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Comparative assessment of bone regeneration by histometry and a histological scoring system

Evaluarea comparativă a regenerării osoase utilizând histometria şi un scor de vindecare histologică

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Abstract

***Objective**: The aim of this research is to evaluate the value of the histological score based on a histological record compared to the histometry for monitoring cranial bone defect healing. **Methods**: We designed a case -control study with a control and a study group. For a number of 60 CD1 mice representing the study group, a bone defect in the cranial bone was surgically induced and grafted with bone grafts obtained by tissue engineering. Bone grafts were obtained using embryonic stem cells seeded on a scaffold obtained from the red deer antler, and osteogenic basal and complex medium was used as differentiation medium. For other 30 CD1 mice representing the control group, a bone defect in the cranial bone was induced and left to heal without grafts. The regeneration process was assessed after 2 and 4 months using the histological healing scoring system and histometry. **Results**: The healing score was statistically significantly correlated with the defect size obtained by means of histometry ($p<0.001$). The evaluation of the parameters comprised in the healing score shows that regeneration of the bone diastasis was the most advanced in the group sacrificed at 4 months after plasty, which employed embryonic stem cells, a complex osteogenic differentiation medium and deer antler as scaffold. **Conclusion**: histological method based on a histological score is a valuable quantification system of bone regeneration comparable to histometry. **Clinical Relevance**: This study proves that the presented histological score can help the clinician in the process of bone regeneration evaluation.*

***Keywords**: tissue engineering, histometry, bone healing, stem cells, histological scoring system*

Rezumat

*Obiective: Scopul studiului de faţă a constat în evaluarea valorii scorului de vindecare histologică, comparativ cu histometria în monitorizarea vindecării ososе la nivelul calotei. **Material şi metodă**: Am realizat un studiu caz-control cu un lot control şi unul de studiu. La un număr de 60 de şoareci CD1 incluşi în lotul de studiu am indus chirurgical un defect osos la nivelul calotei şi am realizat reconstrucţia defectului utilizând grefe obţinute prin*

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inginerie tisulară. Ingineria tisulară a grefonului osos s-a realizat utilizând celule stem embrionare poziționate pe suport matriceal –corn caduc de cerb, iar ca inductor al diferențierii am utilizat mediu osteogenetic bazal și complex. La cei 30 de șoareci CD1 incluși în lotul control am indus chirurgical același defect osos la nivelul calotei, dar nu am realizat reconstrucția osoasă a acestuia. Procesul de regenerare osoasă a fost evaluat la 2 și respectiv la 4 luni utilizând scorul de vindecare și histometria. ***Rezultate****: Scorul de vindecare histologică s-a corelat statistic semnificativ cu dimeniunea defectului obtinută la histometrie ($p<0.001$). Evaluarea parametrilor în baza cărora s-a stabilit scorul de vindecare histologică indică regenerarea cea mai avansată la subiecții din lotul de studiu sacrificați la 4 luni, la care s-a utilizat pentru ingineria grefonului osos celule stem embrionare, suport matriceal corn caduc de cerb și mediu osteogenetic complex ca inductor.* ***Concluzii****: scorul de vindecare histologică este o metoda valoroasă de cuantificare a procesului de regenerare osoasă.* ***Relevanță clinică****: Acest studiu demonstrează că scorul de vindecare histologică prezentat este un instrument util pentru clinician în procesul de evaluare a regenerării osoase.*

Cuvinte cheie*: inginerie tisulară, histometrie, vindecarea osului, celule stem, scor histologic*

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Introduction

Nowadays, bone grafts are widely used in craniofacial, dentoalveolar and orthopedic surgery due to the progress of bone transplantation techniques. In order to reduce intra- and postoperative morbidity and mortality rates and to increase the therapeutic success of bone grafts, surgical strategies are intended to be minimally invasive. This goal was achieved in the '60s, when researchers combined the science of biomaterials with cell biology, opening the gates to a new field - that of tissue engineering (1). The aim of tissue engineering is to regenerate tissue through in vitro implantation of cells on a scaffold (2). The start point of tissue engineering were biomaterials (3), nowadays tissue engineering implies the use of cells seeded on a scaffold before in vivo implantation (4). The engineered tissue must be structurally, functionally and mechanically identical with the tissue aimed to be replaced. The clinical success of the engineered graft essentially depends on a suitable supply of cells, the quality of the scaffold and the presence of signaling molecules involved in cell adhesion and differentiation.

Creating novel bone biomimetic materials is still difficult to achieve in terms of reproducing all required chemical, physical and biological signals at different scales (macro, micro and nanoscale) (5).

The accurate monitoring of the bone healing process at the level of the reconstructed site with auto-allografts or grafts obtained through tissue engineering is also a very important target. Various methods have been used to assess bone healing: conventional radiography (6-8), high resolution CT (9), Cone Beam CT (10, 11), and histology. It is widely accepted that histology remains the golden standard in assessing the process of bone regeneration. Consequently, research regarding the evaluation of the bone regeneration process has compared different methods such as CT or CBCT to histological evaluation, histometry. There is no research on establishing a correlation between histometry and histological evaluation based on a histological score.

The aim of this study was to evaluate bone healing after guided bone regeneration with grafts using a healing score based on a histological record compared to histometry.

Material and method

Study design - Animal model

The study was conducted on CD1 mice aged 8-12 months, weighing 25-40 grams, with balanced gender distribution. All procedures and

the protocol for the animal experiments were approved by The Ethical Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca (Approval number 292, 6/05/2011). We designed a case-control study consisting of a control group (30 subjects) and a study group (60 subjects).

In the control group a bone defect, with a diameter of 5 mm, was surgically induced in the cranial bone. Bone defect reconstruction was not carried out. A number of 15 subjects in the control group were sacrificed after 2 months (group CG-2). Other 15 subjects were sacrificed after 4 months (group CG-4).

In the study group a 5 mm-diameter bone defect was induced in the cranial bone in all subjects. Bone defect plasty was performed in 30 subjects, with engineered grafts using embryonic stem cells positioned on deer antler matrix support in osteogenic base medium. A number of 15 subjects were sacrificed after two months (SG-OBM-2 group). Other 15 subjects were sacrificed after 4 months (SG-OBM-4 group).

In the other 30 subjects in the study group, bone defect plasty was performed with engineered grafts using embryonic stem cells positioned on deer antler matrix support and complex osteogenic medium. A number of 15 subjects were sacrificed after two months (SG-OCM-2 group). Other 15 subjects were sacrificed after 4 months (SG-OCM-4 group).

Bone graft tissue engineering

Scaffold design and selection. As scaffold the internal spongy part of the deciduous red deer antler was used. The dimensions of the scaffold were: thickness between 0.3-0.5 mm, diameter of 5 mm. The porosity of the scaffold was of 0.2-0.6 mm. In order to obtain this scaffold an IsoMet Low Speed Saw, Buehler Ltd. microtome was used. The 5 mm diameter of the scaffold was obtained using the 5 mm diameter plastic scaffold employed to calibrate the bone defect.

3D bone marrow cell culture. Embryonic stem cells were used in engineering the bone grafts. The embryonic stem cells belonged to the R1/E/NA line. The embryonic stem cells were arranged on CD1 murine fibroblast feeder-layers in the presence of LIF. The differentiation of embryonic stem cells (ESC) into bony line cells was triggered using the hanging drop technique. After 2 days of cultivation in hanging drops, the formed embryoid bodies (EBs) were seeded on gelatinized plates in osteogenic medium and cultivated 12 days in these conditions. After that, pre-differentiated ESC were seeded on the surface of deciduous red deer antler scaffolds and cultivated 30 days in the presence of osteogenic medium (basal and complex) and prepared for transplantation.

The basal osteogenic medium consisted of DMEM 4500 mg glucose/mL, 20% fetal bovine serum, penicillin (100 U/mL) – streptomycin (100 μg/mL), 2 mM L-glutamine, 1% non-essential amino acids (NEA), 10 mM β-glycerophosphate, 50 μg ascorbic acid, 1 μg/mL insulin. The complex osteogenic medium has growth factors in addition:

3 ng/mL BMP2, 2 ng/mL TGF-β (12).

Surgical protocol

For all animals, cranial bone defects were surgically induced under general anesthesia (Xylazine 0.75 mg/kg and Ketamine 0.5 mg/kg diluted 10x with saline 0.9%). After the disinfection of the surgical site, a flap was created on the occipital arteries. The cranium was exposed by elevating the flap. The 5 mm diameter bone defect was induced under the Leica M 320 microscope using a 15 scalpel blade. The calibration of the bone defect was accomplished by means of an electronic scale and a double check of the bone defect size using a 5 mm diameter plastic scaffold (13). (Figure 1)

After inducing the bone defect we reconstructed the defect for subjects from the study

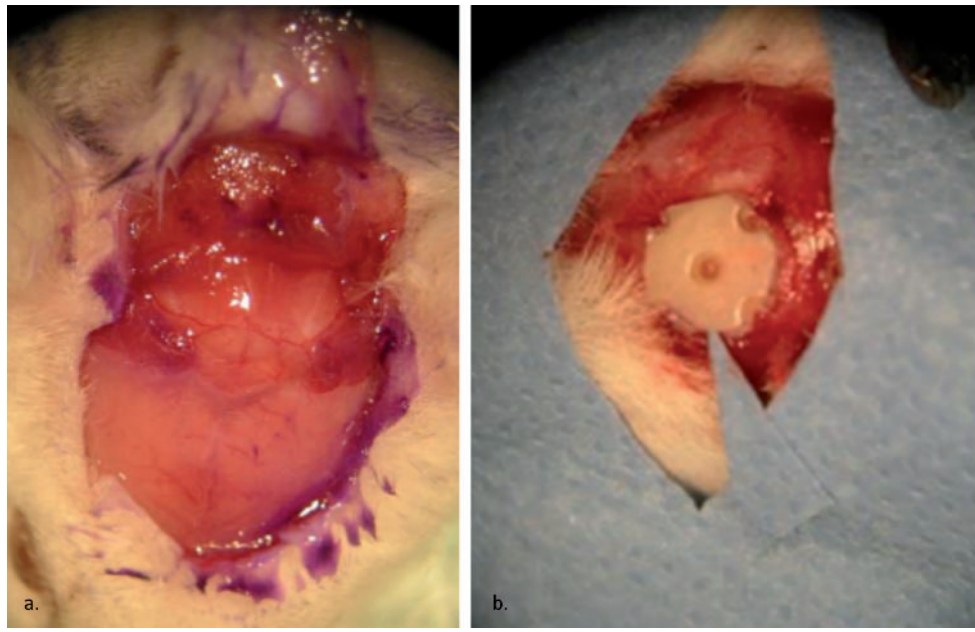

Figure 1. a. Macroscopic aspect of the skull of CD1 mice. b. Macroscopic aspect of the bone defect with the plastic 5 mm diameter scaffold showing the exact calibration of the defect

group by positioning the tissue engineered bone graft at the level of the defect.

The reconstructed site was covered with the flap sutured under the microscope using Ethicon 6.0 resorbable thread. All surgical procedures were carried out by an oral surgeon (OL) with a 10 year experience. Animal survival data for each group are presented in Table I.

Bone regeneration assessment

After the subjects were sacrificed, their brain caps were harvested, collected and immediately immersed in a formaldehyde solution.

To obtain the histological slides, the tissue samples were fixed in formaldehyde 4% (24 h) and introduced in a decalcification solution for bone, after that included in paraffin wax (classic histologic technique). From paraffin blocks were obtain 5 μ slices (with a Leica rotary RM2125 RTS manual microtome). Putted on glass slides, this slices were stained in Hematoxylin-Eosin ant Masson Trichrome (for bone and connective tissue).

Histometry was performed on slides obtained from the subjects sacrificed at 2 and 4 months, and was aimed at establishing the size of the defect. The slides were scanned with the Aperio XT scanner, at Translational Core Pathology Lab, UCLA (Directors Sarah Dry, MD and Jonathan Said, MD). Using Aperio XT, ultrafast

Table I. Summary of survival percentage for each group

Group	CG-2	CG-4	SG-OBM-2	SG-OBM-4	SG-OCM-2	SG-OCM-4
Initial number of animals	15	15	15	15	15	15
Number of animals that survived	7	11	10	8	8	8
Survival rate	46.66 %	73.33 %	66.66 %	53.33 %	53.33 %	53.33 %

high-capacity scanning with the Scan Scope XT 120 slide system was performed. After scanning, the images were interpreted with the Aperio ImageScope v10.2.1.2315 software. Scanning was carried out with a 20x/0.75 Plan Apo objective, with a scanning magnitude of 20x and 40x, at a resolution of 20x: 0.50 µm/pixel and 40x: 0.25 µm/pixel. The image format was TIFF. The resulted image was open in Aperio ImageScope software and using the „Ruler tool", the length of the defect was measured (Figure 2). The results of the histometric measurements were presented as the size of the remaining defect, measured between the edges of mineralised bone.

Histological examination was performed by two experts in histology (DG with 20 years of experience, OS with 15 years of experience) on the histological slides. The examination of slides for histological score, was made on an Olympus BX40 microscope. The two experts in histology also performed descriptive histology on the examined slides. The two experts in histology were not blinded towards the nature of the slides.

The experts received a histological evaluation form with 17 parameters. By adding up the score given to each parameter, the expert obtained the histological healing score for each subject individually. The healing score based on the histological evaluation record was assessed according to Solchaga et al. (14), and other additional parameters that are presented on Table II. The healing score represented the sum of all evaluated parameters, the maximum value being 43.

Data from experts in histology were introduced in an Excel database. The coefficient of concordance Kappa was >0.75, so that the score for each evaluated parameter was the mean of the values obtained from the two experts in histology. Statistical analysis aimed to establish a statistically correlation between the histological parameters (histological healing score) and histometry.

Statistical analysis was performed on independent variables using the SPSS 13.0, Statistica 7.0 and Microsoft Excel applications. Normal distribution was tested with Shapiro-Wilk test. If data were normally distributed we used the t test for independent sample. If data were not normally distributed or categorical, we used the Mann-Whitney test for ranks.

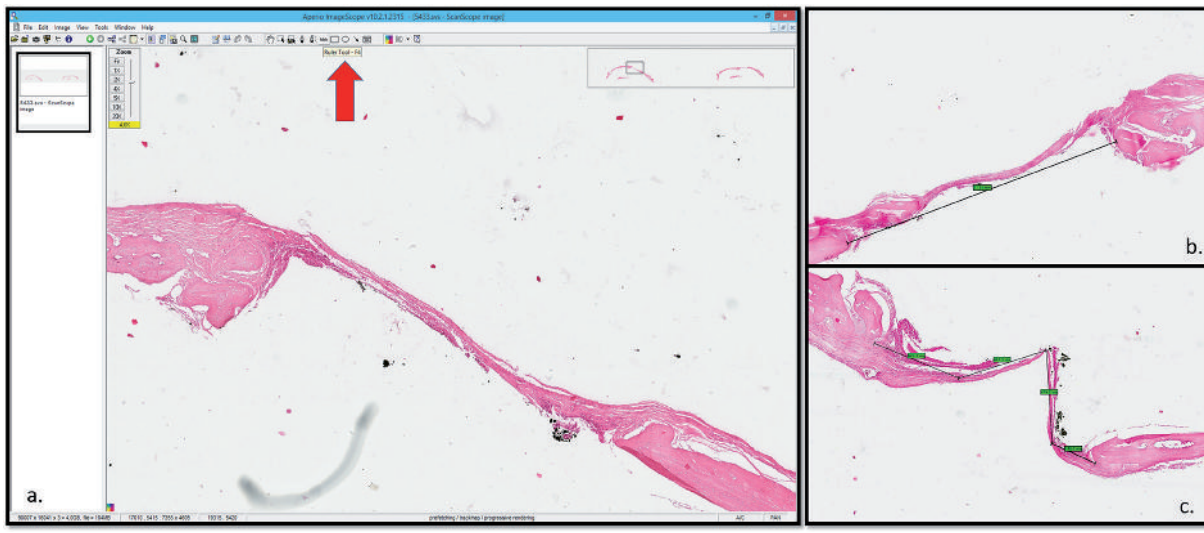

Fig.2.a. The screen windows of Aperio ImageScope software with Ruler Tool activation. b. One step measurement with ruler tool. c. Multiple step measurement with ruler tool.

Table II. Histological evaluation record

Histological score	
1. Bone formation	**10. Scaffold replacement with mature bone**
0 – absent	0 – absent
1 – present at the periphery	1 – present at the periphery
2 – present centrally	2 –present centrally
3 – present centrally and at the periphery	3 – present at the periphery and centrally
2. Bone formation	**11. Bone bridge**
0 – absent	0 – absent
1 – present at the surface of the graft	1 – narrow
2 – present in the depth of the graft	2 – thick
3. Vascularization of the graft	**12. Bone trabeculae**
0 – absent	0 – absent
1 – present at the surface of the graft	1 – present at the periphery
2 – present in the depth of the graft	2 – present centrally
	3 – present at the periphery and centrally
4. Osteoblasts	**13. Haversian canals**
0 – absent	0 – absent
1 – present at the periphery	1 – present at the periphery
2 – present centrally	2 – present centrally
3 – present centrally and at the periphery	3 – present at the periphery and centrally
5. Osteocytes	**14. Inflammation**
0 – absent	0 – present
1 – present at the periphery	1 – absent
2 – present centrally	
3 – present centrally and at the periphery	
6. Osteoclasts	**15. Granulation tissue**
0-absent	0 – present
1 – present at the periphery	1 – absent
2 – present centrally	
3 – present centrally and at the periphery	
7. Immature bone	**16. Neo-formation of blood vessels**
0 – present centrally	0 – absent
1 – present at the periphery	1 – present at the periphery
2 – absent	2 – present centrally
	3 – present centrally and at the periphery
8. Mature bone	**17. Bone tissue**
0 – absent	0 – absent
1 – present at the periphery	1 – present at the periphery
2 – present centrally	2 – present centrally
3 – present at the periphery and centrally	3 – present at the periphery and centrally
9. Osteoclastic degradation of the scaffold	
0 – absent	
1 – present at the periphery	
2 – present centrally	
3 – present centrally and at the periphery	

We set the cut-off value for histometry to less than 2.5 mm diameter remaining defect, which represented a reduction of 50% from the initial lesion. We used a ROC curve in order to calculate a cut-off for the healing score, considering the histometry cut-off value as healing criterion. The cut-off value was chosen where sensitivity and specificity were maximal.

For the estimation of the correlation between two ordinal variables, the Spearman correlation coefficient (r) and the Cohen's Kappa coefficient were used.

The significance threshold for the tests used was set at $\alpha = 0.05$.

Results

1. The correlation between the histologically assessed healing score and histometry

The healing score was statistically significantly correlated with the bone defect measured by means of histometry ($p<0.001$) using the Spearman test. The correlation was negative, the higher the healing score, the lower the bone defect size determined by histometry.

Using histometry as the golden standard, we used a ROC curve to calculate a cut-off value for the healing score. We found an AUC of 0.882, a cut-off value of 16 (sensibility 100% (CI95% 66.4-100) and the specificity of 73% (CI 95% 52.2-88.4).

The concordance Kappa coefficient indicated a good agreement between histometry and the histological score, regarding the healing (kappa=0.583; $p<0.001$).

2. Comparison of the control and study groups based on histological parameters and histometry after 2 and 4 months

There was no statistically significant difference in the histometrically determined defect size between groups CG-2 and CG-4. The mean histometrically determined defect size in control group CG-2 was 3.28±0.72 compared to 3.16±0.13 in control group CG-4. The mean of the ranks for the histometrically determined defect size was lower in group CG-2 (8.71) compared to group CG-4 (10.00), but not significantly lower ($p=0.66$). There was no statistically significant difference in the histometrically determined defect size between groups CG-2 and SG-OBM-2. The mean histometrically determined defect size in group CG-2 was 3.28±0.72 compared to 2.81±0.66 in group SG-OBM-2. The comparison of the mean of the ranks for the histometrically determined defect size between the two groups showed a higher, but not significantly higher value in subjects of group CG-2 (10.57) compared to subjects of group SG-OBM-2 (7.90). No statistically significant difference in the histometrically determined defect size was found between groups CG-2 and SG-OCM-2. The mean histometrically determined defect size in group CG-2 was 3.28±0.72 compared to 2.70±0.39 in group SG-OCM-2. The mean of the ranks for the histometrically determined defect size was higher in subjects of group CG-2 compared to subjects of group SG-OCM-2. (Table III)

At 4 months, there was a statistically significant difference in the histometrically determined defect size between groups CG-4 and SG-OBM-4. The mean histometrically determined defect size in group CG-4 was 3.16±0.13 compared to 2.11±1.15 in group SG-OBM-4. A statistically significant difference in the histometrically determined defect size was found between groups CG-4 and SG-OCM-4. The mean histometrically determined defect size in group CG-4 was 3.16±0.13 compared to 1.75±0.01 in group SG-OCM-4.

3. Characterization of the groups using the healing score and descriptive histology (Table IV)

After 2 months, a slight surface bone formation in the periphery and peripherally mature bone was observed in 29% of the subjects in the control group. (Figure 3)

Healing parameters show only a few changes in the control group recorded after 4 months than

Table III. Healing score and bone defect size by means of histometry for SG-OBM-4 and SG-OCM-4 groups in comparison with the control group CG-4.

Parameters	CG -4 (n=11)		SG-OBM - 4 (n=8)		p	SG-OCM -4 (n=8)		p
	Mean of the ranks	Sum of the ranks	Mean of the ranks	Sum of the ranks		Mean of the ranks	Sum of the ranks	
Histological parameters								
Healing score	6	66	15.5	124	<0.001	15.5	124	<0.001
Histometry	12.36	136	6.75	54	<0.001			<0.001
Histometry	14	154				4.50	36.00	<0.001

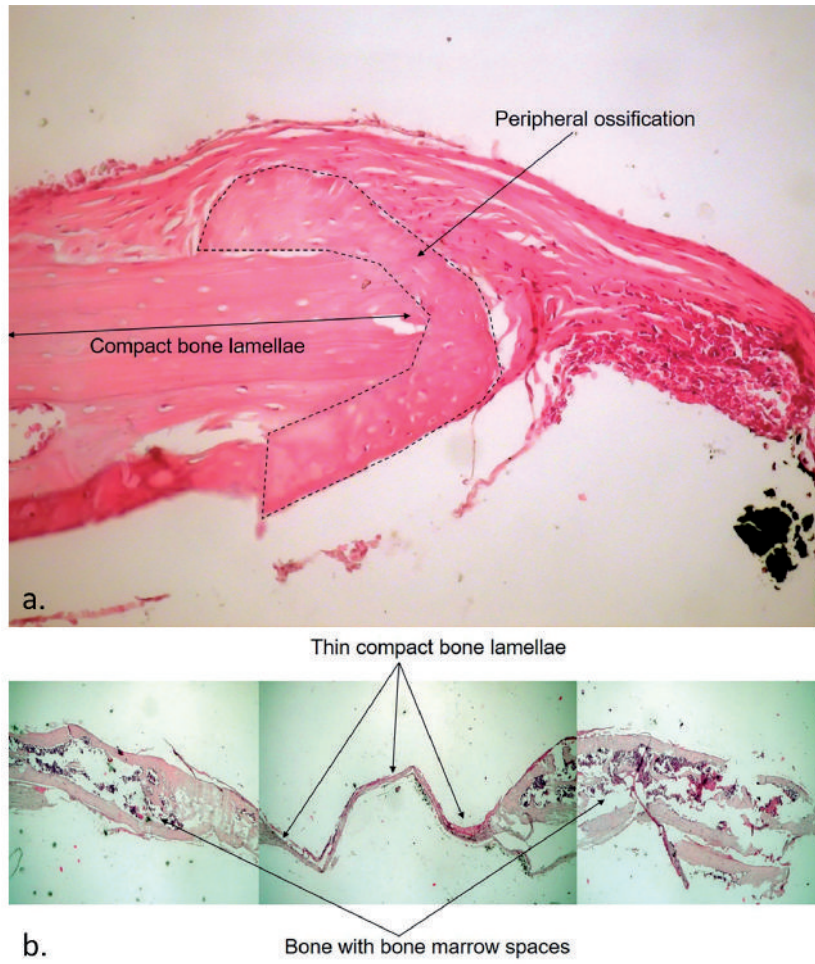

Figure 3. a. Characteristic microscopic aspect for group CG-2, HE stain, 200x
b. Chracteristic microscopic aspect for group CG-4, HE stain, 50x

Table IV. Characterisation of the groups using the healing score (histological parameters)

Histological bone healing parameters		Group frequency (%)					
		CG2	CG 4	SG-OBM-2	SG-OCM-2	SG-OBM-4	SG-OCM-4
Surface bone formation	absent	71	82	0	0	0	0
	peripheral	29	18	80	75	75	0
	central	0	0	0	0	0	0
	central and peripheral	0	0	20	25	25	100
Bone formation in the depth of the graft	absent	0	0	0	0	0	0
	surface	100	91	0	0	0	0
	profound	0	9	100	100	100	100
Immature bone	central	0	0	0	25	75	100
	peripheral	0	9	0	0	0	0
	absent	100	91	100	75	25	0
Mature bone	absent	71	36	0	0	0	0
	peripheral	29	64	80	75	75	0
	central	0	0	0	0	0	0
	central and peripheral	0	0	20	25	25	100
Graft vascularization	absent	86	46	20	25	0	0
	surface of the graft	14	46	0	0	0	0
	depth of the graft	0	8	80	75	100	100
Neo-formation vessels	absent	71	36	0	0	0	0
	peripheral	29	64	100	100	100	100
	central	0	0	0	0	0	0
	central and peripheral	0	0	0	0	0	0
Bone tissue	absent	100	100	0	0	0	0
	peripheral	0	0	100	100	100	100
	central	0	0	0	0	0	0
	central and peripheral	0	0	0	0	0	0
Bone bridge	absent	100	100	40	0	0	0
	thin	0	0	40	0	0	0
	thick	0	0	20	100	100	100
Osteoblasts	absent	100	100	0	0	0	0
	peripheral	0	0	80	75	75	0
	central	0	0	0	0	0	0
	central and peripheral	0	0	20	25	25	100
Osteocytes	absent	100	100	0	0	0	0
	peripheral	0	0	80	75	75	0
	central	0	0	0	0	0	0
	central and peripheral	0	0	20	25	25	100
Osteoclasts	absent	100	82	40	25	25	0
	peripheral	0	9	0	0	0	0
	central	0	9	60	75	75	100
	central and peripheral	0	0	0	0	0	0
Bone trabeculae	absent	100	100	0	0	0	0
	peripheral	0	0	80	75	75	50
	central	0	0	0	0	0	0
	central and peripheral	0	0	20	25	25	50
Haversian canal	absent	100	100	0	0	0	0
	peripheral	0	0	80	75	50	50
	central	0	0	0	0	25	0
	central and peripheral	0	0	20	25	25	50
Inflammation	absent	100	91	0	0	0	0
	present	0	9	100	100	100	100
Granulation tissue	absent	100	91	0	0	0	0
	present	0	9	100	100	100	100
Osteoclast degradation of the scaffold	absent	100	91	40	25	25	0
	peripheral	0	0	0	0	0	0
	central	0	0	60	75	75	100
	central and peripheral	0	9	0	0	0	0
Scaffold replacement with mature bone	absent	100	100	20	0	0	0
	peripheral	0	0	60	50	50	50
	central	0	0	0	0	0	0
	central and peripheral	0	0	20	50	50	50

after 2 months. Surface bone formation is still very low (18%), islands of mature and immature bone appear only at the periphery of the bone defect. Osteoblasts, osteocytes, haversian canals and bone trabeculae are absent. Osteoclasts appear in the center and at the periphery. No bone bridge is detected.

In contrast, bone formation in the study group sacrificed at 2 months was present on the surface (75-80% of subjects) as well as in the profoundness of the graft (100%). Mature bone was detected only at the periphery in this stage. Vascularization was detected only on the surface of the bone graft. Peripheral osteoblasts, osteocytes, bone trabeculae and haversian canals were observed in most subjects with grafts. Central osteoclast degradation was detected in 60-75% of subjects and scaffold replacement with mature bone in 20-50% of subjects from the SG-OBM-2 and SG-OCM-2 groups. (Figure 4)

In SG-OBM-4 and SG-OCM-4 groups, surface bone formation was present in the center and at the periphery, almost 100% compared to SG-OBM-2 and SG-OCM-2 groups where surface bone formation was detected only at the periphery. Mature bone was detected in the center and at the periphery, while immature bone was identified only in the center. Neo-formation vessels which supply the vascularization of the graft were detected in the profoundness of the graft. Osteoblasts, osteocytes, bone trabeculae and haversian canals were detected peripherally and also centrally. Osteoclastic degradation of the scaffold and osteoclast cells was present only in the center. After 4 months, 100% of the subjects with grafts indicated the presence of a thick bone bridge. (Figure 5)

Discussion

This study was conducted on CD1 mice consisting of a control group (30 subjects) and a study group (60 subjects) aiming to evaluate bone healing after guided bone regeneration with grafts using histometry and a healing score based on a histological record.

The results demonstrated the osteogenic potential of tissue engineered grafts using em-

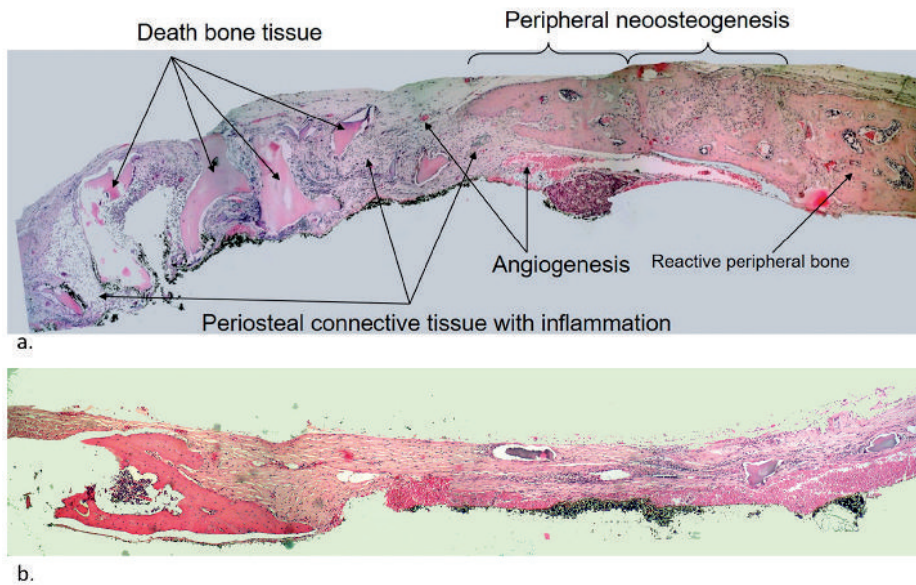

Figure 4. a. Chracteristic microscopic aspect for group SG-OBM- 2, HE stain, 100x
b. Chracteristic microscopic aspect for group SG-OCM- 2, HE stain, 50x

bryonic stem cells seeded on a scaffold obtained from the red deer antler using basic and complex osteogenic medium. CD1 mice in each group in the study had a balanced distribution in terms of age and gender, and 60% of the subjects survived indicating the validity of the animal model.

Knowing that the selection of the scaffold is of paramount importance for the outcome of bone graft engineering (15, 16) we selected the deciduous red deer antler. This scaffold is biocompatible; it promotes cell interaction and tissue development (17). Having a high level of porosity, this scaffold offers a wide surface of interactions with the fluids of the host (18) and facilitates cell affixation, cell migration on the surface and inside the matrix and cell proliferation and differentiation. In our research we did not use the bioreactor for the seeding process of the stem cells on the scaffold. We performed a passive seeding, so that it was very important for the scaffold to have a high level of porosity. to facilitate cell migration inside the scaffold. The red deer antler contains high level of IGF-1, IGF-2, BMP-2, BMP-4 which are responsible for the osteogenesis. This bone matrix provides mechanical support until the regenerated bone has sufficient mechanical integrity to support itself (19). Also the low costs of this scaffold led to the selection of the red deer antler.

The differentiation process was induced by using basic and complex osteogenic differentiation medium. The use of growth factors in osteogenic medium improves bony line differentiation and mineralization process (20-23). TGF β1 is a controversial growth factor because of its dual role in maintaining ESC pluripotency,

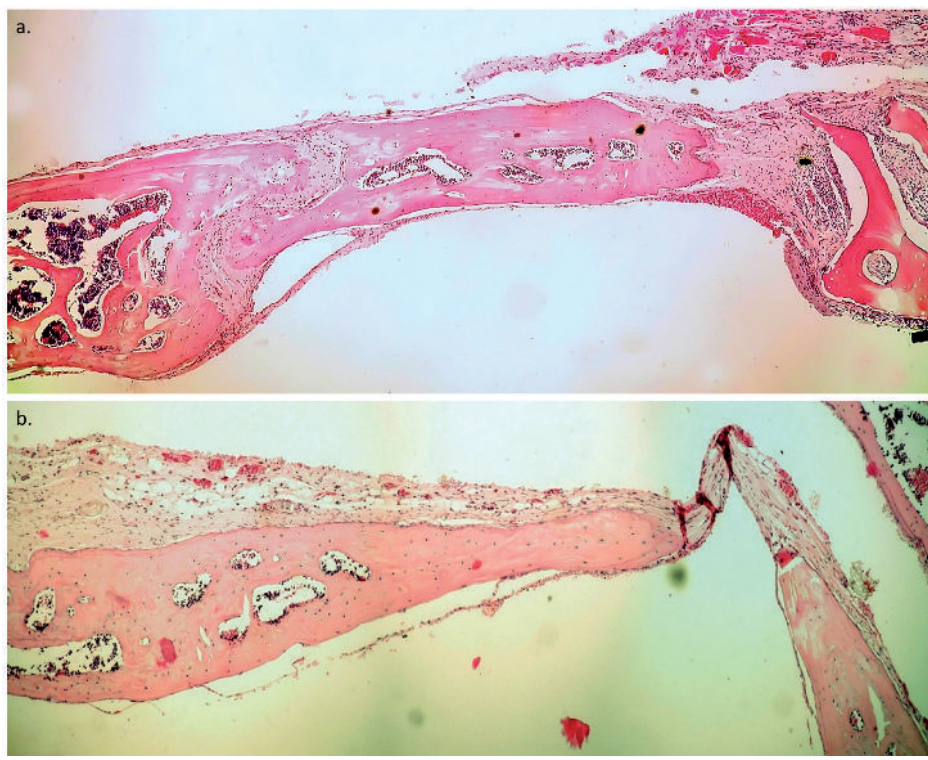

Figure 5. a. Chracteristic microscopic aspect for group SG-OBM- 4, HE stain, 100x
b. Chracteristic microscopic aspect for group SG-OCM- 4, HE stain, 100x

but also because of the induction of chondrogenic cells in a later stage of differentiation in EB-derived cells (24). Growth factor addition to the differentiation medium, such as 1α,25-(OH) (2) vitamin D(3) and all-trans-retinoic acid, are important for their action on signaling pathways, especially Wnt/beta-catenin pathway, an inducer of osteogenic and chondrogenic differentiation (25). EBs are good tools for studying stem cell differentiation in vitro because they revise many aspects of early embryogenesis and they are necessary in order to trigger ESC differentiation into a variety of cell types (26). Nevertheless, the protocols used in ESC differentiation are often inefficient and generate heterogeneous populations (27).

After implantation the tissue engineered bone graft determined angiogenesis and encouraged tissue deposition (Table IV) (28).

The 5 mm bone defect that we induced was a critical size defect, which means that without grafting this defect will not regenerate. Regarding bone regeneration of a critical size non-grafted defect, our study demonstrated by means of histological score and histometry that regeneration takes more than 2 months.

The bone grafts obtained through tissue engineering techniques using embryonic stem cells proved their osteogenic properties. Regeneration and remodeling of bone diastasis was the most advanced in the group employing embryonic stem cells, a complex osteogenic medium and deer antler as a scaffold, the subjects being sacrificed 4 months after plasty (Table IV). This is in accordance with the studies conducted by Wozney et al (21), and Ebara et al (22), both authors emphasizing the importance of BMP2 and TGFβ in the complex osteogenic medium in accelerating bony line differentiation.

A correct determination of bone regeneration is an important parameter. Therefore, the quantitative analysis of the regeneration process by means of a histological record seems to be a valuable tool.

The originality of our study lies in the fact that the quantification of the bone regeneration process was made using the histological score based on a histological record introduced by the authors. Precise assessment of bone healing is vital in both clinical and research settings. Radiographic scoring systems have been developed for clinical use. The research carried out by Tawonsawatruk T et al (29) introduced a radiographic fracture-healing scores in a small animal model but there is none histological healing score validated on an animal model.

The structure of the presented histological record lies in the histological record described by Solchaga et al (14). The potential of the histological score based on a histological record in the assessment of bone regeneration was evaluated on an animal model in comparison with histometry.

Histometry is widely accepted as tool used for bone regeneration quantification (30, 31). The study of de Mello et al (30) evaluated by means of histometry parameters such as vital mineralized tissue (VMT), nonvital mineralized tissue (NVMT), nonmineralized tissue (NMT), and vital mineralized tissue in contact with titanium (VMTCT) for appositional bone regeneration.

In the study of Chakar C et al (32), the authors compare the regenerative potential of preparations containing autologous platelet lysate (APL) and particles of either deproteinized bovine bone mineral (DBBM) or biphasic calcium phosphate (BCP),on an animal model using histometry.

Histometry has also been used for assessing periodontitis in rabbits, for evaluating the bone loss (33).

Bone regeneration has been evaluated comparing histometric measurements with linear radiographic in the study of Hermann JS et al (34). This study demonstrated that histometry is more precise in evaluating bone regeneration.

In 2014, Young-Seok Park et al (35) assessed the relationship between histometric and micro-computed tomographic (CT) data. The authors tried to establish the statistical association between these methods. The results of the research showed there was no significant association between the data from histometry and micro-CT analysis.

All presented studies underline the fact that histometry can be used as a golden standard in assessing bone regeneration. Previous studies have tried to employ correlation between histometry and radiology, micro-CT. There is no study that correlates histometric findings with a histological score.

Our study demonstrated an excellent correlation between the histological score based on a histological record and histometry, performed on an animal model, suggesting the usefulness of further evaluation of this new method.

The results obtained from the two methods (histological healing score and histometry) were numeric data that helped perform statistical data analysis.

The analytical methods used proved to be efficient as a tool for the quantification of bone regeneration. Statistical analysis showed the existing correlations between the histological parameter healing score based on the histological evaluation record and histometry. By using this two methods for the quantification of bone regeneration, the present study demonstrated the validity of the data obtained by the other method. The existence of a correlation between these parameters could also be established. The correlation was negative, the higher the healing score, the lower the bone defect size determined by histometry.

This study, determining the correlation between the investigated parameters, proves that the histological record may be a valuable method to assess bone regeneration but more research should be done including more subjects.

The histological record has a wide clinical implication because it offers more information about the exact stage of the bone regeneration process compared to histometry which only reveals the dimension of the not healed bone defect. Performing the histological record is much easy and implies less costs then histometry.

All procedures and the protocol for the animal experiments were approved by The Ethical Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca (approval number 292 form 6/05/2011) and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

The manuscript does not contain clinical studies or patient data.

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The author declares that there are no potential conflicts of interest.

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