EVALUATION OF CENTRIFUGED OSTEOGENIC BONE MARROW IN FRACTURE CONSOLIDATION IN RABBITS

CARLOS EDUARDO SANCHES VAZ[1], ROBERTO GUARNIERO[2], PAULO JOSÉ DE SANTANA[1]

ABSTRACT

Objective: The purpose of this study was to evaluate the efficacy of a centrifuged osteogenic bone marrow aspirate to stimulate healing in rabbit fibular osteotomies. Methods: Ten white New Zealand rabbits were used. A transverse medial diaphyseal fibular osteotomy was performed on the right fibula, where an absorbable collagen sponge embedded in osteogenic centrifuged bone marrow aspirate, obtained from the ipsilateral iliac bone, was inserted. The left fibula was used as the control group, where the collagen absorbable sponge was inserted without the osteogenic centrifuged aspirate. The rabbits were sacrificed four weeks after surgery to evaluate bone callus formation. Analyses of results were performed with DEXA bone densitometry to evaluate callus mineral mass, multislice computed tomography to evaluate callus volume and histomorphometry to evaluate the relative rate of tissue formation. Results: The employment of centrifuged osteogenic bone-marrow aspirate resulted in a 40.3%, increase of callus bone mineral mass and increased relative quantity of bone tissue formation by 9.4%, without a significant increase in the relative quantities of cartilage, fibrous tissue, or in callus volume. Conclusions: This study shows that the centrifuged osteogenic bone marrow aspirate was able to improve the healing of experimental fibular osteotomies in rabbits.

Keywords: Bone marrow cells. Bone transplantation. Fracture healing. Osteotomy. Rabbits.

Citation: Vaz CE, Guarniero R, Santana PJ. Evaluation of centrifuged osteogenic bone marrow in fracture consolidation in rabbits. Acta Ortop Bras. [online]. 2010;18(6):321-6 Available from URL: http://www.scielo.br/aob.

INTRODUCTION

In recent decades, injuries caused by traffic accidents have become leaders in number of deaths, loss of years of productive activity and cost for health care systems, in all age brackets.[1] In the United States, about thirty-three million people suffer traumatic injuries of the musculoskeletal system on an annual basis, which means an incidence of 13.8 injuries to every thousand individuals. Approximately 6.2 million of these are fractures.[2]

In spite of the development of medical technology and of the better orthopedic handling of fractures in recent years, some of these still consolidate irregularly, while others take a long time to consolidate and some result in pseudoarthrosis.[3]

Although the use of an autologous bone graft is considered the standard technique to stimulate bone consolidation, several complications are associated with this procedure, including damage to the donor site, painful scars, hematomas, infection, claudication and limited supply.[4]

Tissue engineering techniques have recently been presenting promising results seeking to repair, substitute or regenerate specific organs and tissues.[5,6] In the domain of orthopedics, methods for capturing and transplantation of undifferentiated osteoblast precursor cells, use of bioactive matrixes for tissue support and local or systemic introduction of peptide hormones and growth factors are under development.[7]

Friedenstein et al.[8,9,10] demonstrated the efficacy of the transplantation of a culture of undifferentiated precursor cells, obtained from the bone marrow, in bone neoformation.

However, the culture and the transplantation of undifferentiated cells from the bone marrow are complex, costly procedures that involve a methodology restricted to large research centers.[11]

As an alternative, the direct administration of bone marrow aspirates has been clinically and experimentally researched, seeking to stimulate fracture consolidation, treat pseudoarthrosis and fill bone defects acquired due to trauma or surgical bone resections.[12-15]

All the authors declare that there is no potential conflict of interest referring to this article.

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Article received on 6/14/07 and approved on 9/3/07

The aim of this study was to assess the efficacy of a centrifuged osteogenic aspirate, obtained by means of centrifugation of bone marrow aspirates, in stimulating the consolidation of osteotomies in the fibula of rabbits.

METHODS

This study was submitted and approved by the Committees of Ethics in Medical Research of Universidade Estadual de Londrina and Universidade de São Paulo.

Ten white male New Zealand rabbits (Oryctolagus cuniculus) were used, with mean weight of 3 kg. The right hind limb of each animal was selected for performance of the surgical procedure. A transverse medial diaphyseal osteotomy was performed on the right fibula, where an absorbable collagen sponge (Gelfoam®), soaked in osteogenic centrifuged bone marrow aspirate, obtained from centrifugation of the ipsilateral iliac bone marrow, was inserted. The left fibula was used as the control group, being osteotomized in the same manner, yet only the collagen sponge was inserted at this location.

Anesthetic technique

The anesthesia protocol recommended by the Canadian Council of American Care was used in the study. Each animal received 1 mg/kg of atropine sulphate by intramuscular route, ten minutes before the start of the anesthesia, for decrease of vagal tonus. This was followed by the intramuscular application of a solution containing 40 mg/kg of ketamine at 10%, in association with 5 mg/kg of xylazine hydrochloride, in the proximal region of the pelvic limb. The mean anesthetic induction time with this technique was from 5 to 10 minutes, providing duration of approximately 50 minutes. When necessary, the anesthesia was prolonged with the fractionated administration of 1 mg/kg of xylazine hydrochloride, every 30 minutes. The rabbits were identified with numbers tattooed in the ear, soon after the anesthetic induction. A mask with a continuous flow of oxygen was maintained throughout the procedure.

Preparation of the centrifuged bone marrow aspirate

Each animal was submitted to trichotomy of the left iliac crest region. After adequate asepsis and antisepsis, a 16 G needle suitable for the performance of bone marrow punch with a 16 G trocar, was introduced into the posterior iliac crest, with delicate rotation movements, reaching the medullary cavity. The trocar was then withdrawn and a 20 ml disposable plastic syringe, the internal part of which was previously soaked in a solution of heparin at 1:1000, was connected to the needle. By means of suction by firm traction of the syringe plunger, 5 ml of bone marrow were aspirated. The material was then processed under sterile conditions in a chamber with laminar flow, prepared 30 minutes before the beginning of the procedure. The 5 ml sample from the plastic syringe was transferred to a sterile graduated tube, initially by means of 19 G needle, then with a 22 G needle, to disperse the cellular aggregates. This material was taken to a Excelsa®, model 4 centrifugue, where it was centrifuged to 400xg and to 20 ºC, for 10 minutes. After centrifugation, the supernatant, of approximately 2 ml, was discarded with a sterile Pasteur pipette. Of the material that formed a sediment in the tube, 0.5 ml were collected from the buffy-coat, which is formed by the intermediate layer between discarded plasma and the sedimented cells at the bottom of the tube where one expects to find a greater quantity of nucleated cells, which have a higher density. The hematopoietic precursors and cells of the bone marrow stroma, where the mesenchymal and progenitor cells are found, are part of these cells.

The material was homogenized, and a sample of 50 microliters was separated for the cell count and performance of cellular viability test, with the objective of avoiding the administration of dead cells that could result from the centrifugation process.

Surgical technique

The animal remained supine on an appropriate operating table. Trichotomy of the lateral region of the pelvic limb was performed bilaterally. The surgical field was covered with sterile fenestrated drapes, whereas only the region to be operated was exposed bilaterally. A lateral access route was created in the pelvic limb, approximately 2 cm long, in the region of the skin overlying the medial third of the right fibula. The next procedure was dissection by planes, covering the skin, subcutaneous cellular tissue and fascia of the fibular muscles, which were separated, dissecting in the intermuscular plan between the lateral and posterior compartments of the leg. The fibula was then exposed, avoiding the exposure of its periosteum. With the use of a Gigli wire saw, they performed complete transverse medial diaphyseal osteotomy of the fibula. (Figure 1) Afterwards a sample of absorbable collagen gelatin (Gelfoam®) of 0.5 cm^2 was imbedded with the centrifuged osteogenic aspirate prepared in the laboratory (approximately 0.5 ml), and placed in the osteotomy region. (Figure 2) The surgical wound was closed by planes with 4-0 mononylon thread, and covered by an impermeable sterile dressing. The same procedure was carried out on the left fibula, only just the Gelfoam® was applied at the site.

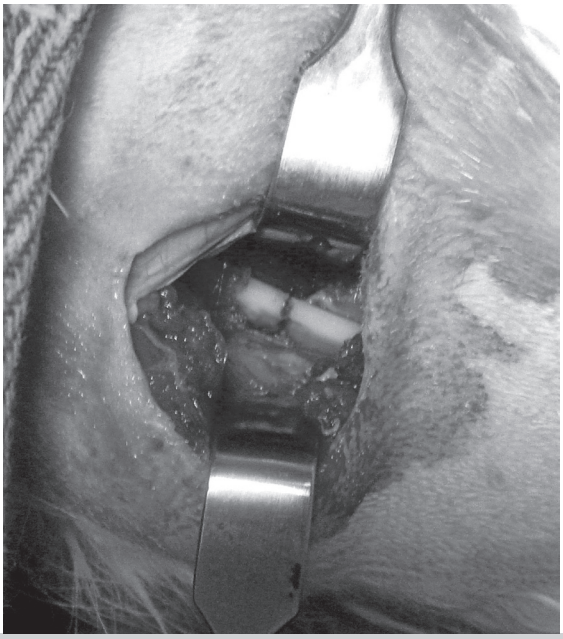

Figure 1 – *Image of the medial diaphyseal fibular osteotomy.*

Postoperative period

Immediately after the end of the surgical procedure, the animal was containerized in an appropriate box, heated and isolated from the others. An intramuscular dose of pencivet® ppu, an antibacterial and anti-inflammatory drug, was administered to the animals, containing the benzylpenicillins G benzathine and procaine, dihydrostreptomycin and piroxicam. After total anesthetic recovery, the rabbits were returned to their cage in the

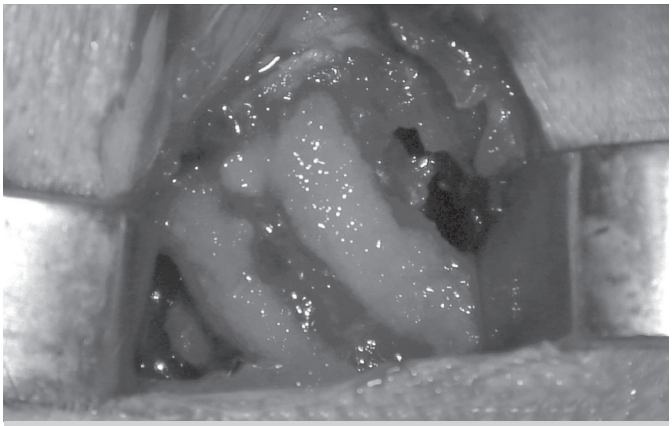

Figure 2 – *Image of the collagen sponge imbedded in the centrifuged osteogenic aspirate and applied at the site of the fibular osteotomy.*

biotherium, receiving water and feed *ad libitum*. The total load on the operated limbs was permitted immediately, without any type of restriction or immobilization. At the end of one week, the dressing and the surgical stitches were removed. The animals were sacrificed thirty days after the date of surgery. They were anesthetized via intramuscular route, in the proximal region of the pelvic limb, with a solution containing 120 mg/Kg of ketamine at 10%, in association with 10 mg/Kg of xylazine hydrochloride, followed by intracardiac administration of a solution of potassium chloride at 20%.The animals' legs were disarticulated at knee and ankle level, with the performance of careful dissection of the piece, removing all the soft parts, taking care not to injure the bone callus. The pieces were placed in suitable containers with fixing solution and were adequately identified.

Evaluation criteria and measuring methods

The results were evaluated by densitometric, volumetric and histological (qualitative and quantitative) measurement of the bone callus formed at the site of the fibular osteotomy, comparing right and left sides (control group).

The mineral density of the bone calluses in g/cm^2 was measured with Lunar DPX-A densitometer with specific software for small animals.

The volume of the bone callus formed was measured with multislice helical computed tomography using a multi-slice scanner with eight rows of detectors, light speed ultra model (General Electric Medical Systems - Milwaukee, WIS, USA). A three-dimensional reconstruction of the bone piece studied was performed, and after the selection of the callus area (Figure 3) its volume was calculated in mm^3.

This was followed by the decalcification and paraffin embedding of the pieces. The paraffin blocks containing the bone callus samples were submitted to histological cuts in a thickness of 3μm, in the coronal direction, with rotating microtome and stained by the Masson trichome technique. The comparative qualitative evaluation of the tissues formed in the osteotomy area was conducted with the help of an Image Analysis System (Kontron Electronic 300, ZEISS®). The results obtained in each field corresponding to the area of the different tissues analyzed (bone, cartilage and fibrosis) were submitted to statistical analysis.

For the analysis of the specific greatnesses (variable), the descriptive statistics of the ordinal samples (quantitative) were performed: mean (M), standard deviation (SD) and standard error of the mean (SEM).

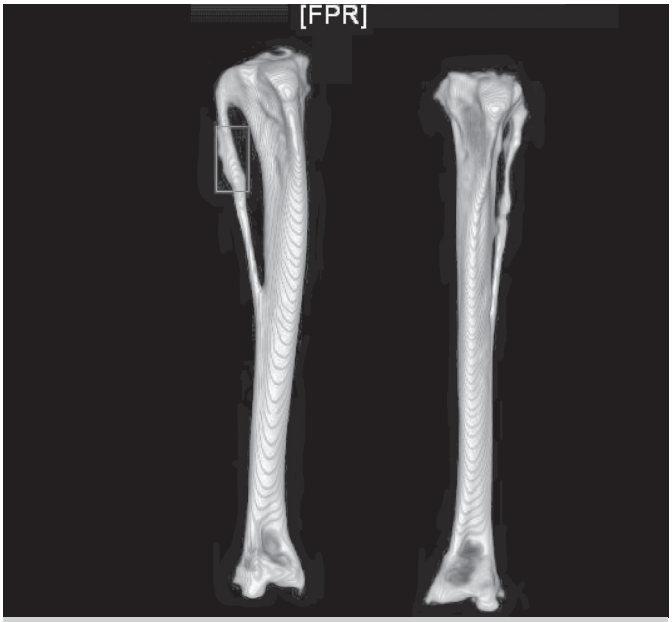

Figure 3 – *Comparative three-dimensional reconstruction of the anatomical parts of the hind limbs of the rabbit.*

The normality of the distributions was tested by the Kolmogorov-Smirnov test(K-S) for continuous variables and by Pearson's Coefficient of Variation(PCV). The distribution of a sample was considered normal when the Kolmogorov-Smirnov test presented p≥0.05 and Pearson's Variation Coefficient was equal to or lower than 30% (PCV ≤ 30%).

The descriptive statistics of the greatnesses (samples) were presented in statistical tables and represented in the form of column graphs (mean ± standard error of the mean): bone mineral density of the bone callus, volume of the bone callus, relative quantity of bone, of cartilage and of fibrosis in the bone callus.

In the comparisons between the fibulas treated with osteogenic preparation and control group, the paired t-test was used for inference on the difference of the mean values of related (paired) and parametric samples and the Wilcoxon test for the related (paired) and non-parametric samples.

Scientific rounding was used. In the tables, the values of the descriptive statistics, the results of the statistical tests and the probability (p) were presented with two decimal places after the point or up to the first significant number.

The differences proved statistically were evidenced by asterisks (*) in the tables.

The confidence level of 5% ($\alpha = 0.05$) was used.

Bilateral or bicaudal tests were adopted (H0 = $\mu_1 - \mu_2 = 0$).

The participants used the statistical program GraphPad Software, Inc.® (1996) Graphpad Prism, version 2.01.

RESULTS

The result of the count of nucleated cells and of the cellular viability test employed after the centrifugation process of the bone marrow aspirates are presented in Table 1.

The comparative analysis by means of bone densitometry revealed greater bone mineral density in the calluses of the osteotomies where the osteogenic centrifuged aspirate was administered. (Table 2 and Figure 4) There was no significant difference in the volumetric measurement of the bone callus. (Table 3 and Figure 5) The quantitative histological analysis of the tissues

Table 1 – *Result of the count of nucleated cells and of the cellular viability test of the centrifuged bone marrow.*

RABBIT	VOLUME OF BUFFY-COAT (ml)	MEAN COUNT (10^7.cells/ml)	NUCLEATED CELL						Cellular viability (%)
			Viable			Non-viable			
			1st	2nd	M	1st	2nd	M	
1	1.0	7.6	16	20	18	1	1	1	94.7
2	0.5	10.0	105	85	95	5	6	5	95.0
3	0.5	3.5	29	36	34	1	1	1	97.1
4	0.5	16.4	144	171	158	4	8	6	96.3
5	0.5	5.1	50	48	49	2	2	2	96.1
6	0.5	6.9	75	52	64	7	2	5	92.7
7	0.5	9.9	113	76	95	3	5	4	95.6
8	0.5	2.8	28	25	27	1	1	1	96.4
9	0.5	9.6	98	92	95	0	1	1	99.0
10	0.5	8.2	83	79	81	1	1	1	98.8

where: 1st = First count 2nd = Second count M = Mean count

Table 2 – *Comparative statistics of the bone mineral density of the bone calluses formed in the fibular osteotomies in the rabbits.*

RABBIT	MINERAL DENSITY OF THE BONE CALLUS (10^{-3}.g/cm^2)	
	Osteogenic Preparation	Control Group
1	72	82
2	79	68
3	80	40
4	87	62
5	56	37
6	70	54
7	93	55
8	85	50
9	85	72
10	99	76
M	80.60	59.60
SD	12.32	15.06
SEM	3.90	4.76
PCV (%)	15.29	25.26
K-S	p > 0.10	p > 0.10
t -paired	t=4.43	p=0.002*

Table 3 – *Comparative statistics of the volume of the bone calluses formed in the fibular osteotomies in the rabbits.*

RABBIT	VOLUME OF BONE CALLUS (mm^3)	
	Osteogenic Preparation	Control Group
1	144	248
2	314	268
3	282	227
4	203	102
5	230	163
6	137	50
7	108	94
8	180	131
9	165	102
10	161	109
M	192.40	149.40
SD	65.63	74.09
SEM	20.75	23.43
PCV(%)	34.11	49.59
K-S	p > 0.10	p > 0.10
Wilcoxon	w=35	p=0.08

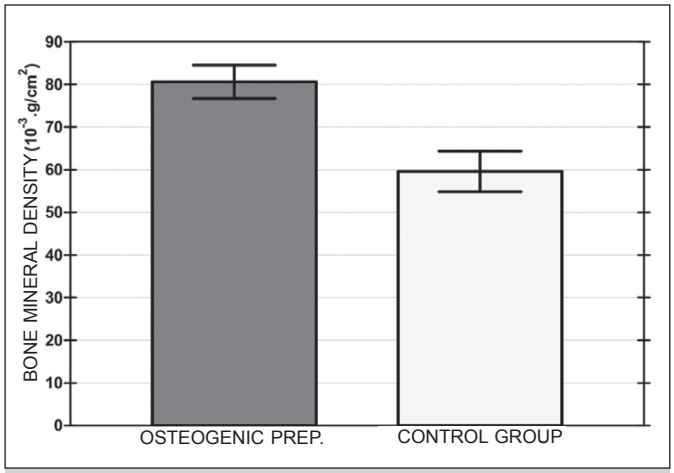

Figure 4 – *Comparison of the bone mineral density of the bone calluses formed in the fibular osteotomies in the rabbits.*

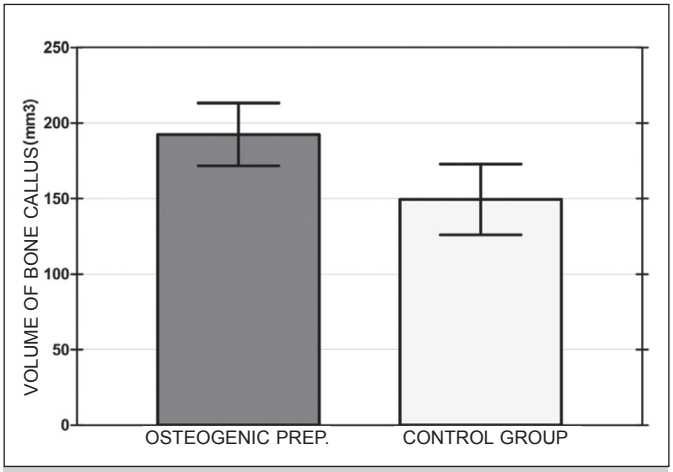

Figure 5 – *Comparison of the volume of the bone calluses of the fibulas treated with osteogenic preparation and control group.*

formed demonstrated a significantly higher percentage of bone tissue in the callus of the osteotomies where the osteogenic centrifuged aspirate was added. (Table 4 and Figure 6)

DISCUSSION

The increasing demand for methods capable of guaranteeing fracture consolidation, filling bone faults and healing pseudo-arthrosis has stimulated a significant number of surveys with a view to the isolation and development of supplements and alternatives to the autologous bone graft.[16]

In spite of the efficacy demonstrated by the therapy of addition of cultures of mesenchymal progenitor cells of bone marrow, this treatment still represents a major technical and logistic challenge, not reproducible at the majority of common treatment centers.[17]

Table 4 – *Comparative statistics of the relative quantity of bone (%) in the bone calluses formed in the fibular osteotomies in the rabbits.*

RABBIT	RELATIVE QUANTITY OF BONE (%)	
	Osteogenic Preparation	Control Group
1	88.90	81.82
2	63.83	43.25
3	97.93	89.13
4	59.63	52.88
5	83.88	96.25
6	100.00	95.85
7	88.17	85.43
8	80.66	70.27
9	85.27	74.26
10	72.85	61.68
M	82.11	75.08
SD	13.31	18.07
SEM	4.21	5.71
PCV(%)	16.21	24.07
K-S	p > 0.10	p > 0.10
t-paired	t=2.65	p=0.03*

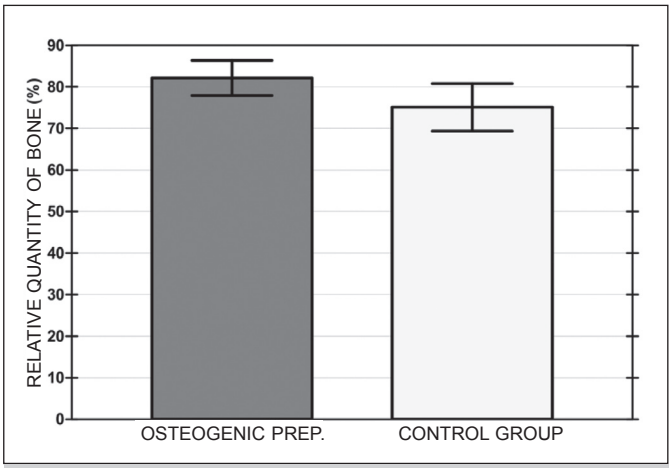

Figure 6 – *Relative quantity (%) of bone in the bone callus of the fibulas treated with osteogenic preparation and control.*

The use of bone marrow aspirate has been applied in this sphere. This technique has the advantage of presenting a much lower morbidity rate than that of the removal of autologous bone graft, and can be added together with an osteoconductive matrix thus forming a composite graft that becomes an effective substitute for the former. However, the unfractionated bone marrow aspirate has only moderate osteogenic potential. Even in healthy adults, it is estimated that one of every 50,000 nucleated cells of the bone marrow is capable of differentiating itself in an osteoblast.

Due to these factors, we seek, in this study, to carry out a bone marrow aspirate centrifugation process[14], aiming to increase the effective concentration of mesenchymal progenitor cells. Our results demonstrated, through the direct counting of cells and the performance of cellular viability tests, that we manage, on average, to triple the number of nucleated cells present in the buff-coat, without causing significant mortality of these cells. Connolly et al.[12,13] compared the number of nucleated cells present in bone marrow aspirates with that obtained after several centrifugation methods. The mean number of nucleated cells in the bone marrow aspirates was 2 X 10^6 cells per milliliter, while the same number after simple centrifugation was 3.6 X 10^6 cells per milliliter. Deploying the different osteogenic preparations obtained after centrifugation in diffusion chambers placed in the peritoneal cavity of rabbits, they proved that osteogenesis inside the chambers is directly proportional to the cellular concentration of the osteogenic solution.

Our results demonstrated that the use of the osteogenic centrifuged aspirate induced greater bone mineral density in the calluses of the osteotomies, but did not lead to the formation of a callus of increased volume. As there was no significant difference in the volumetric measurement of the bone callus, we can infer that the addition of the osteogenic centrifuged aspirate resulted in a qualitative improvement of the bone callus, a fact corroborated by the histomorphometry, which demonstrated a greater percentage of bone tissue in these calluses.

Most experimental studies that evaluate bone consolidation use different radiographic methods for measurement of the bone callus formed, without an acceptable standardization.[14,18,19] Variables such as X-ray exposure time, distance from the tube, film used, quantity of X-ray applied, and others, are not uniform. A simple more prolonged exposure or a higher X-ray charge may mask the true volume of bone callus formed. For this reason we opted for the multi-slice helical tomography, which has the advantage of allowing a three-dimensional evaluation of the structure studied. The sections of the images acquired by the scanner are processed in a computerized workstation that reconstructs the image and allows its visualization in movement in real time. Performing a visual comparison of the callus in the normal radiographies with that obtained with the multi-slice tomography, we verified that in the radiographies this volume always appears lower, since in the initial phases of consolidation the calluses are not yet totally calcified. Accordingly, they are not visualized in full in common radiographies.

Although previous studies value the volume of the bone callus as a factor related to quality of bone consolidation, Boer et al.[20] demonstrated that the relation between the volume of the bone callus and its mechanical properties is more important in the initial phases of bone consolidation, when the callus is still increasing in volume. In the late phases, the natural course of healing leads to a decrease of the total volume, characterized by reabsorption of the periostal callus. Taking into account the

density of the isolated callus, the product of the volume of the callus by its density did not improve the correlation with the biomechanical properties, indicating that the volume of the callus was not an important variable in the determination of resistance of the consolidating bone.

In spite of the positive results obtained in this study, the sample used can be considered insufficient for the transposition of results to clinical practice. However, this was due to the new determinations of the Animal Experimentation Research Committees, which emphasize the need to use as few animals as possible to produce an acceptable statistical result. Although our individual sample is small (10 rabbits), in reality, the use of both hind limbs in a comparative manner functions as if they were 2 separate groups of 10 animals, a "control group" and a "treatment group", thus totaling 20 animals.

Another negative factor of this study was the lack of analysis of biomechanical resistance of the callus formed in the osteotomies, an important factor to consider the quality of bone consolidation.[21] This shortcoming was once again due to the small sample employed, as the performance of the biomechanical tests damages the anatomical piece and thus prevents the performance of the histomorphometry subsequently. Nonetheless, we can consider the study by Blokhuis et al.[22] who, comparing the results of the bone densitometry with DEXA with biomechanical tests, observed a positive correlation between the increase of values thereof with the increase of mechanical resistance of the bone callus. These authors conclude that the increase of mineralization of the callus during the bone consolidation process can be tracked by the bone densitometry, and results in greater biomechanical stiffness of the bone callus.

We sought, with this study, to contribute toward future studies seeking alternatives to the use of the autologous bone graft, and to find routes other than the use of mesenchymal stem cell cultures, the complexity and cost of which render their use on a large scale practically unviable. The osteogenic preparation obtained by simple centrifugation of bone marrow aspirates is a method of easy reproduction, fast and that does not require expensive equipment or specialized labor. As the new bone repair techniques continue to evolve, new studies are necessary to define their efficacy, indications and transportation to daily clinical practice.

CONCLUSION

In this study, the use of centrifuged osteogenic bone marrow favored the consolidation of experimental osteotomies on the fibula of rabbits, resulting in a qualitative improvement of the bone callus.

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