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EVALUATION OF BONE HEALING BY MESENCHYMAL STEM CELLS IN RABBITS (AN EXPERIMENTAL STUDY)

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ABSTRACT

BACKGROUND: Large bone defects are considered a massive challenge in the field of oral and maxillofacial surgery. Cell Therapy using bone marrow-derived mesenchymal stem cells (BM-MSCs), as an alternative technique, has effective potentialfor bone regeneration.

AIM OF THE STUDY: To compare histologically the bone healing rate of critical size defects in rabbit tibia using mesenchymal stem cells versus untreated defects.

MATERIALS AND METHODS: Critical-sized defects were prepared on the tibia of rabbits. Experimental groups were divided into 2 groups: (a) Control group; untreated rabbits left for spontaneous healing, (b) collagen sponge with MSCs treated group. Sacrificing of rabbitswas done at 2 and 6-week intervals.

RESULTS:Bone defects treated with BM-MSCs showed asignificant increase in the healing rate compared to the control group.Histological examination showed immature bone trabeculae lined with plump active osteoblasts with minimal amount of newly formed blood vessels andalmost no inflammatory cell infiltration at the 2-week interval. Furthermore, at the 6-weeks interval the defects were almost healed with mature thickened dense bone trabeculae lined by fattened osteoblasts with no inflammatory infiltration.

CONCLUSION: BM-MSCs are a promissing tool for bone regeneration.

KEYWORDS: Mesenchymal stem cells, Bone healing, Rabbits, Critical size defect.

RUNNING TITLE: Evaluation of bone healing by mesenchymal stem cells.

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INTRODUCTION

The reconstruction of large bone defects poses a great challengefor oral and maxillofacial surgeons because of delayed or incomplete bone healing. When the bone defects exceed the critical size defect, they will be unable to heal spontaneously and will necessitate intervention. Critical size defect (CSD)is statedas the "smallest intraosseous defect that will not heal overthe lifetime of the animal"(1).

Trauma, infection, and tumor resection are major inducers of critical size defectswhich can result in severe maxillofacial dysfunctions and facial deformities. These conditions can dramatically lower the patients' quality of life(2,3) and require bone grafts for reconstruction(4).

The currently used clinical methods include the use of synthetic biomaterials, autografts, allografts, and xenografts. Even though bone reconstruction using autografts produces satisfactory outcomes, they are responsible for certain complications, such as infection at the graft resection site and donor site morbidity (5). Other alternatives like allografts or xenografts lack these adversities, but reveal other disadvantages like the risk of immunological problems, infection, and complicationswith the graft's mechanical and biological qualities (6).

To overcome the drawbacks of bone grafts, a number of experiments on substitute natural and synthetic materials have been carried out(7,8).These substitutes should carry certain properties,

includingbiocompatiblity, osteoinductivity, availability and structuralsimilarity to natural bone. Additionally, they have to pass both in vitro and in vivo safety and efficacy testing(9). Among these substitutes is bone tissue engineering, which has been considered as an innovative tool for the regeneration of tissues and the reconstruction of bone defects in the oral and maxillofacial region(3,10). Initially,BM-MSCs were differentiated toward chondrogenesis for tissue engineering purposes by Johnston et al. and later others used it for osteogenesis as well(11,12). In canine animal models, mesenchymal stem cells have demonstrated efficacy in promoting bone formation (13).

Depending on their intended use, scaffolds for tissue engineering applications must adhere to specific requirements.These scaffolds may beeither natural or synthetic biodegradable materials. The natural scaffolds possess the advantage of biocompatibility and bioactive behavior that can be effective in damaged tissue regeneration. They still have a number of drawbacks, though, including immunogenicity and poor mechanical qualities.Despite synthetic biomaterials lack these drawbacks; they have got their own disadvantages like the lack of ability of biological adhesion(14).

We used the collagen natural scaffolds for the purpose of this study. Collagen was thought to act only as structural support in the healing process; nevertheless, it is now clear that it controls many cellular functions, including protein synthesis, cellularproliferation, differentiation and migration(14).

The proposed hypothesis in this study is that collagen sponge scaffolds seeded with BM-MSCs would accelerate and improve the bone healing quality.

The aim of this study is to evaluate the effectiveness of the mesenchymal stem cells in the formation of new bone histologically and histomorphometrically.

MATERIAL AND METHODS

Ethical considerations

The study was performed after gaining the approval of the Research Ethics Committee, Faculty of Dentistry, Alexandria University with number IORG 0008839-0403-0212022.

The rabbits were preserved under normal ventilated laboratory circumstances of temperature (22-25^{o}C). Rabbits were housed in cages fed by standardized suitable food and tap water. Daily replenishments of the standard diet regimen were made during the duration of the experiment.All operations were achieved with minimal or no stress(15).

Study Design and sample size estimation

The current study was an experimental animal study. The purpose of this experimental study was to evaluate the effectiveness of BM-MSCs in the formation of new bone. The study was held in two phases: the surgical phase, where critical size defects were performed on the rabbits' tibiae and the seeding phase, where BM-MSCs were loaded on a collagen scaffold and implanted in the critical size defect.

This study included 16 New Zealand white male rabbits with a mean age of one year and weighing 3.5-4 Kg with no systemic diseases as well as excluding overweight/underweight, illness/wounds and rabbits that are younger than one year of age. Animals were acquired from the animal house of the Medical Research Institute, Alexandria University.

The sample size was calculated using GPower version 3.1.9.2 and suggested a sample size was to be 8 specimens per group (number of groups=2) (Total sample size=16 specimens) (16).

BM-MSCs isolation

Mesenchymal stem cell isolation, culture and characterization and surgeries that include transplanting the MSCs as well as the histological procedures were performed in the laboratory of the Center of Excellence for Research in Regenerative Medicine and Applications (CERRMA).

BM-MSCs isolation was conducted using 4 MaleSprague Dawley rats(weighed 30-40 g). Rats Bone marrow was flushed from rats' femurs and tibias and cultured in growth media low glucose DMEM Minimum Essential Medium (Lonza, Belgium) with 10% fetal bovine serum (FBS), (Thermo Fisher Scientific, USA), 100 IU ml-1 penicillin and 100mg ml-1 streptomycin (Thermo Fisher Scientific, USA) at 37C in a 5% CO_2incubator till passage 3. For the characterization of cells,immunophenotypingwas done using fluorescent-labeled monoclonal antibodies (mAb) surface markers for CD44, CD73, CD105, CD90 and CD45 (Abcam, Cambridge, UK). Finally, immunofluorescence on cells was analyzed using Becton–Dickinson, FACS caliber flow cytometer equipped with Cell Quest software(14).

Preparation of collagen scaffold

Collagen Plug(RSP1-S) as scaffold (Regenomer@ NIBEC Co., Ltd, South Korea) which is a self-expanding, biodegradable collagen matrix made of porcine atelocollagen (a low-immunogenic derivative of collagen obtained by removal of N- and C-terminal telopeptide components(17), which are known to induce antigenicity in humans. Telopeptides are removed by treatment of collagen with type I pepsin(18).They were adjusted to the size of the defects.

Surgical procedure

The surgical procedureswereexecuted under general anesthesia. All the rabbits were anesthetized by intramuscular injection of (0.15-0.20mg)/Kg ketamine

plus (1-2mg)/Kg lidocaine(19). The surgical area in the right tibiae of all rabbits was shaved before any procedure and the skin was scrubbed with 2% povidone-iodine to avoid contamination.

The rabbits were split into two groups: the control group with absenceof intervention to the induced bone defect, and the collagen scaffold withBM-MSCsgroup where defects were filled with scaffold seeded with 1x 10^6 MSCs.

After anesthesization, a 4-5cm incision was made by surgical blade number 10 in the medial aspect of the rightleg including skin and periosteum. Flap was reflected to expose the right tibia using a periosteal retractor(20). Critical size bone defects 6mm in diameter(21) were made using sterile surgical bur (Trephine bur size (SDTRB-06) 6.0mm, Trimmer kit) with profuse saline irrigation to protect bone from heat generation. The defects were either filled with BM-MSCs seededcollagen scaffolds; or left without treatment. The surgical areas were sutured by 000 nonabsorbable braided sutures.All rabbits received the same course of antibiotics amoxicillin 1gm/Kg body weight every eight hours for five days.

Histological examination

The rabbits were euthanized with an overdose of ketamine at each of the experimental periods 2, and 6 weeks postoperatively. The tibia specimens were obtained. The defect area was dissected out and processed for light microscopic examination. Rabbits were disposed ofby burning by special authorities(22). Specimens were fixed in 10% neutral buffered formalin, washed, and then decalcified in 10% trichloroacetic acid. Following washing, the specimens were dehydrated in increasing alcohol grades, cleared in xylene, and infiltrated and embedded in paraffin wax. Serial sections of 5 µm thick were cut from the paraffin blocks using a rotary microtome and stained with hematoxylin and eosin(23) for qualitative assessment of new boneformation in the defect area.

Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) Quantitative data were described using range (minimum and maximum), mean and standard deviation. Significance of the obtained results was judged at the 5% level. The used tests was Student t-test for normally distributed quantitative variables, to compare between two studied groups.

RESULTS

At the 2-week interval, the histological examination of the control group revealed emptybone defectsreplaced with loose areolar fibrous tissue that displayed thin-walled newly formed dilated blood vessels engorged with RBCs. The surrounding delicate stroma was diffusely infiltrated with inflammatory cells (Figure1 a). At the 6-week interval, despite the diminished vascularity seen in the control group, the bone defect revealed the failure of the healing process, where the defect was almost empty, albite the presence of few thin curved disorganized bone spicules with the persistence of the inflammatory infiltration (Figure1 b).

The healing process in the bone defects treated with bone-marrow derived stem cells was accelerated at the 2-week interval, where the bone defects displayed immature bone trabeculae lined with plump active osteoblasts. The surrounding bone marrow showed a minimal amount of newly formed blood vessels with almost no inflammatory cell infiltration (Figure2 a). With elapsed time, the osteogenic differentiation capabilities of the bone marrow-derived mesenchymal stem cells were accentuated. At a 6-week interval, the bone defects were almost healed with mature thickened dense bone trabeculae lined by fattened osteoblasts and enclosing organized osteocytes seen in their lacunae. The bone marrow was devoid of inflammatory infiltration. Interestingly, stem cells'capacityfor differentiationinto chondrocyte lineage was observed in some defects at 6-week with formation of mature cartilage (Figure2 b). There was a significant difference between the control group and the BM-MSCs group in both the 2-week and 6-week intervals(Table 1 &Figure 3-5).

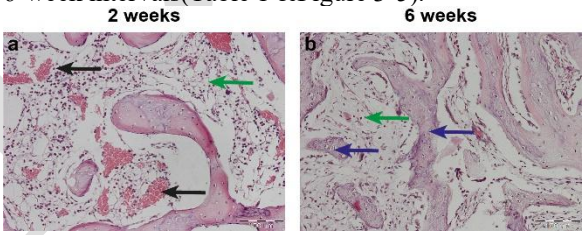

Figure1: H&E-stained photomicrographs of the control group: (a) at 2 weeks (b) at 6 weeks. The black arrows point to the dilated blood vessels engorged with RBCs. The green arrows show the inflammatory infiltration. The blue arrows denote the bone spicules in the bone defects. Scale bar = 100 µm.

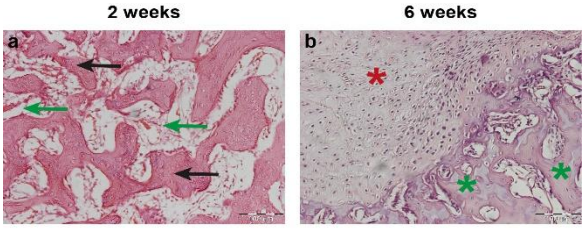

Figure 2: H&E-stained photomicrographs of the bone marrow mesenchymal derived stem cells: (a) at 2 weeks (b) at 6 weeks. The green arrow denotes the blood vessels, the black arrows reveal the newly formed bone trabeculae in the defect area, while green asterisks denote the mature bone trabeculae. The red asterisk points to the cartilage. Scale bar = 100 µm.

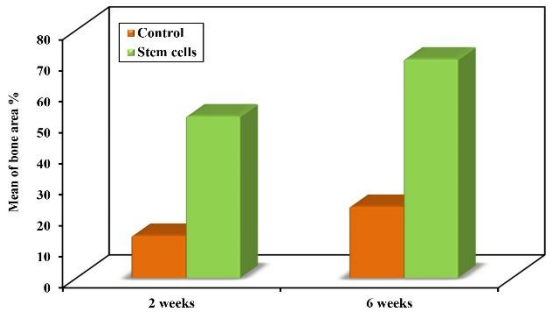

Figure 3: Comparison between the two studied groups according to bone area %

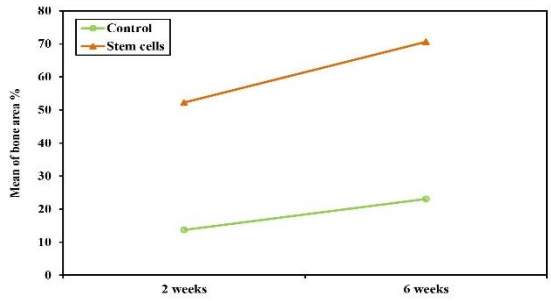

Figure 4: Comparison between the two studied groups according to bone area %

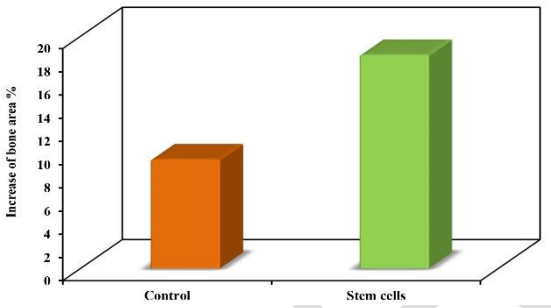

Figure 5: Comparison between the two studied groups according to increase of bone area %

Table (1): Comparison between the two studied groups according to bone area %

Bone area %	Control	Stem cells	t	p
2 weeks Min. – Max. Mean ± SD.	12.68 – 15.21 13.68 ± 1.10	50.94 – 54.06 52.24 ± 1.33	44.735*	<0.001*
6 weeks Min. – Max. Mean ± SD.	20.69 – 25.90 23.07 ± 2.08	66.90 – 74.70 70.63 ± 3.19	24.965*	<0.001*
Increase	9.40 ± 2.42	18.40 ± 3.82	3.982*	0.007*
p_0	<0.001*	<0.001*		

4 replica for each group
SD: Standard deviation
t: Student t-test
p: p value for comparing between the studied groups
p_0: p value for comparing between 2 weeks and 6 weeks in each group
*: Statistically significant at $p \leq 0.05$

DISCUSSION

Owing to the existence of intricate physiological systems such as facial skeletal muscles,cartilage, sensory organs, arteries, veins and lymphatic vessels, engineering the maxillofacial bones faces many challenges including the restoration of aesthetics, adequate mechanical strength, speech-related mobility and masticatory processes(24).Additionally, clinicians must manage bacterial contamination in particularly vulnerable locations, such as the nasal and oral regions.Rigid fixation, bone grafts, microvascular free tissue transfer and other conventional techniques used to repair bone abnormalities in the craniofacial region showed promise in smaller defects. For larger lesions however those techniques have substantial morbidities and are not usually effective for more complicated reconstructive issues(25).

Nowadays,there are many promising future developments for the repair of craniofacial inadequacies.The advancement of research in the field of bone augmentation has greatly aided in the acceptance of tissue engineering as a therapeutic choice in dentistry for conditions involving soft tissue, alveolar bone, and dental implants (26,27).

Bone tissue engineering, a potentially effective substitute technique for bone abnormalities, produces a bone grafting material with osteogeneic, osteoinductive, and osteoconductive capabilities(28). MSCs are a preferred candidate for bone regeneration among adult stem cells simply due to their capacities formultipotency, acquiring immunomodulatory qualities, and releasing trophic factors (29). There are many sources where we can obtain them including adipose tissue and bone marrow. Although Adipose tissue-derived stem cells are easier to obtain from liposuction of human fats, according to several studiesBM-MSCs showed greater in vivo capacity in bone regeneration(30).

Biomedical scaffolds have been made from both natural and artificial materials. Synthetic materials are quickly manufactured and have great mechanical qualities, but their low biological activity when compared to natural materials is a major downside(31).

Therefore, in this study, as a scaffold we have used a collagen spongeplug (Regenomer@ NIBEC Co., Ltd, South Korea).It is generally recommended for the filling of extraction sockets and periodontal defects, in

addition it has shown to have superior functionality and physical/mechanical properties, ideal for cell/tissue culture and cell functional assays.

First, we applied the protocol to isolate and culture rats' BMSCs and then we seeded the cells onto the collagen sponge carrier and placed them into critical-sized defects in the tibia of rabbits comparing them to a control group with empty defects on a span of 2 and 6 weeks and we evaluated the H&E stain quantitative histological results.Critical size defectin rabbit tibia have been described by various studies(32)according to which we decided to go with 6 mm in diameter.

Histological examination was done for qualitative evaluation using hematoxylin-eosin (H&E) and observed under the light microscope; showing the defects filled with BMSC with accelerated healing at 2 weeks while at 6 weeks the defects were almost completely healed compared to non-healed defects in the control group.

An essential component of healing is the inflammatory process. It is present from the beginning, drawing in precursors for the tissue regeneration, and as the process progresses, it gradually reduces its activity. Still, extended inflammation retards the healing process(33).

The control group showed inflammatory indications at the 2-week interval, thin-walled newly formed dilated blood vessels engorged with RBCs with inflammatory cells infiltrate along loose areolar fibrous tissue.

Despite its fading at the 6-week interval the bone defect revealed failure ofthe healing process which shows the effect of prolonged inflammatory response. While the treated group displayed minimal amount of newly formed blood vessels with almost no inflammatory cell infiltration at the 2-week interval, the bone marrow was devoidof inflammatory infiltration at the 6-week interval where the defects were almost healed with mature thickened dense bone trabeculae lined by fattened osteoblasts and enclosing organized osteocytes seen in their lacunae.

It has been documented by some authors that chondrogenic differentiation can contribute bone regenerative capacity of MSC resulting in bone formation through endochondral ossification(34,35) whichwas interestingly observed in some defects at 6-weeks with formation of mature cartilage delineating the capacity of stem cells to differentiate into chondrocytes lineage.

Mesenchymal stem cells (MSCs) have become a promising technique for bone regeneration. Their low immunogenicity, accessibility, and potential for differentiating turn them into a compelling therapeutic toolby promoting cell migration and angiogenesis. Their reparative power is credited to two basic mechanisms; first is cell engraftment, involving the differentiation of cells into the phenotype of damaged tissues and second is through cell empowerment knows as the paracrine effect, that depends on the secretomes of these cells, specifically extracellular vesicles (EVs)(14).

Some studies demonstrated that the formation of new bone may not be solely credited to the osteogenic differentiation of BMSCs as paracrine signals might have stimulated bone regeneration via various mechanisms(36), while others suggested that the functional developments are solely driven by paracrine effects rather than by engraftment(37).

Further investigation of the secretome for its effect through paracrine signals on bone regeneration are needed as Abolgheit et al. demonstrated its effect in skin wound healing(14).

Although the great drawback of MSCs is that they must be cultivated andcultured before being used, the use of MSCs competes with autologus grafting as technique of choice described by some authorsas the "platinum standard" for bone regeneration comparing their results to autologus bone grafting(38).

Studies on the application of autologous bone in combination with stem cells have the potential to set a gold standard for bone regeneration by supplyingthe necessary scaffolding for stem cells, which in turn offer the best conditions for the formation of new bone. It will require more clinical trials to standardize their use. Stem cell therapies are presently only used inlow-prevalence cases due to their high cost compared to other techniques. Consequently, it is essential to conduct new research toimprove processes and enable the routine use of stem cell treatments(39).

CONCLUSION

It was proven that BM-MSCs that were loaded on collagen sponge scaffold have an effective role on bone healing mainly by osteogeneic differentiation. The collagen sponge scaffold showed great biocompatibility.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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