ORIGINAL PAPER

Tissue engineered bone *versus* alloplastic commercial biomaterials in craniofacial reconstruction

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Abstract

This research was developed in order to demonstrate the tissue engineering method as an alternative to conventional methods for bone reconstruction, in order to overcome the frequent failures of alloplastic commercial biomaterials, allografts and autografts. Tissue engineering is an *in vitro* method used to obtain cell based osteoinductive bone grafts. This study evaluated the feasibility of creating tissue-engineered bone using mesenchymal cells seeded on a scaffold obtained from the deciduous red deer antler. We have chosen mesenchymal stem cells because they are easy to obtain, capable to differentiate into cells of mesenchymal origin (osteoblasts) and to produce tissue such as bone. As scaffold, we have chosen the red deer antler because it has a high level of porosity. We conducted a case control study, on three groups of mice type CD1 – two study groups (n=20) and a control group (n=20). For the study groups, we obtained bone grafts through tissue engineering, using mesenchymal stem cells seeded on the scaffold made of deciduous red deer antler. Bone defects were surgically induced on the left parietal bone of all subjects. In the control group, we grafted the bone defects with commercial biomaterials (OsteoSet, ®Wright Medical Technology, Inc., Arlington, Federal USA). Subjects were sacrificed at two and four months, the healing process was morphologically and histologically evaluated using descriptive histology and the golden standard – histological scoring. The grafts obtained *in vivo* through tissue engineering using adult stem cell, seeded on the scaffold obtained from the red deer antler using osteogenic medium have proven their osteogenic properties.

***Keywords*:** tissue engineering, stem cells, biomaterials, bone regeneration.

Introduction

The fundamental premise of tissue engineering is regeneration of tissue and restoration of function through implantation of cells/tissue grown outside the body or stimulating cells to grow into an implanted matrix [1]. Tissue engineering [2] has evolved from the use of biomaterials [3] for damaged or diseased tissue repair to using controlled three-dimensional scaffolds in which cells can be seeded usually before implantation [4]. The living tissue construct is functionally, structurally and mechanically identical with the tissue designed to be replaced. The clinical success of the construct is largely dependent on the quality of the scaffold composition. A suitable supply of cells is of key importance [5] (Figure 1).

In respect of this, we consider that the seeding of bone marrow derived mesenchymal cells on a scaffold obtained from deciduous red deer antler could be an appealing alternative for the engineering of cell-based osteoinductive grafts.

This study evaluated the feasibility of creating tissue engineered bone using mesenchymal cells [6] seeded on a scaffold obtained from the deciduous red deer antler. We have chosen the deciduous red deer antler because it has a high level of porosity, contains high levels of polypeptides like IGF-1, IGF-2, BMP-2, BMP-4, which are responsible for the process of osteogenesis. The structure of this scaffold is very similar to that of human alveolar bone. Mesenchymal stem cells were the option for our research because they are capable to differentiate into cells of mesenchymal origin (osteoblasts) and to produce tissue such as bone. The challenge in this research lies in proving that the grafts obtained through tissue engineering have osteogenic properties, while alloplastic materials have only osteoconductive properties.

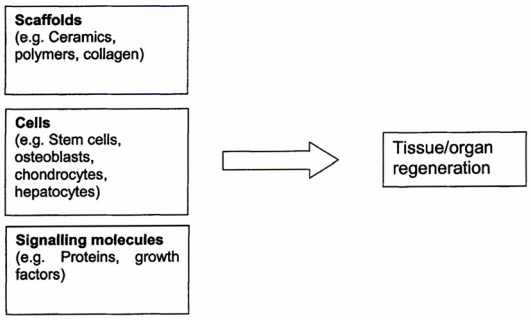

Figure 1 – ***Key factors involved in tissue engineering (Vats A et al., 2003).***

Material and Methods

Design of the study

We conducted a case control study on three groups of CD1 mice – two study groups (n=20) and a control group (n=20), with similar distribution regarding age and gender. We induced left parietal bone defects for each subject from all groups. In the control group (CG) we grafted the bone defects with commercial biomaterials (OsteoSet, ®Wright Medical Technology, Inc., Arlington, Federal USA). For the first study group (SGB) we obtained bone grafts through tissue engineering, using mesenchymal stem cells and basal osteogenic medium seeded on the scaffold made of deciduous red deer antler. For the second study group (SGC) we obtained bone grafts through tissue engineering, using mesenchymal stem cells and complex osteogenic medium seeded on the scaffold made of deciduous red deer antler. The design of the study may be observed in Table 1.

Table 1 – ***Design of the study***

Group	Number of subjects at the beginning of the study	Number of subjects at the end of the study	Material used for the reconstruction of the bone defect	Subjects sacrificed at
Control group (CG2)	10	8	Alloplastic commercial biomaterial containing calcium sulphate, OsteoSet (®Wright Medical Technology, Inc., Arlington, Federal USA).	2 months
Control group (CG4)	10	8	Alloplastic commercial biomaterial containing calcium sulphate, OsteoSet (®Wright Medical Technology, Inc., Arlington, Federal USA).	4 months
Study group (SGB2)	10	10	Bone grafts obtained with tissue engineering protocols, using decidous red deer antler as scaffold, mesenchymal stem cells and basal osteogenic medium.	2 months
Study group (SGB4)	10	8	Bone grafts obtained with tissue engineering protocols, using decidous red deer antler as scaffold, mesenchymal stem cells and basal osteogenic medium.	4 months
Study group (SGC2)	10	8	Bone grafts obtained with tissue engineering protocols, using decidous red deer antler as scaffold, mesenchymal stem cells and complex osteogenic medium.	2 months
Study group (SGC4)	10	10	Bone grafts obtained with tissue engineering protocols, using decidous red deer antler as scaffold, mesenchymal stem cells and complex osteogenic medium.	4 months

Generation and implantation of the bone grafts obtained through tissue engineering

Isolation of bone marrow nucleated cells

The bone marrow derived mesenchymal stem cells were obtained from the femur of CD1 mice. After harvesting the femur and cutting the epiphysis, the medullar channel was washed with DMEM complete medium (DMEM 1000 mg glucose/mL, 20% fetal bovine serum (Sigma), penicillin (100 U/mL) – streptomycin (100 µg/mL), 2 mM L-glutamine, 1% non-essential amino acids (NEA), and the cells were recovered in flasks. The cell suspension was prepared through centrifugation. We performed a cell viability test using Trypan Blue solution and a Thoma counting chamber was used to count the number of cells. The result was a final cell suspension with a concentration of 5×10^6 viable cells/mL. These cells were seeded onto 25 cm^2 Cole flasks containing DMEM complete medium and incubated. After 72 hours of cultivation, the non-adherent cells were removed and fresh culture medium was added. The primary culture was trypsinized when it became subconfluent, and subcultures were made. Changing the culture medium was performed every fourth day.

Design and selection of scaffold

The internal spongy part of the deciduous red deer antler was used in the study as scaffold. Slices were cut using a microtome (Isomet Low Speed Saw, Buehler Ltd.), in order to obtain scaffolds with a thickness varying between 0.3–0.5 mm, with an area of 3–5 mm^2 and a porosity varying between 0.2–0.6 mm.

3D bone marrow cell culture

Osteogenic differentiation was induced by positionning the primary cell culture in 24 well plates. Every well plate contained osteogenic medium and a scaffold obtained from deciduous red deer antler. Two types of osteogenic media were used for this study: basal and complex (Table 2).

Table 2 – ***Basal and complex osteogenic differentiation medium***

Basal osteogenic differentiation medium	Complex osteogenic differentiation medium
DMEM 4500 mg glucose/mL	DMEM 4500 mg glucose/mL
20% fetal bovine serum	20% fetal bovine serum
penicillin (100 U/mL) – streptomycin (100 µg/mL)	penicillin (100 U/mL) – streptomycin (100 µg/mL)
2 mM L-glutamine	2 mM L-glutamine
1% non-essential amino acids (NEA)	1% non-essential amino acids (NEA)
10 mM β-glycerophosphate	10 mM β-glycerophosphate
50 µg ascorbic acid	50 µg ascorbic acid
1 µg/mL insulin	1 µg/mL insulin
	3 ng/mL BMP2
	2 ng/mL TGF-β

Cultivation was carried out during a 30-day period, having the medium changed every two to three days. Loading cells into the scaffold was performed using the passive technique, without using a bioreactor. The culture was examined to identify the surface marker murine stage–specific embryonic antigen (SSEA-1).

Surgical procedure of bone defect reconstruction

For the control group (CG), 3 mm diameter bone defects were created in the left parietal bone of the CD1 mice (Figure 2).

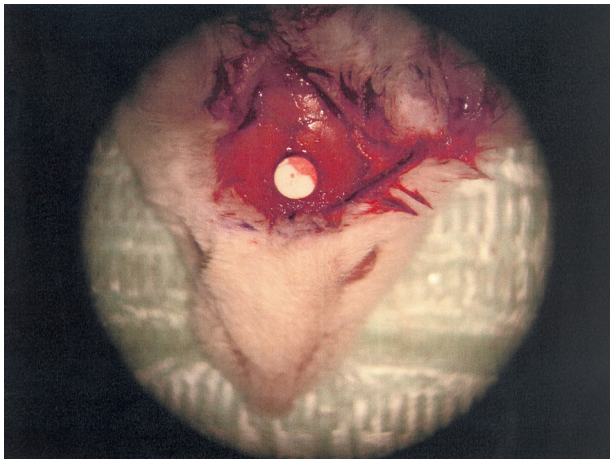

Figure 2 – ***Stereomicroscopic aspect of the skull – grafting of the left parietal bone defect with alloplastic biomaterials, OsteoSet® (Wright Medical Technology Inc., Arlington, Federal USA).***

For the study groups the same induced bone defect was performed. For the first study group (SGB) we grafted the bone gap with the bone tissue obtained through tissue engineering protocols, using deciduous red deer antler as scaffold, mesenchymal stem cells and basal osteogenic medium. For the second study group (SGC) we grafted the bone gap with the bone tissue obtained through tissue engineering protocols, using deciduous red deer antler as scaffold, mesenchymal stem cells and complex osteogenic medium (Figure 3).

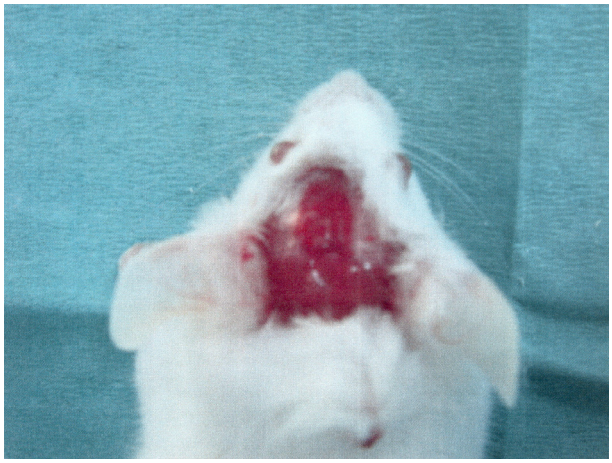

Figure 3 – ***Macroscopic aspect of the skull after grafting the bone defect with bone tissue obtained with tissue engineering protocols using mesenchymal stem cells seeded on a scaffold of deciduous red deer antler.***

Analytical methods

The subjects of control and study groups were sacrificed at two and four months after grafting, using ethanol induced death, and analytical methods were carried out in three stages as follows:

- Cell morphology analysis and immunohistochemical evidence of the differentiation process into osteogenic lineages;
- Quantification of bone formation using the golden standard – histology;
- Statistical analysis using Mann–Whitney test at $p<0.05$ level of significance.

Results

Immunohistochemical analysis

Immunohistochemical analysis of the SSEA-1 surface marker used to stain the fixed samples revealed cells with the specific expression of this surface antigen, which proves the presence of non-differentiated cells among pre-differentiated cells SSEA-1 expression decreases as the cell differentiates.

Cell morphology changed dramatically after medium addition, from spindle-shaped to cube-shaped. Spindle-shape cells formed nodular-shaped colonies starting with the sixth day of differentiation and the presence of some crystals over the cell colonies was observed starting with the fourteenth day. The extracellular matrix was stained for Alizarin Red to reveal the osteogenic differentiation.

Quantification of bone formation using golden standard – histology

The histological evaluation was carried out on the bases of histological records (Table 3) and using descriptive histology.

Table 3 – ***Histological record***

Bone formation	Central	Yes / No	Peripheral	Yes / No
Bone formation	Only at the surface of the graft	Yes	Profound in the graft	Yes
Vascularisation of the graft	Missing	Yes	Penetrated profoundly	Yes
	Limited at the surface of the graft	Yes		
Immature bone	Central	Yes	Peripheral	Yes
Mature bone	Central	Yes	Peripheral	Yes
Interface between host bone and grafted material	Immature bone	Yes	Mature bone	Yes
Central area	Connective tissue	Yes		
	De novo vascularisation	Yes	Bone tissue	Yes
Bone bridge	Narrow	Yes	Thick	Yes
Osteoblasts	Absent	Yes	Present central	Yes
			Present peripheral	Yes
Osteocytes	Absent	Yes	Present central	Yes
			Present peripheral	Yes
Osteoclasts	Absent	Yes	Present central	Yes
			Present peripheral	Yes
Bone trabecules	Absent	Yes	Present central	Yes
			Present peripheral	Yes

Bone formation	Central	Yes / No	Peripheral	Yes / No
Havers channels	Absent	Yes	Present central	Yes
			Present peripheral	Yes
Inflammation	Absent	Yes	Present	Yes
Granulation tissue	Absent	Yes	Present	Yes
Osteoclastic degradation	Absent	Yes	Present central	Yes
Scaffold	Absent	Yes	Present peripheral	Yes
Scaffold replaced with mature bone	Absent	Yes	Present central	Yes

The main histological aspects observed in the control group sacrificed at two months (group CG2) revealed that the bone defect is covered by connective tissue. The tissue layer that covers the bone defect contains granulative tissue and an amorphous biomaterial besides connective tissue. The reaction toward the biomaterial varied among subjects in this group. Against the larger particles of alloplastic biomaterial a granulomatous reaction emerged with giant multinucleated cells (Figures 4 and 5).

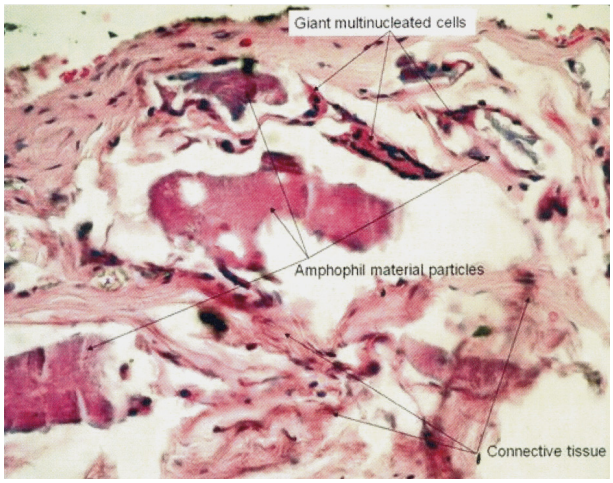

Figure 4 – ***Granulomatous reaction with giant multinucleated cells against the particle of alloplastic biomaterial. Poor inflammatory reaction (HE stain, ×400).***

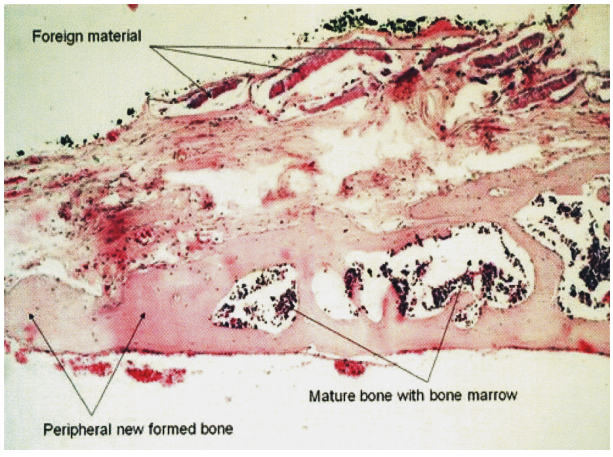

Figure 5 – ***At the periphery of the defect, the destroyed bone creates a reaction of ossification, which covers partially or entirely, like a muff, the destroyed bone. There is no tendency of ossification of the biomaterial.***

Histological analysis of the control group sacrificed at four months (CG4) revealed some differences from the subjects from the CG2: the lack of inflammatory reaction, and presence of mature connective tissue.

The histological aspects observed in the study group sacrificed at two months, with basal osteogenic medium (SGB2) are characterized through dead bone integration into a periosteal connective tissue accumulation. Between the dead bone layers, full myeloid metaplasia (lymphoid series, leukocyte series and erythroid) is rich (Figure 6). Actives osteoclasts caused dead bone injury, determining bone's blade breaking up in small parts, which are under a much more efficient osteoclastic attack. Demineralization appears in some parts of the graft.

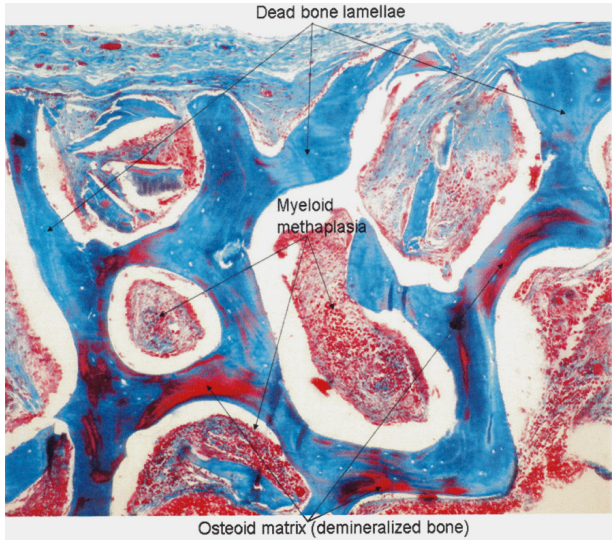

Figure 6 – ***In Masson staining (×100), dead bone lamellae appear in bright blue. Red areas are demineralization areas, osteoid protean (HE stain, ×100).***

At the periphery, neo-osteogenesis is complete and rich, with the exception of one blade from this batch, which presents an approximately completely ossified injury, made with an adult bony plate, delimited however by fibrous pseudoarthrosis.

Subjects from the study group sacrificed at four months, with basal osteogenic medium (group SGB4) revealed the same histological description as batch SGB2, but a thicker layer of dead bone with more advanced osteoclastic destruction can be observed and dead bone small parts are more frequent (Figure 7). Myeloid metaplasia also displays at this point, but incomplete (lymphoid line) and inconstant. This batch also displays a case with an almost completely ossified injury.

Subjects from the study group sacrificed at two months, with complex osteogenic medium (SGC2 group) have the same histological description as subjects from the SGB2 group, but without myeloid metaplasia (Figure 8).

Histological analysis of the study group sacrificed at four months, with complex osteogenic medium (group SGC4) revealed the same aspects as for group SGC2.

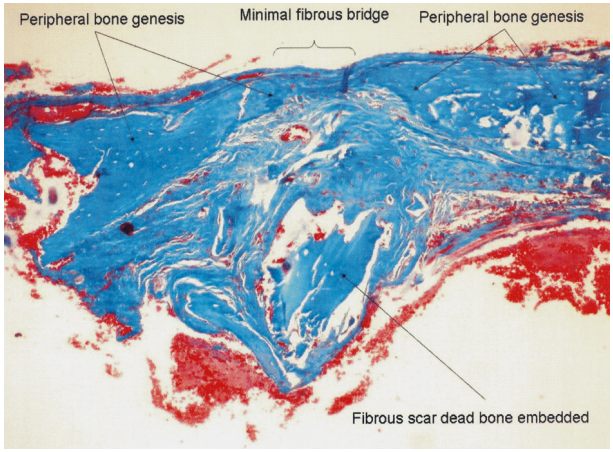

Figure 7 – ***Slide in Masson staining. An approximately complete healing of the defect, except a small dead bone part, which is inside conjunctive scar (HE stain, ×50).***

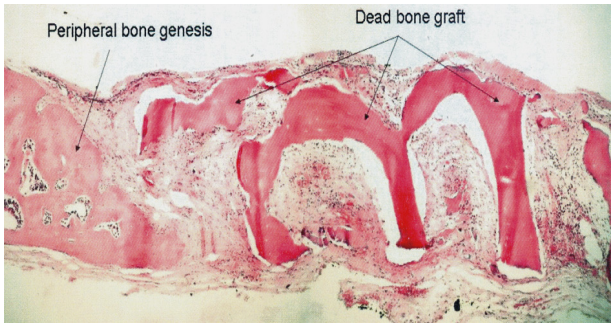

Figure 8 – ***Prototype histological aspect for group SGC2. Panoramic view.***

Statistical analysis

Histological scoring system based on the system described by Solchaga LA *et al.* [7] was used in this study. We scored f or each subject a healing rate by summing up points for each evaluated parameter, as follows (Table 4):

Table 4 – ***Histological scoring system***

Histological scoring	
1. Bone formation 0 – absent 1 – present at the periphery 2 – present centrally 3 – present centrally and at the periphery	*10. Scaffold replacement with mature bone* 0 – absent 1 – present at the periphery 2 – centrally present 3 – present at the periphery and centrally
2. Bone formation 0 – absent 1 – present at the surface of the graft 2 – present in the profoundness of the graf	*11. Bone bridge* 0 – absent 1 – narrow 2 – thick
3. Vascularisation of the graft 0 – absent 1 – present at the surface of the graft 2 – present in the profoundness of the graft	*12. Bone trabecules* 0 – absent 1 – present at the periphery 2 – centrally present 3 – present at the periphery and centrally
4. Osteoblasts 0 – absent 1 – present at the periphery 2 – centrally present 3 – present centrally and at the periphery	*13. Havers channels* 0 – absent 1 – present at the periphery 2 – centrally present 3 – present at the periphery and centrally
5. Osteocytes 0 – absent 1 – present at the periphery 2 – centrally present 3 – centrally and at the periphery present	*14. Inflammation* 0 – present 1 – absent
6. Osteoclasts 0 – absent 1 – present at the periphery 2 – centrally present 3 – centrally and at the periphery present	*15. Granulation tissue* 0 – present 1 – absent
7. Immature bone 0 – centrally present 1 – present at the periphery 2 – absent	*16. Neoformation vessels* 0 – absent 1 – present at the periphery 2 – centrally present 3 – present centrally and at the periphery
8. Mature bone 0 – absent 1 – present at the periphery 2 – centrally present 3 – present at the periphery and centrally	*17. Bone tissue* 0 – absent 1 – present at the periphery 2 – centrally present 3 – present at the periphery and centrally
9. Osteoclastic degradation of the scaffold 0 – absent 1 – present at the periphery 2 – centrally present 3 – present centrally and at the periphery	

A total index of healing score was determined by the summation of the categories 1–17. The maximum value is 43 points. The statistical analysis was carried out using the Mann–Whitney test (Table 5).

Table 5 – ***Results of the statistical analysis of the healing rate using Mann–Whitney test***

Group (n)	Mean rank	Sum of ranks	*p*
CG2 (n=8)	5.50	44.00	0.01
CG4 (n=8)	11.50	92.00	
CG2 (n=8)	4.50	36.00	0.00005
SGB2 (n=10)	13.50	135.00	
CG2 (n=8)	4.50	36.00	0.0002
SGC2 (n=8)	12.50	100.00	
CG4 (n=8)	4.50	36.00	0.0002
SGB4 (n=8)	12.50	100.00	
CG4 (n=8)	4.50	36.00	0.00005
SGC4 (n=10)	13.50	135.00	
SGC2 (n=8)	5.50	44.00	0.003
SGC4 (n=10)	12.70	127.00	
SGB4 (n=8)	6.00	48.00	0.01
SGC4 (n=10)	12.30	123.00	

Significant statistical differences concerning the mean rank of the healing rate appeared between the groups CG2–CG4 (p=0.01), CG2–SGB2 (p=0.00005), CG2–SGC2 (p=0.0002), CG4–SGB4 (p=0.0002), CG4–SGC4 (p=0.00005), SGC2–SGC4 (p=0.003), SGB4–SGC4 (p=0.01) (Table 5).

There were no statistical relevant differences between the mean rank of the healing rate of the groups SGB2–SGB4 and the groups SGB2–SGC2.

The mean rank of the healing rate for group CG2 was lower than that of the groups CG4, SGB2 and SGC2. The mean rank of the healing rate for group CG4 was lower than that of the groups SGB4 and SGC4.

Comparing the mean rank of the healing rate for group SGC2 with SGC4, the result indicated a better healing rate for group SGC4. Comparing the mean rank of the healing rate of the groups SGB4 with SGC4, the result indicated a better healing rate for the group SGC4.

Discussion

In the present study, we demonstrated that grafts obtained through tissue engineering using mesenchymal stem cells seeded on a scaffold obtained from deciduous red deer antler and basal/complex osteogenic differentiation medium are a valuable option in bone reconstruction.

Evaluating bone regeneration on bases of the stages described by Khoury F *et al.* (2007) [8] we have demonstrated that for the subjects from the control group, healing is made by connective tissue, with ossification at the periphery for both subjects sacrificed at two and at four months. Using alloplastic commercial biomaterials is a frequently used option in bone reconstruction, but these materials have only osteoconductive proprieties, so healing of a bone defect grafted with this kind of materials is done in more than four months.

The protocol for harvesting and culturing the bone marrow derived mesenchymal stem cells, described by Baciut M *et al* (2008) [9] and Kotobuki N *et al.* (2004) [10] has proved to be an efficient and reproducible one. The bone marrow mesenchymal stem cells were capable to differentiate into cells of mesenchymal origin (osteoblasts) and to produce tissue such as bone. They demonstrated there multilineage potential as described by Pittenger MF *et al.* (1999) [11].

The design and the selection of biomaterials are critical in the development of engineered tissue as shown by Langer R and Tirrell DA (2004) [12], and Lutolf MP and Hubbell JA (2005) [13]. This is the reason why we selected the deciduous red deer antler for this study. This scaffold material is biocompatible; it consists of bone, promotes cellular interaction and tissue development and possesses proper mechanical and physical properties as demonstrated in the study carried out by Baciut M *et al.* (2007) [9]. The deciduous red deer antler has a high level of porosity in the area of 0.1–0.8 mm; hence, it has a big area of interacting with the fluid of the recipient body, which is a compulsory condition for a scaffold as Rekow D (2003) [14] demonstrated. The deciduous red deer antler contains high levels of polypeptides like IGF-1, IGF-2, BMP-2, BMP-4, which are responsible for the process of osteogenesis. The structure of this scaffold is very similar to that of human alveolar bone. This biomaterial provides an appropriate regulation of cell behavior, such as adhesion, proliferation, migration, and differentiation, in order to promote the development of functional new tissue. This biomaterial possesses appropriate mechanical properties to regenerate tissue with predefined size and shape. Deciduous red deer antler proves to be an efficient scaffold; it facilitates cellular affixation, possibility to migrate on the surface and inside the matrix, cellular proliferation and differentiation as demonstrated by Baciut M *et al.* (2007) [9]. The scaffold created a medium where the cells were able to maintain phenotype and to synthesize the necessary proteins and molecules, compulsory conditions for a scaffold as demonstrated by Lutolf MP and Hubbell JA (2005) [13]. This scaffold provides temporary mechanical support sufficient to withstand *in vivo* forces exerted by the surrounding tissue and to maintain a potential space for tissue development. The mechanical support of the biomaterials was maintained until the engineered tissue had sufficient mechanical integrity to support itself, which is one of the main conditions of a scaffold as described by Haasper C *et al.* (2008) [15].

Although we did not use the bioreactor for the cellular seeding, the passive seeding technique proved to be efficient because of the scaffold porosity, which allows profound cells penetration in absence of bioreactor [12].

When transplanted into an allogenic setting, the bone marrow derived mesenchymal stem cells and the deciduous red deer antler scaffold where not rejected by the recipient immune system, nor did, they stimulate immune or allergic response. They have shown to participate in the regeneration of injured connective tissue such as bone [16].

For subjects from the study group treated with grafts obtained through tissue engineering, using basal differentiation medium, healing occurred by an intense osteoclastic process of the scaffold at the same time with de novo osteogenesis, as described by Gerstenfeld LC *et al.* (2002) [17], Le AX *et al.* (1997) [18], and Probst A and Spiegel HU (1997) [19]. This process was accompanied with myeloid line precursor appearance. This result is not according to those of Labat ML (2001) [20], who underlined that adult mesenchymal stem cells are safer than embryonic stem cells in therapeutic use, are easier to differentiate into a predefined cell lineage because of a reduced capacity to proliferate and to give rise to tumors.

For the subjects from the study group treated with grafts obtained through tissue engineering, using complex differentiation medium, bone injury healing is similar to the previous study group, except for more rapid sequences of the scaffold osteoclastic process and new bone formation, without precursor appearance. Using growth factors (BMP-2 and TGF-β) in osteogenic medium, bony line differentiation was improved and mineralization process accelerated as shown in the researches of Indrawattana N *et al.* (2004) [21], Wozney JM (2002) [22], and Ebara S and Nakayama K (2002) [23]. The same factors accelerate and stabilize the differentiation for the bony line and inhibit different lineage appearance (myeloid metaplasia).

On implantation, the graft obtained through tissue engineering protocols elicited a host-tissue response that initiated angiogenesis, encouraged tissue deposition, and culminated in restoration of structure and function specific to the grafted site, as described by Schimming R and Schmelzeisen R (2004) [24].

Statistical analysis revealed that by using alloplastic commercial biomaterials to graft a bone defect the healing process is more advanced after four months than after two months (Table 4). The evaluation of the healing process at two months revealed that engineered bone tissue using basal and complex osteogenic medium had significantly higher rates than for the groups with alloplastic commercial biomaterial. This underlines once again the osteoconductive potential of alloplastic biomaterials and the osteogenic potential of engineered bone grafts.

Comparing the healing rate at four months after grafting, the bone regeneration process was more advanced for the groups with tissue engineering (Table 4).

Statistical analysis of the healing rate between group SGC2–SGC4, indicates a higher rate for subjects sacrificed at four months. Because same culturing conditions (same osteogenic medium), same source of cells, same scaffold was used, we identified the parameter – time to be the only difference between the two groups. Time influenced the regeneration process, at four month the process being more advanced.

Comparing subjects sacrificed at four months, but having different culturing conditions, basal osteogenic medium and complex osteogenic medium, same cell source and same scaffold, we observed that BMP and TGF-β speeds up the bone regeneration process. Not the same goes for subjects sacrificed at two months, where no statistical relevant difference between the healing rate of this groups was observed.

Conclusions

Using tissue engineering on deciduous red deer antler as scaffold, we obtained *in vitro* bone tissue that could be used in bone defects reconstruction.

The alloplastic commercial biomaterials have proven their osteoconductive properties, none the less bone regeneration using alloplastic commercial biomaterials needed more than four months to be performed. Grafts obtained *in vitro* through tissue engineering using adult stem cell have proven their osteogenic properties.

Grafts obtained using tissue engineering has proven to be more efficient in bone reconstruction than alloplastic commercial biomaterials.

Complex osteogenic medium speeded up the regeneration process.

Although this is a laborious, expensive technique, incontestable advantages impose it as a future alternative of bone reconstructive techniques.

Acknowledgements

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