

Microdensity and morphometric analysis of autologous bone grafts cells

H. P. PETRESCU^{1)*}, G. DINU^{2)*}, DELIA BERCEANU-VĂDUVA³⁾,
M. BERCEANU-VĂDUVA¹⁾

¹⁾Department of Orthopedics and Trauma,
"Victor Babeș" University of Medicine and Pharmacy, Timisoara

²⁾Department 14 – Orthopedics and Anesthetics,
"Carol Davila" University of Medicine and Pharmacy, Bucharest

³⁾Department of Microbiology,
"Victor Babeș" University of Medicine and Pharmacy, Timisoara

*The authors have contributed equally to this work.

Abstract

Bone transplantation as a mean to reduce the fracture healing time in large defects was attempted for the first time more than 300 years ago, with nowadays several techniques and methods of assessment of its efficacy. The bone graft was longtime thought as initiating the osteogenesis from the recipient, but new data show that cells from the graft contribute to osteogenesis and to its incorporation into newly formed bone. There is no accurate assessment of the microdensity of bone graft cells in evolution so far, the only studies published recently referring to newly formed bone area. We have performed bone transplantation on seven dogs and have analyzed the microdensity and the morphometric features of bone graft cells. We have found that the cellular microdensity increases both in the graft and in the recipient bone, simultaneously with a decrease of cell size and circularity during maturation.

Keywords: bone transplantation, bone graft, osteocytes, morphometry, bone cells microdensity.

Introduction

Bone transplantation as a mean to reduce the healing time or to restore functionality in fractures with large defects has been first attempted in 1668 by Job Janszoon van Meekeren, a Dutch surgeon [1], and ever since was performed using a variety of techniques and sources for bone tissue, ranging from iliac crest autotransplants to different *in vitro* synthesized replacement for bone tissue [2], augmented nowadays by the availability of highly purified growth factors and cytokines which stimulate bone formation and contribute to rapid fracture healing.

Depending on the pathology and on the size of the defect, several types of bone transplants may be used, from local vascularized pedicle bone grafts in conditions associated with avascular necrosis [3, 4] or in post-tumor resection with large musculoskeletal defects [5, 6] to grafts with angiogenesis and osteogenesis stimulated by cytokines (VEGF and FGF-2) [7], impacted spongy bone autotransplants [8, 9] or even synthetic materials followed by cancellous bone implants stimulated by growth factors and cytokines [10].

The ability of bone marrow and periosteum to induce bone formation is well documented, but the osteocytes from cancellous bone autotransplants were supposed to undergo apoptosis, while leaving it only as a scaffold for newly formed bone from periosteum or bone marrow. This theory has recently been contradicted by

Komiyama H *et al.*, who showed that bone cells from GFP transgenic rats are able to migrate and populate the recipient site and also to differentiate into HSP47-positive macrophages [11].

Apart from this model, several methods have been used to assess the ability of the bone graft to incorporate in the recipient site and to stimulate the formation of new bone between them. Most of the studies use different radiographic scores or computer tomography to evaluate the efficiency of the bone grafting [12–14], while the assessment using scanning electron microscopy [15] or histological analysis is rarer. There are only two studies published so far that use computer-aided morphometric analysis of the bone formation in different experimental settings, measuring the area of newly formed bone [16, 17].

To the best of our knowledge, this is the first study that uses computer-aided morphometric analysis to quantify the cellular density and shape during experimental bone transplantation.

Materials and Methods

After obtaining the approval of the Ethical Board of the Faculty of Veterinary Medicine, University of Veterinary Medicine and Agricultural Sciences, Timișoara, Romania, seven dogs were performed autologous iliac crest bone transplants to the lower third of the femoral

shaft under general anesthesia with 20 mg/kg body weight Inactin completed with analgesia (Morphine 0.2 g/kg body weight). The grafts contained periosteum, compact bone, cancellous bone and bone marrow and were fixed to the receptor site previously prepared by excision of bone tissue with screws. After completion of surgery, the limbs were immobilized until we have sacrificed the animals.

The sampling of the specimens for histological diagnosis was performed at intervals of 7, 14, 21, 45, 60 days to 6–8 months. They were fixed in 10% buffered formalin, followed by decalcification and usual primary preparation. For the histological study, we used the method of decalcification with trichloroacetic acid in concentrations of 5% and 10%.

The usual primary preparation consisted in embedding in paraffin and sectioning the decalcified samples at 5 μ m thickness. After sectioning, the slides were dewaxed and rehydrated, then stained with Hematoxylin–Eosin as well as Masson's trichrome method for the evaluation of the cells and bone matrix.

Low magnification images were obtained using a Nikon Eclipse i80 microscope equipped with a Nikon DS-U2 CCD camera. The morphometric analysis was performed with ImageJ software. Basically, the color images were transformed in black and white, than an intensity and diameter threshold was applied semi-automatically, using bony areas as regions of interest and then counting and measuring the cells in the bone matrix lacunae, in both the transplanted tissue and the receptor, after calibration of the image. We have then compared the density of the cells and their morphometric features in evolution, at seven days and 60 days after the transplant.

Statistical analysis was performed on exported data using Microsoft Excel 2007 from the Microsoft Office bundle.

Results

Histological evolution of the transplanted bone

Representative images of the transplanted bone-receptor complex can be seen in Figures 1 and 2.



Figure 1 – Macroscopic image of the entire bone with the transplanted bone-receptor complex at 25 days after the transplant.

We have noticed the presence of the transplanted bone on sections from all experience animals, predominantly as spongy bone, with trabeculae of different shapes and sizes, surrounded by hyperplastic periosteum that fills the space between it and the receptor bed almost completely.

Dilated blood vessels were also noticed on histology, although not significant in all of the cases.

The lacunae in the transplanted bone tissue, as well as in the receptor bed, were dilated, with osteocytes present and increased in number, with an apparent higher density in the transplanted fragment.

Morphometric analysis

Two representative binary images along with their Masson's trichrome stained counterparts are presented in Figures 3–6, respectively. In all images, the graft is in the upper part, while the receptor bed is in the lower part of the image.

The morphometric parameters measured, were, apart from the number of bone cells, their circularity, their area and their perimeter.

The microdensity of bone cells in the transplanted tissue and in the receptor bed, in immediate vicinity of the graft, has increased approximately three fold during the evolution of the bone formation process, between seven days and 60 days after transplant.

The average microdensity of bone cells per section was 1107.5 ± 92.63099 at seven days, while at 60 days the cellular density was 3357.6 ± 273.6503 . The difference was statistically significant ($p=0.036178$ in paired t -test).

The values of the areas occupied by the cells followed an inverse trend, with a decrease from $36.68554604 \pm 22.29280038 \mu\text{m}^2$, to $27.68149389 \pm 8.932180137 \mu\text{m}^2$, at seven days and at 60 days, respectively ($p=0.83$), not statistically relevant.

The average circularity of the cells, measured as the ratio between the biggest and the smallest diameter, was 0.798380944 in the seven days after the transplant, and 0.35850727 in the 60 days after the transplant, respectively, which is consistent with the maturation of the cells and therefore changing in their shape.

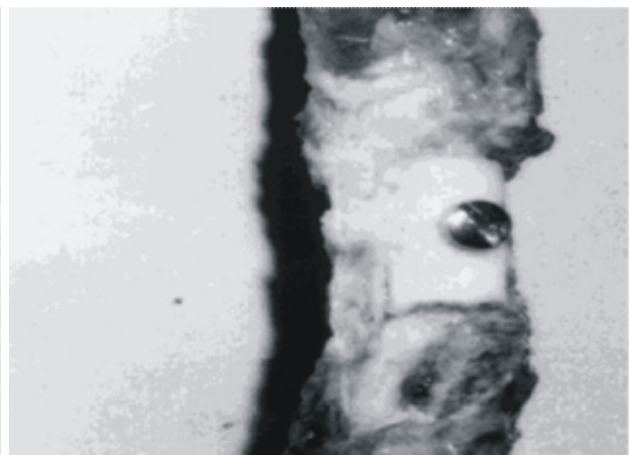


Figure 2 – Macroscopic image of the transplanted bone-receptor complex at 25 days after the transplant. Side view.

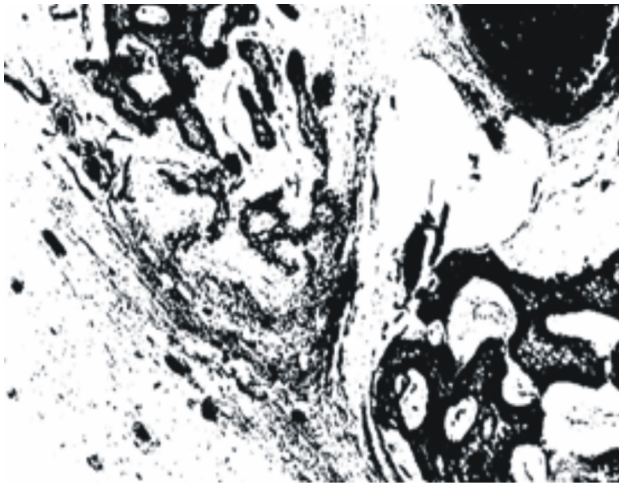


Figure 3 – Binary image of the graft (upper left) and the receptor bed (lower right) at seven days of evolution, 40 \times .

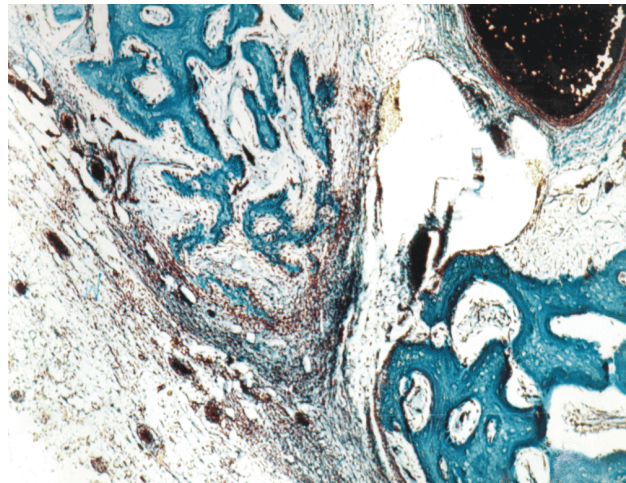


Figure 4 – Masson's trichrome stained slide of bone graft and receptor bed. The hyperplastic periosteum of the graft can be easily spotted, 40 \times .

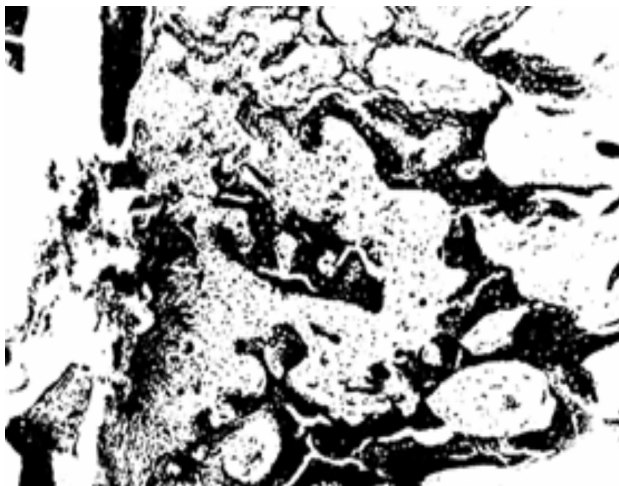


Figure 5 – Binary edited image of a late evolution of the transplanted bone tissue. New bone formation is identified in the middle of the image, bridging the transplant to the receptor bed, 40 \times .

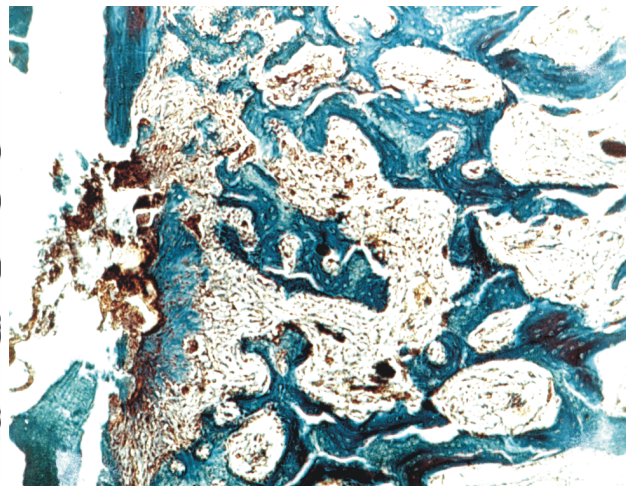


Figure 6 – Late stage evolution of the graft and the receptor bed. There is active bone formation from the graft. Masson's trichrome staining, 40 \times .

Discussion

The purpose of this study was to perform the morphometric characterization of transplanted bone cells and receptor bed in experimental conditions.

The former theories postulated the resorption of the bone transplants, which, according to Duriez J [18], serve only as a scaffold for the formation of new bone from periosteum and from the receptor bed. In our experiments on dogs where we performed autograft, the presence of preserved periosteum is certainly an asset for the early healing and accelerated bone formation, as the stem cells present in its internal layer undergo mitosis and differentiation into osteoblast and begin to synthesize bone matrix, thus contributing to the generation of new bone bridges between the graft and the receptor.

The homotransplants are not always suitable for this use because of local inflammation, which may appear even in aseptic conditions. A balance between pro-inflammatory cytokines that contribute to the integration of the graft into the receptor and to the remodeling of the newly formed immature bone tissue and anti-inflammatory ones that decrease inflammation in later stages thus

maintaining the new bone and preventing its lysis is difficult to obtain in practice. Allografts may be an alternative, especially when using frozen bone tissue [19]. Although the authors of the study showing the migration and differentiation ability [11] did not calculate the micro-density of GFP-positive bone cells from the transplant and its evolution in time, our findings support their conclusions on the fate of bone graft cells. The micro-density of bone cells in the transplanted tissue and in the receptor bed, in immediate vicinity of the graft, has increased approximately three fold during the evolution of the bone formation process, between seven days and 60 days after transplant, which is statistically significant. There are mainly two ways of the assessment of the integration of the bone transplants into the host tissue: the measurement of the area of new bone matrix along with the histochemical demonstration of immature collagen and early mineralization, or the study of the cell fate during transplantation. We have not assessed the area of bone formation, as did other authors [16, 17] as this was not the purpose of our study; however, at 60 days, there was extensive bone formation and

integration of the transplanted tissue, both macroscopically and microscopically. A recent study has used computer-assisted morphometry to highlight the different percentages of the areas occupied by bone matrix in representative images from biopsies of the femoral head of patients with necrosis of the femoral head and different risk factors and found no statistical difference between the groups of patients [20]. This approach of measuring the area of newly formed bone is suitable in later stages, although it would be impossible to precisely identify each component's contribution to the bone formation.

The assessment of the histopathological aspect of the bone transplant interface with the host is more useful in early stages of the bone integration as it can show the aspects of the cells from both components. The hypertrophic osteoblasts can be noticed even in the areas of inflammation and necrosis, as a recent published study shows [21]. The proliferation index of the bone cells in the bone lacunae as determined by Ki-67 immunohistochemistry may be a useful approach to demonstrate their involvement in new bone formation, as well as the gene and protein expression of matrix organic components, such as collagen I. Their morphometric aspect is also highly suggestive for their activation, as upon dividing and maturation their microdensity increases, and they become hypertrophic and change their shape and their circularity, as we have found out in our study.

✉ Conclusions

All of the experience animals that we have examined had viable and active transplanted bone structures. Both the osteocytes and the hyperplastic periosteum of the graft contribute to osteogenesis and to the integration of the graft into the receptor bone. The microdensity of bone cells in the graft increased significantly during the healing of the experimental fracture. The morphometric parameters of the bone cells from the graft were consistent with shape changing of those cells during maturation.

References

- [1] Hernigou P, Homma Y, *Tissue bioengineering in orthopedics*, Clin Cases Miner Bone Metab, 2012, 9(1):21–23.
- [2] Correia C, Grayson WL, Park M, Hutton D, Zhou B, Guo XE, Niklason L, Sousa RA, Reis RL, Vunjak-Novakovic G, *In vitro model of vascularized bone: synergizing vascular development and osteogenesis*, PLoS One, 2011, 6(12):e28352.
- [3] Fishman FG, Adams SB, Easley ME, Nunley JA 2nd, *Vascularized pedicle bone grafting for nonunions of the tarsal navicular*, Foot Ankle Int, 2012, 33(9):734–739.
- [4] Jones DB Jr, Rhee PC, Shin AY, *Vascularized bone grafts for scaphoid nonunions*, J Hand Surg Am, 2012, 37(5):1090–1094.
- [5] Tanaka K, Maehara H, Kanaya F, *Vascularized fibular graft for bone defects after wide resection of musculoskeletal tumors*, J Orthop Sci, 2012, 17(2):156–162.
- [6] Schwarz GS, Disa JJ, Mehrara BJ, Healey JH, Cordeiro PG, *Reconstruction of oncologic tibial defects in children using vascularized fibula flaps*, Plast Reconstr Surg, 2012, 129(1):195–206.
- [7] Willems WF, Larsen M, Friedrich PF, Shogren KL, Bishop AT, *Induction of angiogenesis and osteogenesis in surgically revascularized frozen bone allografts by sustained delivery of FGF-2 and VEGF*, J Orthop Res, 2012, 30(10):1556–1562.
- [8] Patil N, Hwang K, Goodman SB, *Cancellous impaction bone grafting of acetabular defects in complex primary and revision total hip arthroplasty*, Orthopedics, 2012, 35(3):e306–e312.
- [9] Rychlik D, Wójcicki P, *Bone graft healing in alveolar osteoplasty in patients with unilateral lip, alveolar process, and palate clefts*, J Craniofac Surg, 2012, 23(1):118–123.
- [10] Taylor BC, French BG, Fowler TT, Russell J, Poka A, *Induced membrane technique for reconstruction to manage bone loss*, J Am Acad Orthop Surg, 2012, 20(3):142–150.
- [11] Komiya H, Arai Y, Kajikawa Y, Yoshida A, Morihara T, Terauchi R, Kida Y, Fujiwara H, Kawata M, Kubo T, *The fate and role of bone graft-derived cells after autologous tendon and bone transplantation into the bone tunnel*, J Orthop Sci, 2012 Nov 14.
- [12] Lewis SJ, Kulkarni AG, Rampersaud YR, Jhaveri S, Quraishi N, Bacon SA, Magana SP, *Posterior column reconstruction with autologous rib graft after en bloc tumor excision*, Spine (Phila Pa 1976), 2012, 37(4):346–350.
- [13] Wei LC, Lei GH, Sheng PY, Gao SG, Xu M, Jiang W, Song Y, Luo W, *Efficacy of platelet-rich plasma combined with allograft bone in the management of displaced intra-articular calcaneal fractures: a prospective cohort study*, J Orthop Res, 2012, 30(10):1570–1576.
- [14] Wangsrimongkol T, Manosudprasit M, Pirmsinthavee S, *Comparison of a clinical method with two radiographic methods for assessing quality of alveolar bone grafts*, J Med Assoc Thai, 2011, 94(Suppl 6):S1–S8.
- [15] Zielak JC, Mathias SA, Giovanini AF, Mathias AL, *Oral bone grafting in a rat model and the use of scanning electron microscopy for tissue morphology evaluation*, Scand J Lab Anim Sci, 2007, 34(3):201–210.
- [16] Albert R, Vászárhelyi G, Bodó G, Kenyeres A, Wolf E, Papp T, Terdik T, Módos L, Felszeghy S, *A computer-assisted microscopic analysis of bone tissue developed inside a polyactive polymer implanted into an equine articular surface*, Histo Histopathol, 2012, 27(9):1203–1209.
- [17] Nissan J, Marilena V, Gross O, Mardinger O, Chaushu G, *Histomorphometric analysis following augmentation of the anterior atrophic maxilla with cancellous bone block allograft*, Int J Oral Maxillofac Implants, 2012, 27(1):84–89.
- [18] Duriez J, *Evolution histologique du greffon hétérogène compact utilisé dans l'arthrodèse vertébrale pour scoliose*, Acta Orthop Belg, 1967, 33(4):649–654.
- [19] Guerrero JS, Al-Jandan BA, *Allograft for maxillary sinus floor augmentation: a retrospective study of 90 cases*, Implant Dent, 2012, 21(2):136–140.
- [20] Kamal D, Trăistaru R, Alexandru DO, Kamal CK, Pirici D, Pop OT, Mălăescu DG, *Morphometric findings in avascular necrosis of the femoral head*, Rom J Morphol Embryol, 2012, 53(3 Suppl):763–767.
- [21] Kamal D, Alexandru DO, Kamal CK, Streba CT, Grecu D, Mogoantă L, *Macroscopic and microscopic findings in avascular necrosis of the femoral head*, Rom J Morphol Embryol, 2012, 53(3):557–561.

Corresponding author

Horățiu Pompiliu Petrescu, Assistant Professor, MD, PhD, Department Orthopedics and Trauma, "Victor Babeș" University of Medicine and Pharmacy, 2 Eftimie Murgu Square, 300041 Timișoara, Romania; Phone +40722–239 771, e-mail: lalusha87@yahoo.com