Assessment of Healing in Calvarial Bone Defect by Allogenic Demineralized Bone Matrix and Adipose Derived Stem Cells

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ABSTRACT

Background: The critical size calvarial bone defect is a challenging problem in the craniomaxillofacial field. Till now, the golden standard for its reconstruction is the autografts which harbors multiple drawbacks as availability and morbidity. So, the use of Allogenic Adipose Derived Stem Cells seeded on Demineralized bone matrix (DBM) scaffolds offer an already made new tissue regenerate that can be stored in bone banks and used when needed.

Material and Methods: 32 adult male albino rats with surgically created Calvarial bone defect (8mm) in the Rt. Parietal bones were divided into 4 groups; group I, control; group 2; reconstructed by DBM; group III; ADSC seeded on DBM and group IV; ADSCs seeded on prolene mesh. Evaluated 8 weeks post-operative by Gross Evaluation Score (proposed in this study), 3D CT scans with objective analysis by a software (ImageJ 1.47v) and histologically.

Results: Bone healing is evident 8 weeks post implantation with bone formation 75-100% in 62.5% of the defects that is reconstructed by ADSCs and DBM.

Conclusion: This study presents a beneficial method for reconstruction of critical size calvarial bone defects by an already made non-immunogenic new tissue regenerate.

Key Words: ADSCs – DBM – Allogenic – Stem cells – Critical size defect – Calvarial-bone healing.

INTRODUCTION

The Craniomaxillofacial surgeons are facing frequently critical sized calvarial bone defects that present a challenge for reconstruction [1]. It can result secondary to congenital or acquired causes like; infection, trauma, post tumor excision or deforming skeletal diseases [2].

Multiple treatment options are available to solve this problem. It can vary from bone grafts, bone substitutes or bone transport methods [2]. But till now the golden standard is the autograft. They offer minimum immunological rejection, complete histocompatibility and can provide the best osteoinductive, osteoconductive and osteogenic prop-erties [3]. The limitations in using autografts are the limited graft availability, bone resorption and the need for an additional surgery with resulting donor site morbidity [4].

Bone allograft is another option. It is obtained from a cadaver of the same species. It has osteoinductive ability by releasing bone morphogenic proteins (BMPs). It has also osteoconductive properties but lacks the osteogenic properties due to the absence of viable cells [5]. Despite its wide availability in various shapes and sizes without sacrificing host structures and extra donor morbidity. It fails to meet the reconstruction needs due to the difficult preservation and possible infection transmission [6,7].

A further step forward in bone reconstruction was the bone tissue engineering (BTE). Tissue engineering is to restore damaged or degenerated tissues with a functional living construct from cell development [8]. It is a new field in the bone reconstruction armamentarium that builds up a new thinking for bone replacement without extraneed for the traditional bone grafting surgeries with its complications [9].

Basically, a triad of stem cells, scaffold and growth factors should interact to regenerate a new bone reconstruct [9,10]. Multiple studies in this field were greatly described to synthetize a novel tissue engineered bone with multiple designs by seeding diverse types of stem cells on different scaffolds.

PATIENTS AND METHODS

This study was done between October 2014 and December 2017. It was conducted at The Medical Research Center associated with Ain Shams' Faculty of Medicine and approved from

the Research Ethics Committee (REC) of Faculty of Medicine, Ain Shams University (No: FMASU 1969/2014).

A total of 42 Male albino rats were used after approval of CARE (Committee on Animal Research and Ethics), Ain Shams University, Faculty of Medicine. It was divided into two models: Model 1: (Donor rats for adipose derived stem cells); 10 rats of young (5-6 weeks) male albino rats were used. Model 2: 32 adult (6-7 months) male albino rats weighing 260-340 grams were divided into four groups; Group I (n=8): Control group where the surgically created calvarial bone defect will be left without repair; Group II (n=8): Reconstructed by allogenic DBM without cell seeding; Group III (n=8): Allogenic DBM seeded by ASCs; Group IV (n=8): Reconstructed by prolene mesh seeded by ASCs.

- *Preparation of prolene mesh Scaffold:*

A prolene mesh sized 30x30cm was cut into small, symmetrical, circular Segments (scaffolds) of 8mm diameter by using 8mm biopsy punch (Fig. 1). Each segment was separately sterilized and stored.

- *Preparation of DBM Scaffold:*

Rats were anaesthetized using intramuscular injection of ketamine (1-2 mg/kg) which was maintained as required. Under sterile conditions, the surgical incision site (Right parietal area) was shaved with razor and lubricant then scrubbed by povidone iodine and 70% alcohol and the rat was draped by a disposable waterproof drape. A 2cm incision was made along the sagittal suture, the skin; musculature and periosteum were dissected to expose the Right parietal bone. A circular defect measuring 8mm diameter was then made using the 8mm biopsy punch with care to avoid injury to the dura matter. The bone harvested from Group I (control) and Group IV (reconstructed by prolene mesh and ADSCs) were demineralized and used as a donor for Group II (reconstructed by DBM without cell seeding) and III (reconstructed by DBM with cell seeding) respectively. Demineralization was done by immediate bone transfer into a solution of (0.6 N HCL) for 72 hours at 4ºc. The HCL was changed every 24 hours. The acid was then removed by washing the bone segments with distilled water for 8 hours with continuous stirring [23]. Each segment will be separately stored in small plastic tubes at minus 70ºc while still immersed in alcohol (Fig. 2).

In this study, an 8mm biopsy punch is preferred than trephine burr due to many reasons. It is easier,

accurate, disposable and less expensive especially when compared with the trephine burr. It also preserves the extracted bone as a block that can be demineralized and reused as a scaffold with the same defect dimension so, no need for hardware fixation.

- *Harvesting inguinal pad of fat:*

Two bilateral inguinal folds Incisions were done. The inguinal bad of fat were carefully dissected, isolated and immediately transferred to a sterile petri dish. The wounds are closed with a 4.0 absorbable suture (vicryl) (Fig. 3).

- *Tissue processing:*

The harvested fat was washed with phosphate buffered saline (PBS) and cut into pieces of approximately 1-2mm diameter inside a laminar flow. The tissue will then be rinsed three times in PBS for 5 minutes.

Isolation of ADSCs:

The minced fat was digested by adding a 0.2% collagenase type I and vigorous constant shaking in a water bath shaker for 40 minutes at 37ºC. The collagenase effect is neutralized by equal volume of complete culture medium (CCM). The CCM is reconstructed by adding 500mL DMEM, 65mL FBS (final conc. 13%), and 1.5% antibiotic and anti-mycotic mixture Penicillin G (10,000 units /mL), streptomycin sulfate (10,000µg/mL) and Amphotericin B (25mcg/mL) in a solution of 0.85% NaCL (modified by medical research center team instead of 10% FBS & 1% antibiotic/antimycotic as described by Lu et al., 2008) [24]. CCM is filtered through 0.22µm sterile filter unit then divided into aliquots and stored at 4ºC. Before the experiment, the aliquots were warmed to 37ºC. The cell suspension was centrifuged. The formed cell pellet was re-suspended in a 10ml of CCM to lyse the red blood cells.

Culture:

Cell pellet will be cultured in a culture flask 25cm^2 with CCM in the CO_2 Incubator at 37ºc, 5% CO_2 and 100% RH. The medium was replaced every 72 hours. The non-adherent cells were discarded, and the adherent cells were washed by PBS.

Expansion:

The cell expansion was followed by the inverted microscope. The cells were harvested at 80 to 90% confluence at the 12th day from passage zero (Fig. 4).

The cultured cells were detached from the culture flasks with 0.25% trypsin-EDTA and micro-scrubber.

- *In vivo implantation:*

The scaffolds (DBM and prolene mesh) wet by DMEM solution were seeded by a $3x10^6$ cells. Group III with surgically created calvarial bone defect was repaired by allogenic DBM with cell seeding. The dimensions of the defect and the DBM are equal so, press fit is enough for fixation. Group IV was reconstructed by prolene mesh seeded with ADSCs (Fig. 5).

Postoperatively, Analgesics and antibiotics were administered. Wounds were followed daily for signs of inflammation, disruption, hemorrhage and exudation. Euthanasia was done 8 weeks post implantation.

- *Biopsy harvest:*

The biopsies were taken as blocks containing the reconstructed defect and a rim of the surrounding calvarium.

- *Evaluation:*

In this study, 3-objectively based analytical steps was used to evaluate the results; Gross evaluation at autopsy, Radiological assessment by 3D CT scans with software analysis for the surface area of the newly formed bone (ImageJ 1.47v, National Institute of Health, USA) and histological evaluation.

Gross evaluation at autopsy:

The reconstructed defects were exposed and examined. there no universal guidelines for data analysis in a well settled rating scale. So, in this current work, a proposal for a scoring system for gross evaluation of healing at autopsy is offered. It includes the four main parameters which is subdivided into multiple rating points covering the entire range or shades of healing possibility in a numerical scale in which the minimum is 4; indicates the worst healing and the maximum is 11;

the best. This score was extremely helpful in this study (Table 1).

Table (1): Gross evaluation score for evaluation of bone healing at autopsy.

Gross evaluation score at autopsy	
Palpation:	
No healing	1
Soft healing	2
Hard healing	3
Transillumination:	
Trans-illuminant	1
Non-trans-illuminant	2
Mobility with pressure:	
Mobile	1
Minimal mobility	2
No mobility	3
Integration with the surrounding bone:	
Absent	1
Incomplete	2
Complete	3
Score: (/11) Minimum 4	
Maximum 11	

Radiological Evaluation:

A 3D reconstructed CT Scanning was performed for the heads of all study groups immediately after euthanasia using a CT scanner (SOMATOM® Definition Flash 128 Dual source, Siemens Medical Solutions, Germany). The relation between the surface area of the newly formed bone to the surface area of the surgically created defect was analyzed using a software (ImageJ 1.47v, National Institute of Health, USA) (Fig. 6).

Histological evaluation:

The specimens were fixed in 10% formaldehyde for 2 days, decalcified in 25% H_2SO_4 for 10 days and mounted in paraffin. Serial sections 5µm-thick were cut by a microtome in the sagittal plane and including the defect and rim of the surrounding calvarium then stained with hematoxylin and eosin and examined by light microscopy to evaluate the qualitative bone healing.

Fig. (1): The preparation of prolene mesh; Prolene mesh was cut by punch into multiple small pieces (scaffolds).
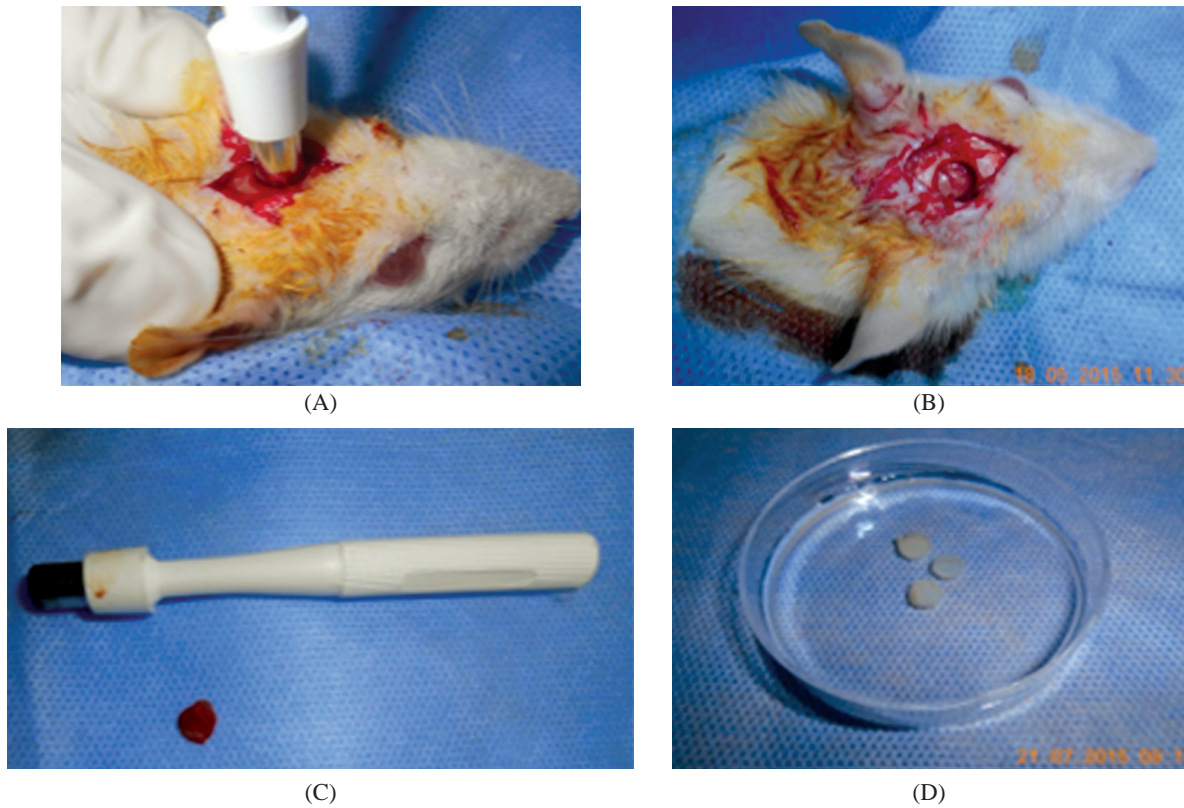
(A)

(B)

(C)

(D)

Fig. (2): Harvest of an 8mm circular bone from rats calvaria; (a) Circular bone excised by 8mm biopsy punch. (b) Dura intact after bone harvest. (c) Harvested bone. (d) The Demineralized bone scaffolds are immersed in alcohol.
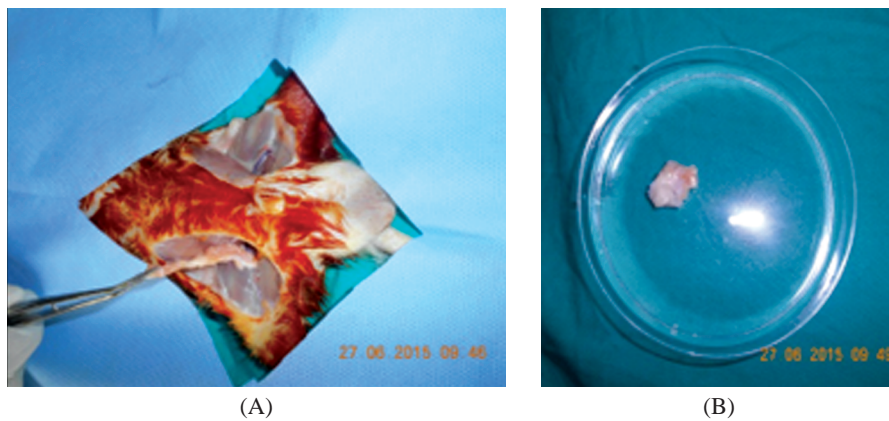

(A)

(B)

Fig. (3): Harvest of inguinal pad of fat: (a) Both inguinal folds are incised and dissected with isolation of the inguinal pad of fat. (b) The harvested inguinal pad of fat collected in a petri dish.
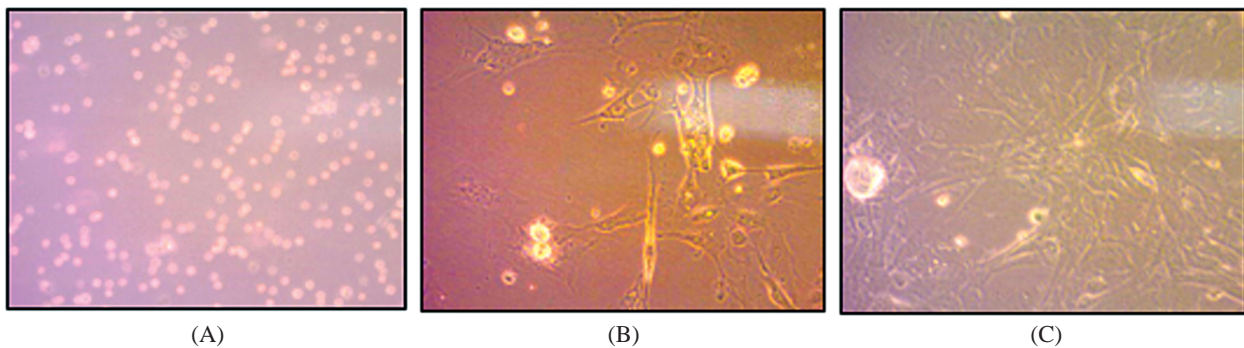

(A)

(B)

(C)

Fig. (4): Cell Expansion (X100) under inverted microscope; (a) Passage zero; the cells appearance under the inverted microscope (b) Day 3: The cells appeared as spindle shaped cells (c) Day 5: The cells are more confluent.
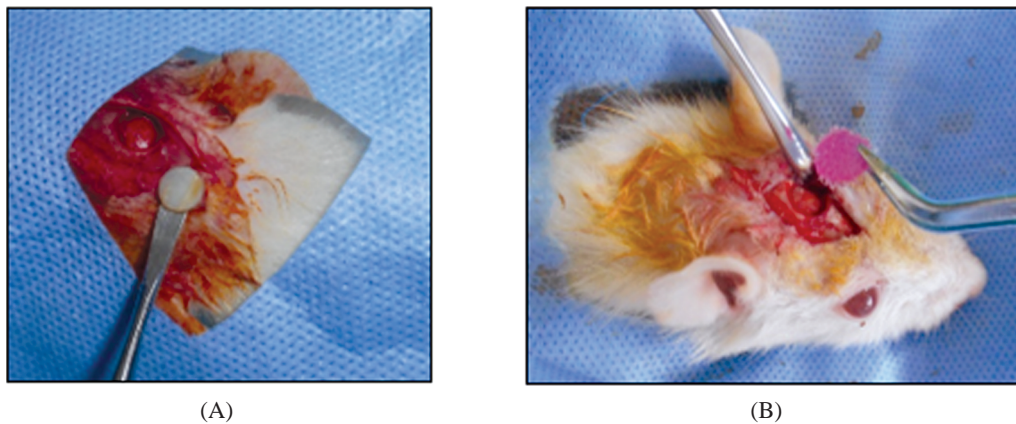
(A)   (B)

Fig. (5): In vivo implantation; (a) Group III: Allogenic DBM seeded with ASCs; (b) Group IV: Prolene mesh seeded with ADSCs.

(A)   (B)   (C)
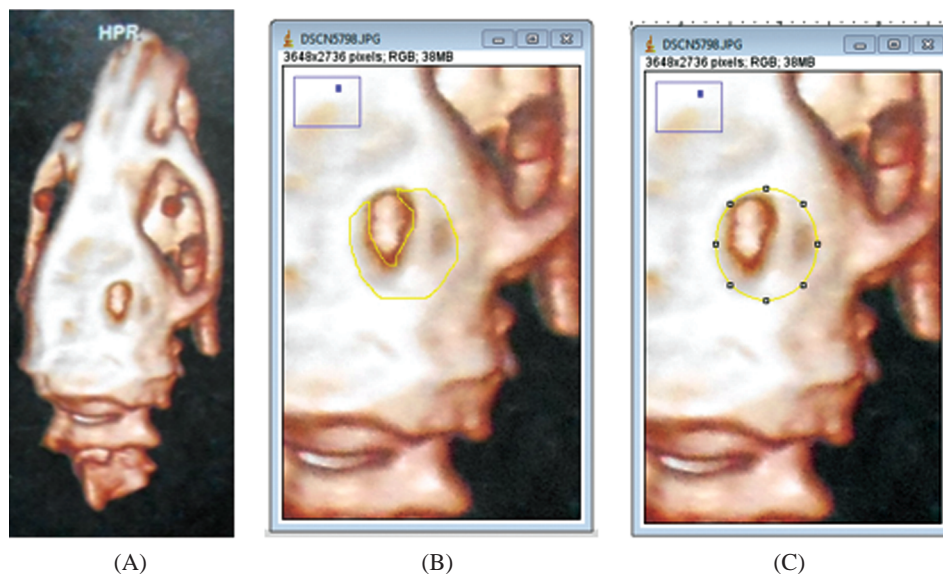
Fig. (6): A- 3D CT scan for group IV. B- The surface area marking of the newly formed bone by ImageJ 1.47v. C- The surface area marking of the defect by ImageJ 1.47v.

RESULTS

The rat's survival at all studied groups (Model 2) was uneventful apart from Four mortalities during surgical creation of the critical size defects (1 in group I, 1 in group II and 2 in group IV). Theses rats were replaced.

Statistical methods:

The collected data were coded, tabulated, and statistically analyzed using IBM SPSS statistics (Statistical Package for Social Sciences) software version 22.0, IBM Corp., Chicago, USA, 2013.

Descriptive statistics were done for quantitative data as minimum & maximum of the range as well as means (standard deviation) for quantitative normally distributed data, while it was done for qualitative data as number and percentage.

Inferential analyses were done for quantitative variables using independent *t*-test in cases of two independent groups with normally distributed data. In qualitative data, inferential analyses for independent variables were done using Fisher's Exact test for differences between proportions with small expected numbers. The level of significance was taken at *p*-value <0.050 is significant, otherwise is non-significant.

A- *Gross evaluation at autopsy:*

Gross evaluation score was highest in group III (10.5/11), followed by Group II (10/11), then group IV (9.3/11) and least in group I (2.1/11). Group III was significantly higher than groups I& VI (Table 2) (Fig. 7).

Table (2): Comparison between study groups using a gross evaluation score. Group III is significantly higher.

Findings	Group-I	Group-II	Group-III	Group-IV
Mean ± SD	5.1±0.8	10.0±0.8	10.5±0.8	9.3±1.2
Range	4.0-6.0	9.0-11.0	9.0-11.0	8.0-11.0
Comparison between group-III and other groups				
		Group-I	Group-II	Group-IV
p		<0.001*	0.207	0.023*
Difference:				
Mean ± SE		5.4±0.4	0.5±0.4	1.3±0.5
95% CI		4.5-6.2	−0.3-1.3	0.2-2.3

#Independent *t*-test.
Difference: Group-III relative to other groups.
CI : Confidence interval.
SD: Standard Variation.
SE: Standard error.

Fig. (7): Comparison between study groups using a gross evaluation score.

B- *Radiological evaluation:*

a- *Radiological Extent of bone healing:*

High grades of bone formation were most frequent in group-III (62.5% healing of 75-100% of the defect and 37.5% healing of 50-74%), followed by group-IV (62.5% healing of 25-49% of the defect and 37.5% healing of 50-74% of the defect), then group-II (12.5% healing of 25-49% of the defect) and least in group-I (no healing). Difference were significant with all groups (Table 3) (Fig. 8).

Table (3): Comparison between study groups regarding radiological extent of bone healing. Group III is significantly higher.

Extent of Bone formation	Group-I	Group-II	Group-III	Group-IV
0-24%	8 (100.0%)	7 (87.5%)	0 (0.0%)	0 (0.0%)
25-49%	0 (0.0%)	1 (12.5%)	0 (0.0%)	5 (62.5%)
50-74%	0 (0.0%)	0 (0.0%)	3 (37.5%)	3 (37.5%)
75-100%	0 (0.0%)	0 (0.0%)	5 (62.5%)	0 (0.0%)
Comparison between group-III and other groups				
		Group-I	Group-II	Group-IV
p		<0.001*	<0.001*	<0.006*
RR (95% CI)		–	–	–

#Fisher's Exact test.
RR: Relative rate (group-III relative to other groups regarding 75-100%).
CI: Confidence interval

Fig. (8): Comparison between study groups regarding radiological extent of bone healing.

b- *Radiological bridging:*

High grades of radiological bridging the defect length with new bone were most frequent in group-III (75% entire length bridging and 25% partial length bridging), follow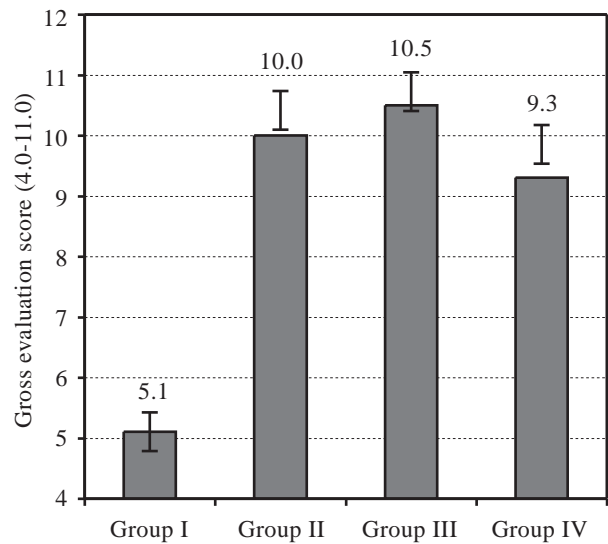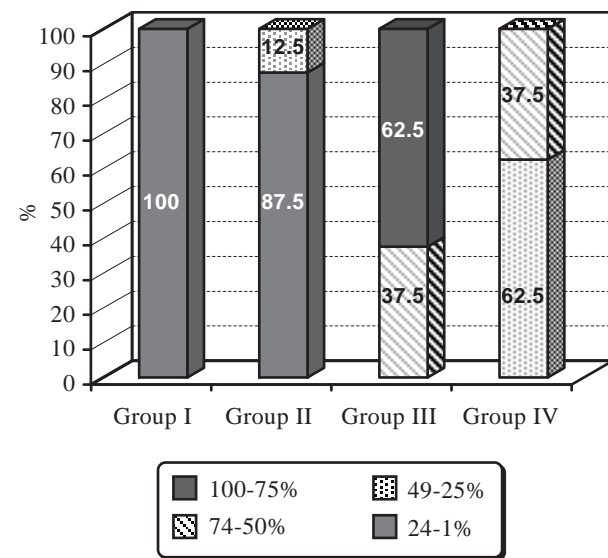ed by group-IV (37.5% partial length bridging and 62.5% just formed bone over defect boarders), then group-II (100% just formed bone over defect boarders), and least in group-I (50% no healing and 50% just formed bone over defect boarders). Difference were significant with all groups (Table 4) (Fig. 9).

Table (4): Comparison between study groups regarding radiological bridging. Group III is significantly higher.

Bridging	Group-I	Group-II	Group-III	Group-IV
No bone formation	4 (50.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Just formed over defect borders	4 (50.0%)	8 (100.0%)	0 (0.0%)	5 (62.5%)
Partial length bridging	0 (0.0%)	0 (0.0%)	2 (25.0%)	3 (37.5%)
Entire length bridging	0 (0.0%)	0 (0.0%)	6 (75.0%)	0 (0.0%)

Comparison between group-III and different group			
	Group-I	Group-II	Group-IV
p	<0.001*	<0.001*	<0.003*
RR (95% CI)	5.000 (1.448-17.271)	5.000 (1.448-17.271)	5.000 (1.448-17.271)

#Fisher's Exact test.　　　　CI : Confidence interval.
RR: Relative rate (group-III relative to other groups regarding).

Fig. (9): Comparison between study groups regarding radiological bridging.

C- *Histological evaluation:*

The new bone formation was evident in group-III, where multiple Islands of new irregular bone formation over dead necrotic bone that suggest the presence of the DBM remnants. The bone cells are seen inside their lacuanae resting over dead bone with small amount of Fibrous Tissue. In group-IV, a homogenous wide spaces resembling prolene mesh was seen and surrounded by fibrous tissue and newly formed bone. The amount of bone is more than fibrous tissue. In group-II, 3 zones could be identified; first zone is Dead bone with necroti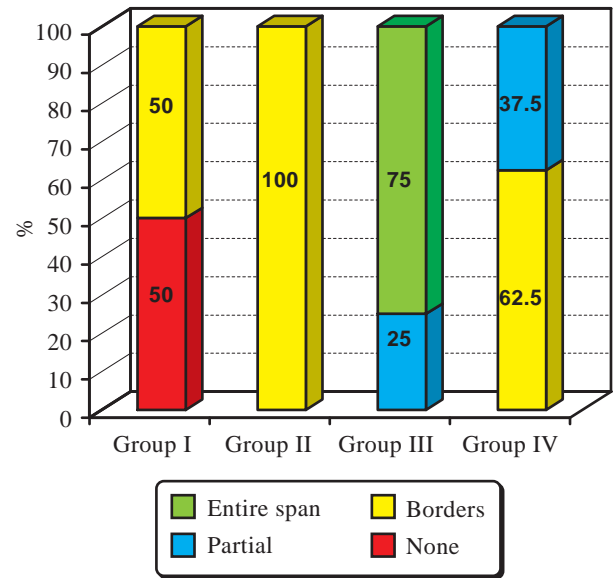c bone lamellae and absent cells which is sugessting the prescence of DBM; second zone is junctional zone that shows a new bone formation where blood vessels and osteocytes are seen inside their lacuanae; third zone is the surrounding rim of the calverial bone. The control group (group-1), showed minimal islands of bone formation with greater amount dense fibrous tissue (Fig. 10). Interestingly, there were no abnormal cellular infiltration or immune reaction rather than normal cells included in normal healing process. This result is comparable to the result reached by Khaled, 2008 [28] and is considered another prove for the non–immunogenicity of the allogenic ADSC and for DBM.
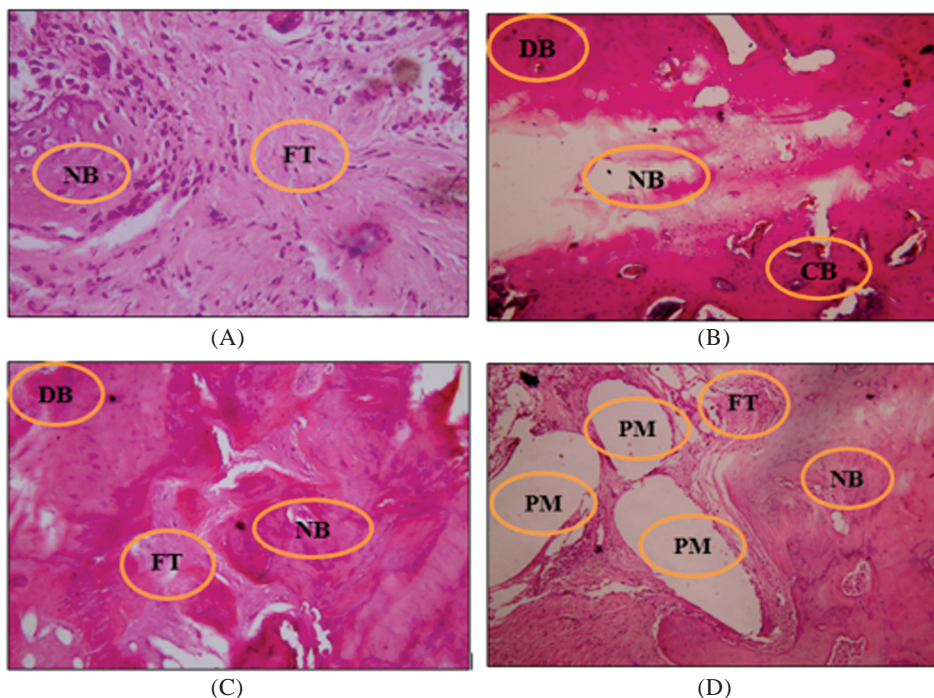

Fig. (10): Histological evaluation (H&E x 400). A- Group I; showing island of bone formation (NB) with osteocytes seen inside lacuanae surrounded by dense fibrous tissue (FT). B- Group II; 3 zones; Dead bone(DB) with necrotic bone lamellae and absent cells; second zone is junctional zone with a new bone formation; third zone is the surrounding rim of the calverial bone (CB). C- Group III; Islands of new irregular bone formation over dead necrotic bone. D- Group IV; Homogenous wide spaces resembling prolene mesh (PM) and surrounde by fibrous tissue and newly formed bone.

Bone tissue healing was most frequent in group-III (62.5%), followed by group-IV (25%), fibrous tissue healing was more evident in group-I (62.5%) then in group-II (50%). Difference were significant with groups I& II (Table 5) (Fig. 11).

Table (5): Histological evaluation; Comparison between study groups regarding type of tissue healing.

Type of tissue healing	Group-I	Group-II	Group-III	Group-IV
Mostly fibrous	5 (62.5%)	4 (50.0%)	0 (0.0%)	0 (0.0%)
fibrous > bone	3 (37.5%)	4 (50.0%)	3 (37.5%)	6 (75.0%)
bone > fibrous	0 (0.0%)	0 (0.0%)	5 (62.5%)	2 (25.0%)

Comparison between group-III and other group			
	Group-I	Group-II	Group-IV
p	0.006*	0.007*	0.315
RR (95% CI)	3.667 (1.397-9.624)	3.667 (1.397-9.624)	2.333 (0.662-8.219)

#Fisher's Exact test.   CI : Confidence interval.
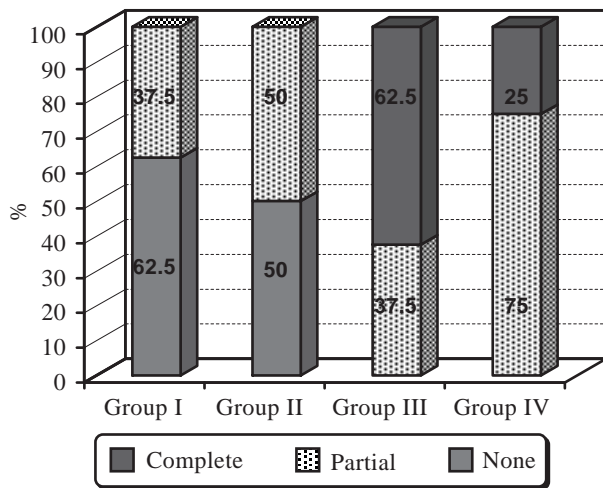RR: Relative rate (group-III relative to other groups regarding complete filling).

Fig. (11): Histological evaluation; Comparison between study groups regarding type of tissue healing.

DISCUSSION

The incidence of bone defects represents a major burden for the individual, families and the whole society. In USA, more than 1,600,000 bone grafts are implanted per year and 6% from this figure are craniofacial bone grafts [25]. Unfortunately, in our country, A statistical description for the magnitude of the critical size calvarial defects and the need for reconstruction is lacking.

Many reconstructive options are available from bone grafts, bone substitutes or bone transport methods [2]. The autograft is the golden standard, but, its main drawback is limited donor, extramorbidity and resorption [4].

Moreover, there is a high demand in reconstructive field for an already made bone that is available for urgent and extensive reconstruction where the needs are more than the autografts can meet. So, multiple studies were started to synthetize a novel tissue engineered bone and Researchers in this field tried to design a new bone tissue regenerate by seeding diverse types of stem cells on different scaffolds.

Though the lipoaspirate was considered as a waste for many years, it is a very rich source for ADSCS. This type of stem cells is clonogenic i.e. can form colonies in culture conditions [11,12]. Also, it has an osteogenic potential and surprisingly Its osteogenicity isn't greatly affected by age factor in contrast to BMSCs [13]. Furthermore, every processed 300mL of lipoaspirate can produce between 1×10^7 and 6×10^8 ADSCs [14-17] and every 1gm of surgically excised adipose tissue, yield approximately 5×10^3 stem cells [18]. Its harvest and expansion are greatly easier than BMSCs [19-20]. Also, ADSCs are more superior to BMSCs in bone regeneration as it could be maintained in vitro for longer periods with a constant doubling, more proliferative capacity and lower senescence [11,12].

Despite the diversity of multiple scaffolding in the field of BTE, the DBM is considered an ideal scaffold. It is osteoconductive and non–immunogenic as the demineralization process exposes the proteins and other various growth factors, which are present in the extracellular matrix, to be available for the host environment and it destroys the antigenic surface structure of the bone [21]. The DBM is also osteoconductive [22]. It could be prepared and preserved easily. It is commercially available in various ranges of shapes and forms as such as morselized particles and struts.

In this study, the DBM scaffold is compared to prolene mesh scaffold which was used by Khaled, 2008 [28] and seeded by BMSCs. The advantages of this type of scaffolding are the availability, low cost, resistance to infection, malleability, biocompatibility and the ability for cell adherence and support. But its main disadvantages are the non-degradability; that might affect the mechanical property of the new tissue regenerate. Also, it is non osteoconductive nor osteogenic. On the other hand, the DBM scaffold biocompatible, biodegradable, non-immunogenic and at the same time,

osteoconductive and to some extent osteogenic 26,27. So, seeding the DBM with ADSCs can cover nearly all phases of bone healing i.e. The osteogenesis, osteoconduction and osteoinduction.

The results obtained from this work advocates the use of Allogenic Demineralized Bone Matrix and Adipose Derived Stem Cells as a reconstructive tool for bone regeneration. But further clinical studies are needed to evaluate the rule of ADSCs seeded over DBM in the unfavorable conditions as this study and the previous studies were conducted in normal healthy conditions, but the clinical situation is a little bit different as many local factors as osteomyelitis, osteoradionecrosis and soft tissue scarring may exist or general factors like smoking, diabetes, obesity and osteoporosis may also affect healing. So, this work recommends Further clinical studies to produce an evidence based clinical application of bone tissue bioengineering in reconstruction of craniomaxillofacial bone defects.

Conclusion:

This study presents a beneficial method for reconstruction of critical size calvarial bone defects by preparing an already made non-immunogenic new bone by seeding DBM with ADSCs.

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