new bone 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 remodeling phase of fracture healing was noticed also proximally former to distal segment. These observations were in agreement with that of Amer et al. [19] and El-keiy et al. [34].

The obtained results after sequential radiography showed a promising bone defect healing. The use of Ch-gel alone without MSCs Group-B gave moderate bone healing. On the other hand the use of a Ch - gel seeded with MSCs Group-C gave a promising bone healing as was reported by Miranda et al. [14] who used the porous Chitosan gel seeded with MSCs for alveolar bone regeneration. These results may be attributed to the osteogenic properties of Chitosan as a porous structure for osteogenic cell seeding and differentiation. The good healing results of Group-C could be as a result of addition of MSCs that have osteogenic properties. In general the aforementioned results were nearly similar to those obtained by Amer et al. [19] and Bruder et al. [25].

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. These results were in accordance with that reported by Amer et al. [19] and El-keiy et al. [34].

Gross examination of the harvested femora was performed before the histological investigation to confirm the radiographic findings at each successive observation period. The fibrous connective tissue (FCT) with the periosteal reactivity firstly was covering the bone segments proximally and distally and observed in all animals of all groups. Furthermore; the bone stability after plate removal was more in Group-C than other groups. The defect appeared filled with semi-hard fibrocartilagenous connective tissue. This may attributed to the differentiation of MSCs seeded on the Ch - gel scaffold into osteogenic cells in both Group-C and B that resulting in the osteoid tissue formation and hardening [19, 25]. Later on; the periosteal reactivity was almost disappeared proximally but still present at distal segment. The bone-defect interfaces were not detected. The segments were stable with internal hard callus within the defect in Group-C. These results in agreement with Amer et al. [19], Bruder et al. [24], Kirker-Head et al. [36] and Heino and Hentunen [37].

The histological examinations of tissue sections from harvested femora at different observation periods proved that Ch-gel scaffold either seeded with BM-MSCs or alone may have an osteoconductive and osteoinductive properties with some differentiations. These results in agreement with those obtained by Amer et.al. [19].

At 1.5 months P.O; there was variability in cellular type and differentiation in all groups. In case of Group-A and Group-B; organized FCT with some inflammatory cells infiltration were encountered all over the field. On the other hand in Group-C the fibrocytes was found at different maturation stages and fewer inflammatory cells. Few osteoblastic activity and vascularized FCT were found at the bone defect interfaces especially proximally. The advanced cellular differentiation in Group-C than other groups may be attributed to the presence of MSCs seeded on the used scaffold. These result were in accordance with those reported by Amer et al., [19].

The osteogenic activity was progressively increased with the formation of some trabecular bone in the proximal bone-defect interface with mature bone cell aggregations were detected within the defect intermixed with few FCT in case of Group-A than Group-B. The former histological findings with cellular differentiation may be attributed to the presence of BM-MSCs that were suggested to enhance bone tissue formation and cellular differentiation as reported by Amer et al. [19], Marie and Fromigue [37] and Heino et al. [38].

Concerning the morphohistological findings, one can say that, the use of Chitosan seeded with MSCs scaffold was found better than Chitosan alone in healing of critical size defect. These findings were nearly similar to those obtained by Sinibaldi [33]; El-Keiy et al., [34] in using the segmental cortical bone grafting and Amer et al. [19] in using amniotic membrane scaffold augmented with mesenchymal stem cells.

In conclusion, the clinical, radiological and morphohistological studies motivated together in assessment of the present study. It can be said that the rate of bone defect healing is a relatively a slow process due to the specific structure of bone tissue itself especially in case of critical bone defect. However, after the use of scaffold (Ch-gel) seeded with MSCs, the bone healing process was more stimulated and improved than the use of scaffold (Ch-gel) alone compared with the control group. The promising preliminary results of the experiment and the techniques of the operation may encourage the gradual use of Ch-gel

scaffold seeded with mesenchymal stem cells for treatment of critical size defect for bone healing in clinical cases.

Further long-term studied may be needed for more bone healing assessments. Also other studies concerning the immunogenicity and biomechanical properties of the scaffold should be conducted but work funding is limited.

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