Effect of autologous stem cells on regenerated bone during distraction osteogenesis by Ilizarov technique in the radius of dogs. Histomorphometric analysis[1]

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ABSTRACT

PURPOSE: To investigate by histomorphometry the distraction osteogenesis by Ilizarov technique in dog radius with the use of autologous stem cells in regenerated bone.

METHODS: Ten dogs (20 radiuses) underwent the osteotomy of 20% of extension of their radiuses, and osteogenic distraction using the Ilizarov technique after this procedure at rate of 1mm per day divided into 0.5mm every 12 hours. The elongation was performed until the regeneration reached 20% of the total length of the radius. The stem cells were isolated, concentrated and injected in the regenerated bone, when it reached 10% of the length of the entire radius. The regenerated bone was evaluated using histomorphometric analysis when the elongation was 20% the size of radius.

RESULTS: The bone formation was evidenced by histomorphometric indices were significantly greater in the study group. In the histology evaluation the type of healing was mixed in 80% (intra membrane and endocondral) in both groups; the osteoblastic activity from moderate to intense was greater in the study group; and the space occupied by the newly-formed bone tissue was more evident in the study group.

CONCLUSIONS: The histomorphometric indices in this study expressing the microarchitecture, trabecular thickness, trabecular spacing, number of trabecula and quantity of bone that was significant in the group study. These data suggest that the use of undifferentiated stem cells autologous bone marrow in the regenerate bone induces osteogenesis and bone quality.

Key words: Ilizarov Technique. Osteogenesis, Distraction. Bone Regeneration. Stem Cells. Dogs.

Introduction

The correction of faults and shortenings bone remains a complex problem and difficult to solve[1-5], it requires long-term treatment, multiple surgeries, high complication rates and functional outcomes questionable[1,6,7].

Ilizarov in 1951 revolutionized the treatment of musculoskeletal disorders through the concept of distraction osteogenesis using circular external fixator[8-11]. The technique uses biological and mechanical principles. After a corticotomy followed by slow and progressive distraction of the osteotomized bone segments, through a bone distractor generating a mechanical stress voltage at the fracture (osteotomy), which stimulates the formation of regenerated bone, and hence the growth of adjacent soft tissue, filling the space created by the osteotomy[8,12].

Theoretically, there is no limit to the bone stretches by the Ilizarov technique[4,9,10,13]. However, one of the most limiting factors of this technique is the long-term required to consolidate the newly formed bone. This occurs mainly in large bone defects and shortening in that prolonged with the external fixator is associated with complications such as joint stiffness, subluxation or luxation, soft tissue contracture, infection, instability and loosening of the fixator pins or wires[9].

The autologous cancellous bone of the bone marrow is considered the best material for bone graft, not only for its osteoinductive and osteogenic properties, but also because it is not immunogenic, do not transmit infectious diseases and have lower cost of production[14].

Nevertheless, the large bone defects, the required amount of autologous bone or the donor area morbidity prevents and limits its use[15,16]. Aiming to replace or assist the autologous bone graft substitutes were devised, such as graft heterologous and homologous bioceramics, whose use revealed complications such as rejection, instability and infections[17].

Studies aiming to improve the biological characteristics of bone allografts are described in the literature[18,19]. Among which is the enrichment of allograft with autologous bone marrow is rich in undifferentiated precursor cells osteoblasts[20]. Bone marrow stem cells have the potential to differentiate in cell lines involved in the repair of bone lesions. These cells play an important role in osteogenesis or phagocytosis, depending on the conditions of the medium[21]. However the use of autologous bone marrow is unable to fill large bone defects, and in those cases, associate it with grafts or bone substitutes[10].

The treatment of large bone defects gained new strand at the end of last century and early this century, with the adjunctive use of stem cells obtained from bone marrow, growth factors platelet-derived and bone morphogenetic proteins. These biological agents commonly are used in order to promote or accelerate the consolidation process or osteointegration, in association with bone grafts or substitutes, not as autologous grafts and bioceramics[19].

The objective of this study was to analyse by histomorphometry the distraction osteogenesis by Ilizarov technique in dog radius using autologous stem cells in regenerated bone.

Methods

The project was approved by Ethics Committee on Animal Research, Federal University of Pernambuco (UFPE), nº 23076.020444/2008-49. Experimental study accomplished in the Center for Experimental Surgery, UFPE, in the period from September 2006 to January 2008.

Twenty two radius of the right forearm and left 11 adult crossbred dogs were used, with weight varying from 15 to 20 kg, average age of 4.5 years and clinically healthy. One dog was excluded from the study during the control procedure by failure of osteosynthesis material (breaking a Kirschner wire), compromising the regenerated bone during distraction osteogenesis phase.

The groups were formed by the forearms dogs (radius), determining by lot, which, for each dog, the group I (control) was constituted by the right forearm and the left as group II.

Preoperative planning

Panoramic radiographs in anteroposterior and lateral animal's forearm were performed before the surgery to determine the extention of osteotomy of 20%, after the end of surgery and the every each seven day, for monitoring and to determine the total time of distraction osteogenesis (TTDO). It was assumed an elongation of 20% of the total length of the radius of each dog at the rate of 1 mm/day.

Anesthetic and surgical procedures

Anesthesia was induced with sodium pentobarbital (12.5 mg/kg body weight) intravenously, following general anesthesia conducted with ketamine hydrochloride (2.5 mg/kg body weight) and pancuronium bromide (0.1 mg/kg body weight 0.2 mg/kg body weight). The antibiotic prophylaxis was performed using the protocol of Rahal *et al.*[7] The assembly of Ilizarov apparatus was composed of four rings of stainless steel 100mm diameter, two on

each end, connected by four threaded rods

The circular Ilizarov external apparatus was fixed at each end of the forearm bones, according to Ilizarov technique modified by Catagni *et al.*[10]. Then there was a transverse osteotomy at the diaphysis of the radius and ulna[10].

Postoperative procedures

After seven days was initiated elongation of the bones in the forearm rate of 1 mm/day, divided into 0.5 mm every 12 hours.

In the control group, the distraction was carried out continuously until the regenerated obtain a length of 20% of the total size of the radius. In the study group at the time at which the same completed 10% of the total length of the radius, i.e., 50 % elongation provided the animal was prepared for collection of bone marrow and subsequent inoculation of autologous stem cells (TIC) within the regenerated bone.

Stem cells procedures

Bone marrow was collected by percutaneous puncture of the femoral medullary canal of the animal studied, internal condyle of the femur to the contralateral forearm previously operated. After puncture, proceeding to the aspiration of bone marrow 50mL in a 60mL syringe containing 10mL of low molecular weight heparin.

The bone marrow aspirate was conducted to the laboratory for primary isolation and concentration of autologous stem cells. The concentrated CTI, volume of 2mL, was injected into the center of the regenerated bone through the left radius by Jelco 20-gauge.

After the consolidation of the bone segment lengthened the both, right and left radius, the animal underwent a new surgical procedure for resection in block of the regenerate bone, which was preserved in 10% formalin for preparation of histological slides with three cross-sections of 5μm thick, with respect to the longitudinal axis regenerated. The obtained sections were stained with hematoxylin and eosin (HE) and Masson Trichrome (TM) for histmorphometric study.

After collecting part (regenerated bone), the compression was performed at the site of bone defect using the same fixator. This was removed after clinical and radiographic pseudarthrosis site created previously. The animals were donated to institutions for animals.

Histologic and morphometric procedures

The capture of images for histomorphometric analysis was performed using a Motic digital cameras (Micro-Optic Industrial Group Ltd.COTM), attached to a Nikon microscope trinocular®, connected to a laptop computer. The histological images were processed and analyzed by morphometry program ImageJTM 1:42 version, with specific plug-ins.

The program Adobe Photoshop™ version CS4 Extended, Adobe™, was used to assist the processing of images, as well as segmentation and selection of areas of interest with the histological mask tool, allowing the measurement of histomorphometric parameters.

Histomorphometry was analyzed using 10 fields (corresponding to 869.65 μm per photo) in each histological section with x10 magnification, and 20 fields with x40 magnification.

The following histomorphometric indices were calculated: *Percentage of new bone mineralized* [TOTAr (%)], *Bone Volume* [BV/TV (%)], *Trabecular Thickness* [Tb.Th (μm)], *Number of Trabeculs* [Tb.N (mm)], *Trabecular Spacing* [Tb.Sp (μm)] and *Osteoblastic Surface* [Ob.S/BS (%)]. The percentage of areas of fibrous tissue [FTAr (%)], cartilaginous tissue [CgAr(%)], the total area of tissue [T.Ar(%)], percentage of hypertrophic osteoblasts [OHT (%)] and hyperplastic osteoblasts [OHP (%)] were measured to assist in further histological evaluation[22].

The type of bone, osteoblastic activity and proliferation of connective tissue areas were analyzed in the histological sections by optical microscopy with x20, x40 and x100 magnification. To classify the type of bone healing were evaluated 20 fields with x40 magnification in slides stained with T.M. were measured into account the percentage of CgAr(%), FTAr (%) relative to the analyzed total area [T.Ar(%)]. In the type of bone joint was predominantly considered OE, when the percentage area of the cartilaginous tissue was at least 5% larger than the area of the membranous tissue. The types of osseous consolidation were considered: endochondral (OE), intramembranous (OI) and mixed (endochondral and intramembranous).

The evaluation of osteoblastic activity was performed by counting hypertrophic osteoblasts and hyperplastic osteoblasts (OHT) and total number of osteoblasts (OT), in 20 random fields with x100 magnification in slides stained with HE.

The osteoblastic activity was considered high when the sum of the percentage of OHP and OHT was greater than 50% of all osteoblasts sample; moderate activity, while the percentage of the sum of OHT and OHP was less than 50% and 10% greater; the low activity when the sum of the percentage of OHT and OHP

was less than 10% of the total count of osteoblasts. The percentage occupancy of connective tissue, bone or not, was calculated with respect to the total area of the tissue [T.Ar.(%)] of 20 random fields of stained slides with HE. The TAr took excellent consideration, and the percentage was equal to or greater than 90%; good when less than 90% but equal to or greater than 50%, regular, when less than 50% and equal to or greater than 10%, and bad when T.Ar was less than 10%[22].

The histomorphometric data were stored directly in Microsoft Excel spreadsheets. As of quantitative variables (histomorphometric indices and healing time clinical and radiographic - TCCT) were expressed as mean and standard deviation (descriptive statistics). Test was used for statistical t-Student for paired samples. As qualitative variables (osteoblastic activity, occupation of newly formed tissue, bone and type of bone healing) were distributed in contingency tables. These variables were analyzed using Stuart-Maxwell nonparametric test.

The statistical program used was the Statistical Analysis System (SAS), version 8.0. The significance level for rejection or acceptance of the null hypothesis was 5.0%.

Results

The total time of Clinical and radiographic healing (TCCR) of the regenerated bone in the control group varied from 62 to 155 days (mean of 117.4 ± 28.0 days), and in the study group ranged from 47 to 78 days (mean 64.3 ± 8.2 days) (Figure 1).

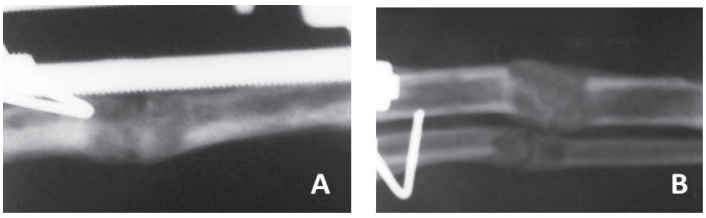

FIGURE 1 - Radiographs in AP two months after the osteotomy (**A**) and 43 days after inoculation of undifferentiated cells of bone marrow (**B**).

The average values of the indices related to histomorphometric number of trabecular [Tb.N (mm)], bone valume [BV/BT (%)], trabecular thickness [Tb.Th (μm)], trabecular spacing [Tb.Sp (μm)], and osteoblastic surface [SO (%)], the control and study groups are in the Table 1.

TABLE 1 - Histomorphometric rates of regenerate bone per groups.

Histomorphometric Rates	Groups				p[(1)]
	Control (n=10)		Study (n=10)		
	Average	Standard deviation	Average	Standard deviation	
Number of trabeculae [Tb.N (mm)]	6.86	1.97	14.30	4.86	0.0019
Bone Volume [BT/TV (%)]	36.23	12.16	59.18	5.63	0.0005
Trabecular Thickness [Tb.Th (μm)]	94.23	41.25	161.46	36.132	0.0116
Trabecular Spacing [Tb.Sp (μm)]	173.67	65.46	112.73	33.69	0.0231
Osteoblastic Surface (%)	42.31	18.13	70.52	16.24	0.0073

Observation: [(1)]Test t of Student

The type of bone healing was similar in the control and study groups. In the control group, the type of endochondral ossification purely occurred in one (10%) radio, intramembranous ossification, also in 1 (10%) radio, and mixed ossification was seen in eight (80%) radius (Table 2, Figure 2).

In the study group, there was one (10%) radio with OI, one (10%) case with OE, and eight (80%) radios with mixed ossification, among which three (30%) had a prevalence of OI, three (30%), predominance of OE. In the other two (20%) radios, there was no predominance of one type of ossification on the other (p = 0.9999).

TABLE 2 - Distribution of the types of ossification of the regenerated bone in control and study groups.

Type of Healing	Groups			
	Control (n=10)		Study (n=10)	
	N	%	n	%
OE	1	10	1	10
OI	1	10	1	10
Prevalence of *OE*	2	20	3	30
Prevalence of *OI*	2	20	3	30
Without prevalence	4	40	2	20
TOTAL	10	100	10	100
p(1)		0.9999		

Observation: (1)Stuart-Maxwell Test[2]
Legend: OE = endochondral ossification; OI = intramembranous ossification

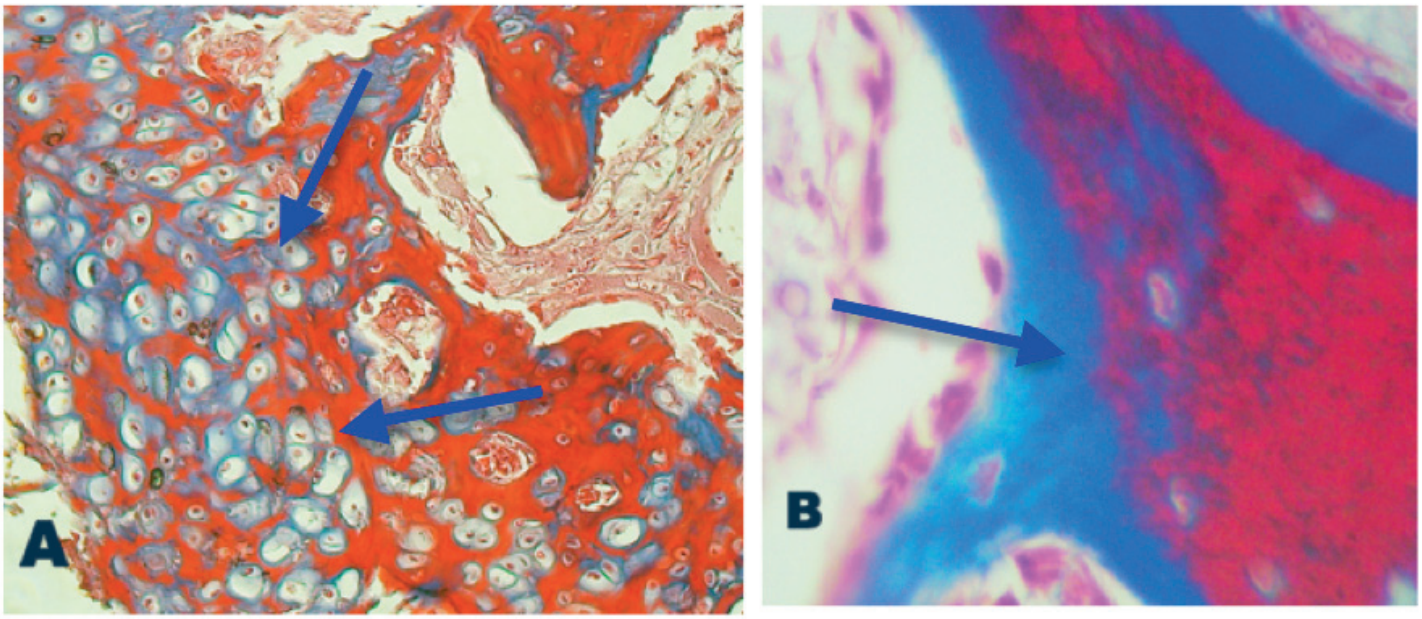

FIGURE 2 - Histological aspects of ossification. **A.** Endochondral. **B.** Intramembranous (x100).

The osteoblastic activity in all radios in the study group was distributed equally between moderate and high. In the control group, prevailed moderate osteoblastic activity, present in seven (70%) cases diagnosed as being high in one (10%) cases, and poor in two (20%) cases (Table 3, Figure 3). These differences in osteoblastic activity between the regenerated radius, control and study group were not significant (p = 0.9999).

TABLE 3 - Distribution of osteoblastic activity in bone regenerated radios in the control and study groups.

Osteoblastic Activity	Groups				p(1)
	Control (n=10)		Study (n=10)		
	n	%	n	%	
Higher	1	10	5	50	
Average	7	70	5	50	
Lower	2	20	-	-	
Total	10	100	10	100	0.9999

Observation: (1)Stuart-Maxwell Test

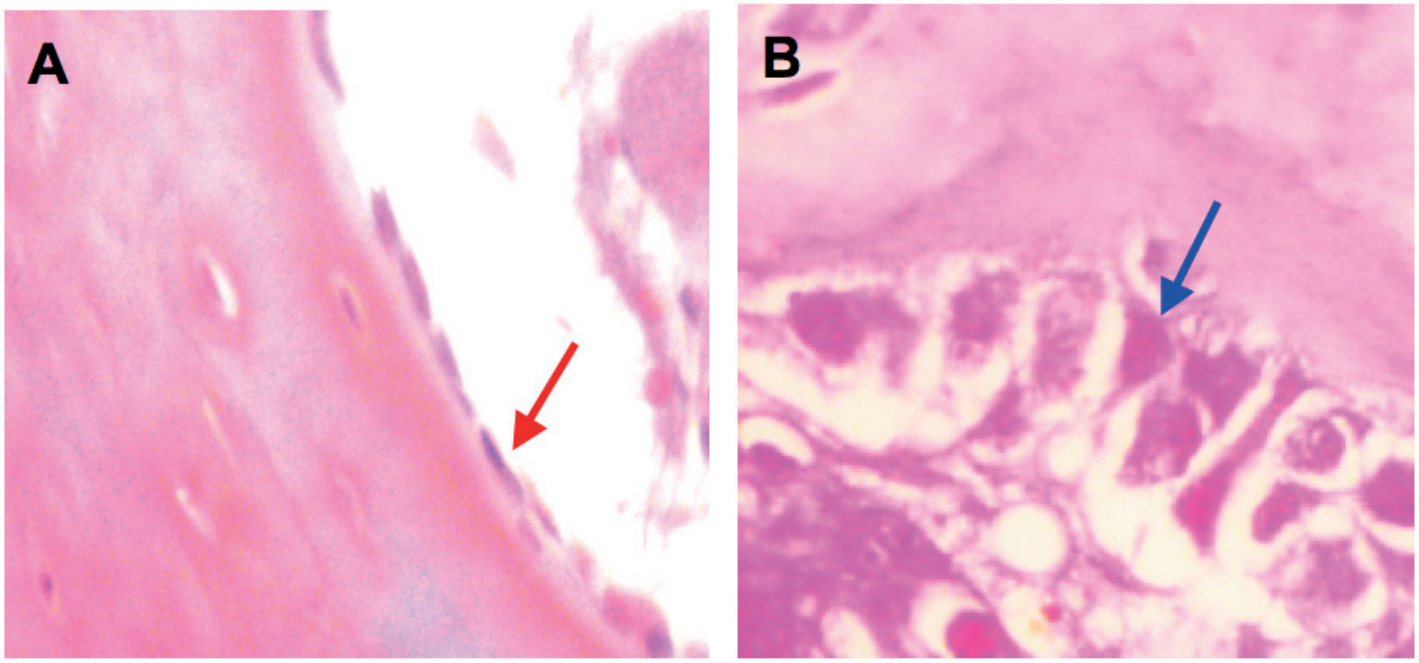

FIGURE 3 - Osteoblasts (x100 magnification - HE). **A.** Osteoblasts fusiform (red arrow). **B.** Osteoblasts hypertrophic/hyperplastic (blue arrow).

The percentage of the total area of the occupation in fields of histological tissue (bone and conective tussue) are described in Table 4.

TABLE 4 - Total area of occupancy of the fields by connective tissue in the control and study groups.

Percentage of total area of occupancy	Groups				p[1]
	Control (n=10)		Study (n=10)		
	n	%	n	%	
≥ 90	4	40	7	70	
50 ├ 90	4	40	3	30	
10 ├ 50	1	10	-	-	
≤ 10	1	10	-	-	
Total	10	100	10	100	0.705

Observation: [1]Stuart-Maxwell Test

In the control group, four radius, the percentage of occupation by bone and connective tissue, was greater than 90%, in four radius was in the range of 50% to 90%, in one (10%) if the occupation was lower 50% and at one (10%), less than 10%. In the study group, the occupation with bone and other connective tissues exceeded 50% in the ten radius, among which seven (70%) were occupied by more than 90% of the analyzed area and in three (30%) radios, the occupation was between 50% and 90%. In a significance level of 5%, with no significant difference between groups (p = 0.705).

In the observational study of the fields with respect to the histological type of tissue proliferated, it was found that areas not filled by bone tissue were occupied by fibro-cartilage, bone marrow or gaps still remained. The fibrous tissue and cartilage predominated in central regenerated. Only in a radius (control group) was observed a central area with significant amount of granulomatous tissue, rich in fibroblasts. There was no microscopic field with tissues or cells that characterize the infectious process, neoplastic, or teratogenic formation of heterotopic tissue areas analyzed.

Discussion

Data from this study suggest that inoculation of undifferentiated cells of bone marrow can reduce the total time of consolidation of the regenerated bone during distraction osteogenesis by Ilizarov technique, and promote bone formation in greater quantity and quality, as evidenced by these data histomorphometric indices. Additionally, the technique is relatively safe as secondary infections and neoplastic degeneration. It was also observed that the greater the distraction frequency, the better the outcome[8].

The histomorphometric rates (the number of trabecular per millimeter [Tb.N (mm)], bone volume [BV/BT (%)], trabecular thickness [Tb.Th (μm)], trabecular spacing [Tb.Sp (μm)], the average percentage of osteoblastic surface [SO (%)]), in the group that received the stem cells were higher of than in the control group. These differences were significants which demonstrates the effectiveness of undifferentiated cells of bone marrow as a factor that induces bone regeneration.

The histomorphometric rates for the number of trabecular per millimeter [Tb.N (mm)] was higher (14.3 ± 4.86 mm) and than in the control group (6.9 ± 2.0 mm). This difference was on average 9.4 mm greater in the study, representing a significant difference (p = 0.0019) represents a good quality of the regenerated bone in the group treated with stem cells in the bone marrow.

The average value of bone volume [BV/BT (%)] was lower in radius in the control group was (36.2 ± 12.2%) than and in the study group (59.2 ± 5.6%). The difference of 47.0% between these averages was significant (p = 0.0005). Radius in the study group, the average of trabecular thickness [Tb.Th (μm)] in the radius of the study group was higher (161.4 ± 36.1 μm) and *than* in the control group (94.2 ± 41.2 μm). The difference between these averages (67.2 μm) was significant (p = 0.0116).

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The average of trabecular spacing [Tb.Sp (μm)] in radius in the control group was (173.67 ± 65.46 μm) was higher than and in the study group (112.73 ± 33.69 μm). The difference of 60.94 μm between these averages was significant (p = 0.0231). The average percentage of osteoblastic surface [SO (%)] of the radius in the study group was (70.52 ± 16.24%) was higher than and in the control group (42.31 ± 18.13%). The difference between these averages of 28.21% was significant (p = 0.0073).

The average of trabecular spacing [Tb.Sp (μm)] in radius of control group was (173.67 ± 65.46 μm) it was higher than and in the study group (112.73 ± 33.69 μm). The difference of 60.94 μm between these averages was significant (p = 0.0231). The average percentage of osteoblastic surface [SO (%)] of the radius in the study group was (70.52 ± 16.24%) was higher than and in the control group (42.31 ± 18.13%). The difference between these averages of 28.21% was significant (p = 0.0073).

All results from the methods applied in this study to assess bone regeneration indicated that the concentrated of autologous stem cells of bone marrow induces better quality of the regenerated in less time.

Comparing results this study with those of others in which the biological agent (stem cells obtained from bone marrow, growth factors platelet-derived and bone morphogenetic proteins) was introduced at the beginning or after the end of distraction osteogenesis, and the effects were similar[19]. Despite, this comparison should be made with care by the difference in methodology in relation to the biological agent used. The use of autologous undifferentiated stem cells promoted similar histological response to a cost-effective and less favorable.

In the present experiment, the type of consolidation was regenerated mixed (endochondral and intramembranous ossification) in 80% of radios in both the control group and the group study. There was no predominance of one type of ossification on the other (p = 0.9999).The endochondral finding ossification during distraction osteogenesis were assigned by Ilizarov[8] to the frame instability of the fixator. However the findings of endochondral ossification in this study were not attributed to this factor, because we followed the biomechanical criteria followed in other experiments, which used similar settings[2,4,6].

Fink *et al*.[20], after histomorphometric studies of the type of ossification of the regenerate bone in distraction osteogenesis by Ilizarov technique that underwent 24 dogs, concluded that the formations endochondral and intramembranous occurred at a ratio of 5:1, and not were due to adverse situations as instabilities Ilizarov fixator[19,20]. Among the cases of mixed ossification, five cases occurred predominantly in one type of ossification, being three endochondral and two cases intramembranous. In the other three (30%) radios, there was no predominance of one type of ossification over the other.

The autologous adult stem cells obtained from bone

marrow have some advantages with respect to fetal stem cells, such as lower tumorogenecidade, technique of obtaining easier and less ethical problems[23]. This was confirmed in this experiment, since the regenerated bone in which the stem cells were introduced, there was no formation of heterotopic or neoplastic tissues. Moreover, the absence of histological features of these radios infectious process suggests that the techniques used to obtain, in the preparation and inoculation of these stem cells in regenerated bone during distraction osteogenesis are relatively safe.

The use of stem cells in large bone lesions tends to be a promising technique, associated with the techniques of bone distraction with external fixation, with high cost / benefit ratio, and can thus be incorporated in the routine treatment of orthopedic injuries.

Conclusions

The histomorphometric indices expressing the microarchitecture (trabecular thickness, trabecular spacing and number of trabecular) and quantity of bone were significant in the group study. These data suggest that the use of undifferentiated stem cells autologous bone marrow induces osteogenesis and bone quality.

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