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Adipose Derived Stem Cell on the Regeneration of Irradiated Mandible of Adult Rabbit Submitted to Distraction Osteogenesis

M. M. Soares, H. Segreto, A. Aloise, and L. M. Ferreira

Department of Plastic Surgery, Federal University of Sao Paulo, Rua Napoleão de Barros, Sao Paulo-SP 04024-900, Brazil

Abstract: Purpose: To evaluate the effect of adipose derived mesenchymal stem cell (ADSC) on the regeneration of irradiated mandible submitted to distraction osteogenesis on adult rabbits. Methods: Ten adult rabbits were selected and the dorsal fat was harvested for isolation, dissociation and culture of adipose derived adult mesenchymal stem cells. The mandibles were irradiated on a single dose of 20 Gy. After 30 days the animals underwent mandibular lengthening by distraction osteogenesis. After 10 mm of lengthening, the animals were distributed into two groups. Saline solution (1 mL) was injected the control group, and supplemented with ADSC, on the study group. After 4 weeks the mandibles were removed and evaluated by tomography and histomorphometric analysis. Histomorphometric was obtained and statistical analysis done by Mann-Withney test ($p > 0.05$). Results: The tomography shows a significant improvement on the Hounsfield scale for lateral fibrovascular zone (114.5 ± 9.7 control group to 148 ± 24, $p = 0.0045$) and central fibrovascular zone (37 ± 13.4 control group to 96 ± 41.8 on study group $p = 0.0045$). The Histomorphometric analysis demonstrated an increase area of new bone formation. The mineralized area was of 61.2 ± 9.9% in the study group and 31.3 ± 10.6% in the control group ($p = 0.0045$) for the central fibrovascular zone, 79.1 ± 9.1% in the study group and 39.1 ± 14.7 on control group for anterior fibrovascular zone ($p = 0.0001$) and 67.7 ± 12.9% in the study group and 38.4 ÷ 12.9% in control group for posterior fibrovascular zone ($p = 0.0001$). Conclusions: The injection of adipose derived mesenchymal stem cell was effective to improve bone regeneration distraction callus on the irradiated mandible.

Key words: Stem cells, bone regeneration, osteogenesis, distraction, mandible, rabbits.

1. Introduction

Distraction osteogenesis is a technique that through which the application of tension forces under osteotomized areas, is able to promote tissue regeneration, creating a new formed bone [1-3]. Due to the capability of lengthening bones, distraction osteogenesis is an important tool in the surgical correction of several orthopedic and cranio facial deformities [4-6]. Despite the range of clinical applications [7-10] of distraction osteogenesis, some clinical conditions can be an obstacle for bone maturation on distraction osteogenesis. Radiotherapy has been shown to reduce angiogenesis, leading to a failure on bone regeneration at distraction gap, resulting on lack of fixation, pseudarthosis, infection and osteonecrosis [7-11]. In order to prevent these complications, hyperbaric therapy [12], laser therapy, injection of growth factors [13, 14] and others have been reported in order to reduce complications and morbidity of distraction on irradiated mandibles.

Several studies have demonstrated that adult mesenchymal stem cells are able to stimulate angiogenesis [15, 16] and to differentiate into osteocytes and osteoblast [17], improving regeneration on bone fracture, bone grafts [18] and non irradiated mandibles submitted to distraction osteogenesis [19-22]. The aim of this study was to investigate the influence of adult mesenchymal stem cell derived from adipose tissue on irradiated mandible submitted to distraction osteogenesis.

Corresponding author: Marcelo Melo Soares, DDS, Ph.D., research fields: maxillofacial surgery and tissue engineering.

2. Materials and Methods

This investigation was conducted on the Laboratory of Cell Culture, Department of Plastic Surgery at Federal University of Sao Paulo, approved under the Ethic Committee protocol number 0333/12.

2.1 Materials

This study 10 adult's male rabbits (New Zeland, average weight 3.8 kg) were selected. The animals were submitted to sedation by intramuscular injection of 40 mg/Kg ketamine (Dopalen®, Brazil) and 2 mg/Kg of midazolan (Dormine®, Brazil) and local anesthesia by infiltration of 2 mg/kg of xylocaine with fenilpresina 1/1000 on the dorsal area. The dorsal area was shaved and under aseptic conditions an incision was done on a perpendicular line that runs from the midline of the interscapular line to caudal with 2.5 cm of length. A careful blunt dissection was done and the fat panicle was gently removed and sent on a FalconBD® tube (Becton, Dickinson and Company, New Jersey, EUA) with 10 mLof Hanks solution to the laboratory. The tubes were taken to the Laboratory of Cell Culture of the Department of Plastic Surgery of the Medical School of Federal University of Sao Paulo (UNIFESP) for mesenchymal stem cell separation, identification and culture. All rabbits were submitted to adipose tissue harvest in order ted all then to the same surgical stress.

After the harvest of fat tissue, and still under sedation , the animals were transferred to the Department of Radiotherapy of UNIFESP, where were submitted to calibration of dose on a Acuity®/Varion device, and irradiation by a single dose of 20 GY on the nuclear accelerator device Varion 6 VM model 600 CD®. The animals were maintained in cage with rabbit food and water *at libidun* for 30 days. The surgical wounds were cleaned on a daily bases with Chlorohexidine (Rioquimica, São José do Rio Preto-SP, Brazil) until complete healing.

2.2 Methods

2.2.1 Adipose Derived Adult Mesenchymal Stem Cell (ADSC) Culture

The adipose tissue removed from the dorsal panicle fat was processed in a laminar flow cabinet (Class III B3, Forma Scientific) weighted and 4 g of adipose tissue was selected for the cell isolation and culture. The adipose tissue was fragmented into small pieces (average of 1 mm) and washed on PBS solution, antibiotic and fungicide (A5955 Sigma Aldrich Missouri USA, penicillin and streptomycin 1%) and the process was repeated six times. Then, the fragments were dissociated by digestion with Type II collagenase for 30 min at 37 ^{o}C in an orbital agitator (Agitator Vortex Orbital Tecnal TE 420®). After the enzymatic process, the digested tissue was centrifuged at 300 G at a temperature of 22 ^{o}C for 10 min (Centrifuge 206 BL, Fanem, São Paulo, Brazil) for the rupture of adipose cells. The supernatant was discarded and the pellet resuspended on Dulbecco's modified Eagle's medium with nutrient F12 mixture (DEMEM F-12) medium with 10% bovine fetal serum (CULTILAB, Curitiba-PR, Brazil). The cells were checked for cell viability (dye trypan blue) and counted on automated cell counter (2906-SIGMA ALDRICH, CO, Misouri, USA). The solution was inserted on a culture vial and incubated at 35 ^{o}C with 5% CO_2. Each 48 h, the culture medium was changed. When 80% of confluence was achieved no adherent cells were removed. Cells were used after the third passage, when culture had reached confluence. The cells were frozen at -80 ^{o}C for storage until one week before their use (GAIBA).

2.2.2 Distraction Procedure

Distraction osteogenesis was conducted by the same anesthetic procedure of adipose tissue harvest surgery. By a submandibular approach and careful layer dissection the mandible body was exposed and mentalis nerve identified for osteotomy reference. A

complete trans-mandibular osteotomy was performed with piezzo surgery (Osada Enac OEW-10, Nagaya, Japan) anterior to the mentalis foramen, from the inferior border to the alveolar process until complete the mobilization of the fragments. The distraction device was fixed by four self drill 1.6 mm screws. The fixation and stability of the distraction device was tested and the surgical wound was closed by layers with polivicryl 4-0 and the skin by mononylon 4-0. The animal was maintained with intramuscular injection of amoxicillin (ABL, Cosmópolis, Brazil) cetoprofeno (MSD, Cruzeiro, Brazil) and 2 cc of tramadol (Pfizer, Guarulhos-SP, Brazil) for three days. The surgical wounds were washed each two days with toothbrush moistened with chlorohexidine and water until the end of the experiment.

The distraction protocol was: latency period of 5 days, activation on a rate of 1 mm twice a day for 5 days (2 mm/day of lengthening). At the last activation, 1 mL of 0.9 % saline solution (control group) and 10^6 stem cells diluted on 1 mL of 0.9% saline solution (study group) were randomized (using randomizer software) selected and injected on the distraction gap (fibrovacular zone) of the lengthened mandible. After the maturation period of 4 weeks, the animals were sedated (Ketamine 40 mg/kg and midazolan 2 mg/Kg) and sacrificed with a lethal endovenous dose of sodium thiopental. The mandibles were dissected, removed and immersed on ethanol 70%, for tomographic evaluation.

2.2.3 Determination of the Population of Adult Mesenchymal Stem Cells Derived from Adipose Tissue

In order to determine the population of the AMSC, three trials were conducted to determine the differentiation potentials:

(1) Adipogenic differentiation

The cells were grown on six plates with TPP containing 2 mL of DEMEM/f12 culture medium supplemented with 10 mol/L of dexamethasone (Sigma Aldrich) for 15 days. After that, they were kept on a humid incubator at 37 ^oC under 5% CO_2, and the culture medium replaced every 3 days. After this period, the medium was removed and the cells were washed twice with PBS. Immediately after being washed, 2 mL of paraformaldehyde fixing solution (0.4%) in PBS (Electron Microscopy Science) was used. After 30 min, the fixing solution was removed by suction and the cells were washed three times with PBS as follows: once in PBS containing 0.1 mol/L of glycine for 10 min and twice with only PBS for 2 min.

Subsequently, the cells were incubated with 0.5% oil red O dye solution (Sigma Aldrich) at room temperature for 30 min. The dye solution was carefully removed using a 2 mL disposable pipette and washed five times with 2 mL of running water to remove the excess dye. Then, the cells were incubated with 1 μg/mL of dye (4',6-diamidino-2 phenylindole), dihydrochloride (DAPI Sigma Aldrich) in PBS (0.1% bovine serum albumin [BSA], Sigma Aldrich) and 0.2% saponin (Cal-biochem) for 2 min. The cells were then washed again three times in PBS. The slide with the fixed and dyed cells was observed under a Nikon Ti-U epifluorescence microscope and photographed using NIS-Elements software, version 3.2 (Nikon Instruments).

(2) Osteogenic differentiation

For osteogenic differentiation, the cell cultures were performed on six-well plates (TPP) containing 2 mL of complete DMEM/F12 culture medium supplemented with 50 μmol/L of ascorbic acid (Sigma Aldrich), 0.1 μmol/L of dexamethasone, and 10-2 mol of β gyl-cerophosphate (Baker Analyzed reagent) for 21 days. They were then kept in a humid incubator at 37 °C under 5% CO_2, and the culture medium was replaced every 3 days. After this period, the medium was removed by suction and the cells washed twice with PBS. Soon after, 2 mL of a 0.4% paraformaldehyde fixing solution in PBS was used. After 30 min, the fixing solution was removed by suction and the cells were washed three times with PBS as follows: once in PBS containing 0.1 mol/L of

glycine for 10 min and twice with only PBS for 2 min.

Subsequently, the cells were incubated with a 40 mmol/L solution of alizarin red sodium (pH 4.1) (Sigma Aldrich) at room temperature for 30 min. The dye solution was carefully removed using a 2 mL disposable pipette and washed five times with 2 mL of running water to remove the excess dye. Then the cells were incubated with 1 μg/mL of DAPI dye in PBS (0.1% BSA and 0.2% saponin) for 2 min and again washed three times in PBS. The slide with the fixed and dyed cells was observed under a Nikon Ti-U epifluorescence microscope and photographed using NIS-Elements software.

(3) Chondrogenic differentiation

For chondrogenic differentiation, the cells were grown on six-well plates (TPP) containing 2 mL of complete DMEM/F12 culture medium supplemented with 10 μmol/L of insulin, 0.1 μmol/L of dexamethasone, 50 μmol/L of ascorbic acid, and 10 mg/mL of TGF-β1 (Cell Signaling Technology) for 21 days. They were then kept in a humid incubator at 37 °C under 5% CO_2, and the culture medium was replaced every 3 days. After this period, the medium was removed by suction and the cells washed twice with PBS. Immediately after, 2 mL of a 0.4% formalin fixing solution in PBS was used. After 30 mi, the fixing solution was removed by suction and the cells were washed three times with PBS as follows: once in PBS containing 0.1 mol/L of glycine for 10 min and twice with only PBS for 2 min. Subsequently, the cells were incubated with a 0.1% toluidine blue solution (Sigma Aldrich) at room temperature for 30 min. The dye solution was carefully removed using a 2 mL disposable pipette and washed five times with 2 mL of running water to remove the excess dye. The slide with the fixed and dyed cells was observed under a Nikon Eclipse Ti-U epifluorescence microscope and photographed using NIS-Elements software (Nikon INC. New York, USA).

2.2.4 Determination of the Population of Adult MSCs with Flow Cytometry (Immunophenotyping)

After isolation, the cells from the second passage were trypsinized and the suspension was centrifuged at 300 G for 4 min. The supernatant was discarded and 1 mL of PBS was added. This procedure, that is, the washing of the cell suspension, was repeated twice. After the second washing of the cells, antibodies CD16, CD31, CD34, CD45, CD73 and CD105 (Becton, Dickicon and Company, New jersey, EUA) were added. The tubes containing the cells were incubated for 20 min at room temperature and protected from light. The immunophenotyping of the samples were obtained by flow cytometry analysis on Guava easyCyte® (FACS Calibur, Bacton Dicknson Company, New Jersey, EUA) for identification of the specific fluorescence channels of each antibody.

2.3 Computed Tomographic Analysis

Three-dimensional radiological volumetric analysis of the lengthened bones was performed on a NewTon 3G-NIM s.r.l. (Verona, Veneto, Italy) tomography device. The samples were fixed for the measurements. The central zone was identified as the lowest gray scale found in the fibrovascular zone. From this landmark, two others points were identified 2 mm distant from the central point one anterior and other distal.

2.4 Histomorphometric Analysis

Decalcification of the fragments taken from the lengthened bones was made by submersion in 10% EDTA for 10 days. With the decalcified mandible, 7-μm sections from the vestibular to lingual face of mandible were embedded in paraffin blocks, and quantitative analysis was conducted using an optical microscope after staining with Malory Tricrome blue.

All the images were taken with a CCD digital camera (RT Color, Diagnostic Instruments) coupled to a light microscope with 1.25× magnification, combined in a single image using Photoshop Elements, version 2.0 software (Adobe Systems), and evaluated by, previously calibrated blinded examiner using imaging

software imageJ (Image Pro Plus 4.5, Media Cybernetics). Parameters of NFMT (new formed mineralized tissue) were taken from three sections of the lengthened bone. One on the CFVZ (central fibro vascular distraction zone), another 2 mm was measure from the AFVZ (anterior fibro vascular zone) and PFVZ (posterior fibro vascular zone). The parameters of new formed mineralized tissue were measured, stated as percentages of the total defect area, and compared.

2.5 Statistical Analysis

The quantitative data were analyzed using SPSS-V17 software (SPSS). The Man-Whitney test for non-parametric data was used to compare the mineralized tissue on tomographic image and the percentage area of mineralized tissue on the histomorphometric analysis obtained from the control group and stem cell group (SG). The differences for $p < 0.05$ were considered statistically significant.

3. Results

3.1 Differentiation Assays

The trilineage differentiation of ADSCs toward adipocyte, osteoblast and chondrocyte was observed on all cultures.

3.2 Immunophenotyping

The immunophenotyping was obtained by flow cytometry analysis and showed negative marking for CD16, CD31, CD34 and CD45 and positive for CD73 and CD105 surface markers.

3.3 Clinical Evaluation

The rabbits underwent the experiment uneventfully. An average of 9.3 mm of bone lengthening (ranging from 8.3 to 9.6 mm) was observed. A laterognathia and class III malocclusion with deviation of midline incisor was observed on all rabbits. No compromise for the distraction device stability was observed. Macroscopic evaluation shows that, even though all animals presented clinical regeneration on the lengthened mandible, the fibro vascular distraction zone was more evident on the control group.

3.4 Computed Tomographic Analysis

The tomography image of both groups showed bone continuity of the lengthened bone, which made impossible to identify the osteotomy line. The distal and central fibro vascular zone showed a statistical difference on bone density on the SG compared to the control group (LFVZ 114.5 ± 9.7 in CG, 148 ± 24 in SG $p = 0.0045$* and CFVZ 37 ± 13.4 CG and 96 ± 41.8 SG $p = 0.0045$* (Fig. 1).

3.5 Histomorphometric

The lengthened mandible was majorly repaired by intramembranous ossification in all rabbits (Fig. 2). The new formed mineralized tissue was significantly higher all fibro vascular zones. The AFVZ increased from 39.1 ± 14.7% on the CG to 79.1 ± 9.1% on the study group $p = 0.0001$* the PFVZ increased from 38.4 ± 12.9% on the CG to 67.7 ± 12.9% on the study group $p = 0.0001$* and the CFVZ increased from 31.3 ± 10.6% to 61.2 ± 9.9% $p = 0.0045$* (Fig. 3).

4. Discussion

Since the introduction of distraction osteogenesis by McCarthy et al. [23] for correction of mandibular microssomia, it has been largely used with successful outcomes in the management of cranio facial deformities. Although the high rate of success and predictability of distraction, complications like non-union, infection and failure during distraction have been reported.

Among several of the factors which can compromise distraction bone healing, radiotherapy is able to decrease or even inhibit the maturation of the lengthened new bone. Irradiation on the mandible is able to decrease bone maturation by increasing the presence of fibrous tissue and decreasing angiogenesis and vasculogenesis on the lengthened area [9, 12, 24-26]. Kesemenli et al. [15] demonstrates in an animal model that even when irradiation is applied on a distal

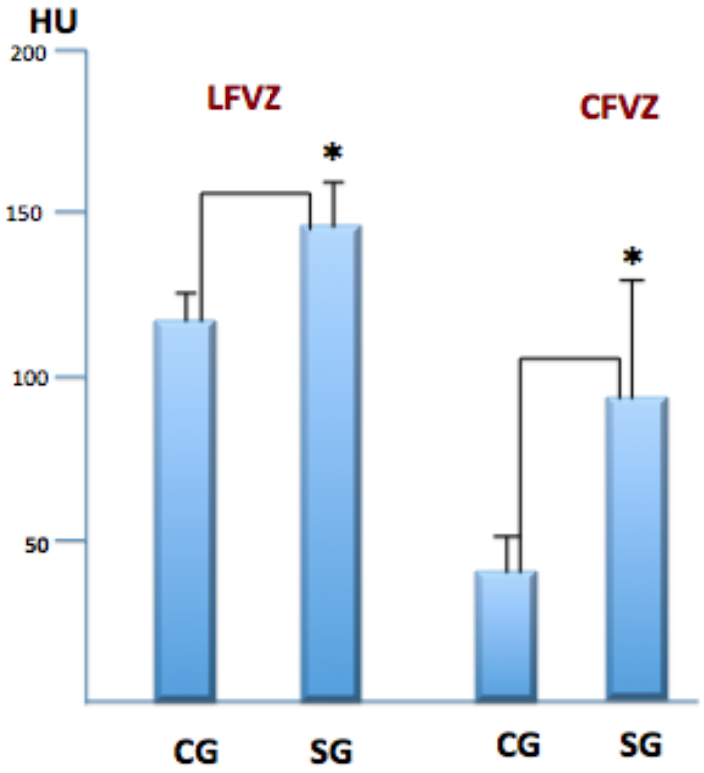
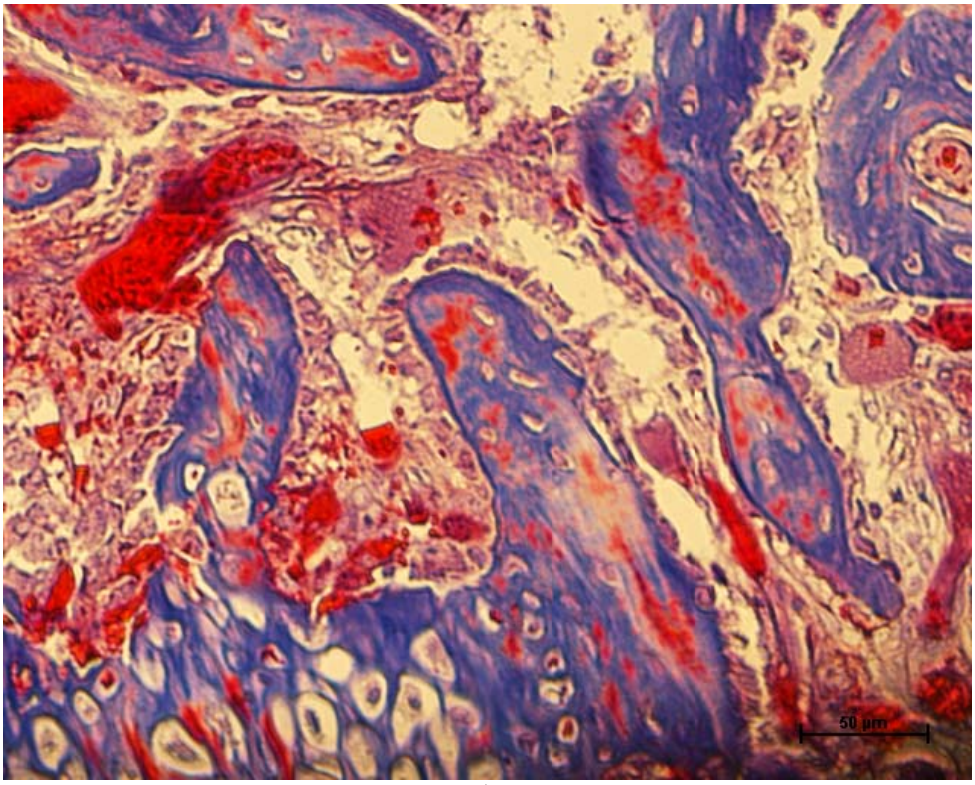
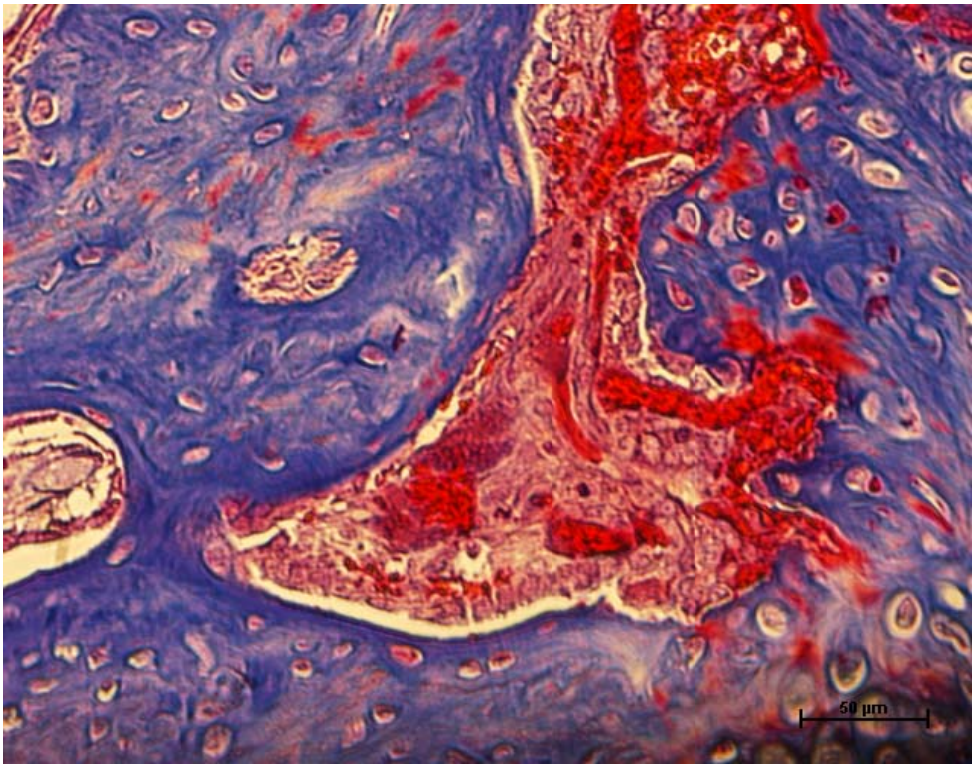


Fig. 1 Tomographic evaluation of scale of HU (Hounsfield unit) for LFVZ 114.5 ± 9.7 on CG (control group) and 148 ± 24 on SG (study group) $p = 0.0045$* and for CFVZ 37 ± 13.4 on CG and 96 ± 41.8 $p = 0.0045$* on SG.



A



B

Fig. 2 Histologic evaluation rabbits mandibles after irradiation and lengthened by distraction osteogenesis (Malory Tricrome bule). A: Control group shows immature and unorganized trabecular bone surrounded by non mineralized tissue. B: Study group, injected with adipose derived adult stem cells, shows mature and organized trabecular bone with predominance of mineralized tissue.

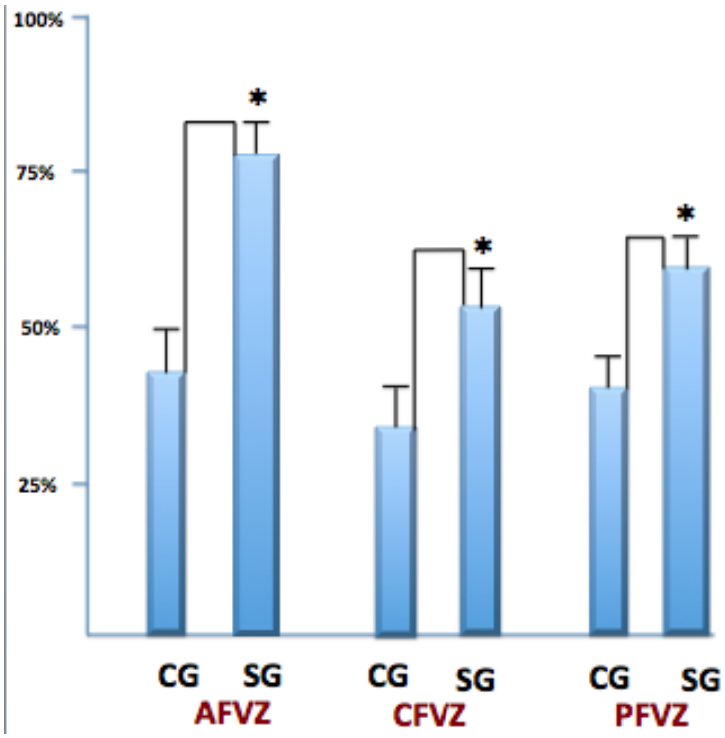


Fig. 3 Results for AFVZ 39.1 ± 14.7% on the CG and 79.1 ± 9.1% on the SG *p* = 0.0001* the PFVZ increased from 38.4 ± 12.9% on the CG to 67.7 ± 12.9% on the SG *p* = 0.0001* and the CFVZ increased from 31.3 ± 10.6% to 61.2 ± 9.9% *p* = 0.0045*.

bone (tibia) the mandible is compromised. Despite the fact that distraction osteogenesis is regarded as an endogenous tissue engennering technique [27], the prolonged treatment time and damages caused by irradiation reduce the DO clinical application after radiotherapy.

In order to reduce complication and improve the bone healing, several regenerative techniques have been associated with DO protocols. Low level laser therapy has been showed to reduce the time for bone regeneration of the DO in rabbits, gene therapy like addition of BMPs on the lengthened callus are reported to be able to accelerate bone maturation with different levels of success and cost [13, 28] .

Despite DO increases angiogenesis [29], the irradiation generate immature bone with failure on mineralization [25, 27, 29, 30] due to reduction on vascularization, reduction on the expression of growth factors [11, 30, 31] and increase of osteoblast apoptosis [32], resulting on a nobe quth poor quality for osteointehration and low resitence for mastigatory loading.

Several papers have demonstrated the capability of stem cells to improve bone healing on fractures, and to increase graft integration and the bone maturation on the lengthened mandibles [22, 33-35] however, all tehse investigations studied the regeneration on health bones, situation rarely found on clinical applications of DO. The results reported with the uses of stem cells on non irradiated mandibles, stimulate the authors to investigate their potential on unhealth bones, being irradiated lengthened bones an excelente model of dameged bone. Felice et al. [31] have reported an increase up to 90% of gross bone union on bone healing on irradiated murine mandible submitted to DO associated with deferoxamine, showing, result which is similar to the findings reports on this study. Deshpande et al. [25] showed that bone marrow derived stem cells implanted in a collagen scaffold after the osteotomy, is able to stimulate the murine irradiated mandible reestablishing the ability to heal, Zhang et al. [36] injected transfected mesenchymal stem cells with BMP into the rabbits irradiated mandibles lengthened by DO, reporting the increase on the amount of trabecular and on bone volume on histological micro-CT evaluation.

The mechanotransduction produced by DO, increases the expression of several growth factors (tissue growth factor beta, bone morphogenetic growth factor vascular endothelial growth factors), increasing the generation of a neoformed bone on the distracted gap [1, 2, 30, 37] and the differentiation of stem cells on osteoblasts attracting and signalizing the stem cell for differentiation on the healing bone [1, 38-40]. This investigation does not focus on the elucidation of the mechanism of action on the mineralization of the lengthened areas, so, more investigations are required in order to elucidate the mechanism in which stem cells act on the healing of distraction process.

This investigation follow surgical and cell culture and injection describe on previously studies [12, 34, 41-43], although all showed increase on the regeneration, none shows the path of the cells after being injected.

Despite the improvement reported by previous studies [5, 20, 36], the researchers do not studied the mineralization on different lengthened áreas. Since the maturation occurs, on distracted bones, from the extremity to the center, the crucial point of fragility is the central zone. The use of several measurement areas can be a bias on the interpretation of the bone density, since it the extremity bones will show more mineralization then the central, increasing the average mineralization. In order to evaluate the more critical zone (FVCZ) and to show a more complete picture of the mineralization process, the measurements were taken from three areas for tomography and five for histomorphometric evaluation. The results have shown that despite the fact that all areas showed statistically significant difference on bone maturation, the number on the central zone presented a higher difference, showing that stem cells were able to increase bone density, reducing the damage caused by irradiation on

the lengthened mandible.

5. Conclusions

The radiotherapy causes severe damage on bone repair, restricting or even contraindicated distraction osteogenesis for the reahabilitation of these patients. This investigation shows that stem cells were able to improve the bone regeneration of distraction osteogenesis in irradiated mandibles sugesting that the distraction mechanotransduction is able to signalize and activeted the sten cells, improving the bone healing. More studies must be conducted in order to establish dose, mechanism of action of stem cells and proteomics involved on the process, in order to elucidate cell therapy principles and stablish bases for clinical protocols.

References

[1] Lee, D. Y., Cho, T. J., Kim, J. A., et al. 2008. "Mobilization of Endothelial Progenitor Cells in Fracture Healing and Distraction Osteogenesis." *Bone* 42 (5): 932-41.

[2] Lee, D. Y., Cho, T. J., Lee, H. R., et al. 2010. "Distraction Osteogenesis Induces Endothelial Progenitor Cell Mobilization without Inflammatory Response in Man." *Bone* 46 (3): 673-9.

[3] Stogov, M. V., Tushina, N. V., and Emanov, A. A. 2015. "Serum Concentration of Growth Factors in Dogs under Different Condition of Distraction Osteogenesis." *Bull Exp. Biol. Med.* 2: 213-5.

[4] Andrade, N. N., Reshamwala, S. M., and Subburaj, K. 2011. "Mandibular Reconstruction through Transport Distraction Using Intraoral Appliance." *J. Oral. Maxillofac Surg.* 69 (8): 2260-9.

[5] Pichelmayer, M., and Zemann, W. 2012. "Bilateral Vertical Segmental Distraction in a Patient with Bilateral Cleft of Lip, Alveolus, and Palate." *Cleft Palate Craniofac J.* 49 (3): 365-8.

[6] Rachmiel, A., Srouji, S., Emodi, O., et al. 2012. "Distraction Osteogenesis for Tracheostomy Dependent Children with Severe Micrognathia." *J. Craniofac Surg.* 23 (2): 459-63.

[7] Chrcanovic, B. R., Reher, P., Sousa, A. A., et al. 2010. "Osteoradionecrosis of the Jaws—A Current Overview—Part 1: Physiopathology and Risk and Predisposing Factors." *Oral. Maxillofac. Surg.* 14 (1): 3-16.

[8] Hee-Kyun, O. H., Chambers, M. S., Martin, J. W., et al. 2009. "Osteoradionecrosis of the Mandible: Treatment Outcomes and Factors Influencing the Progress of Osteoradionecrosis." *J. Oral. Maxillofac. Surg.* 67: 1378-86.

[9] Jereczek-Fossa, B. A., and Orecchia, R. 2002. "Radiotherapy-Induced Mandibular Bone Complications." *Cancer Treat Rev.* 28 (1): 65-74.

[10] Kesemenli, C. C., Kaya, H., Memisoglu, K., et al. 2015. "Distraction Osteogenesis Is Inhibited by Cancer Radiotherapy with Co 60." *Clin. Invest Med.* 32: 376-82.

[11] Shao, Z., Liu, B., Liu, Y., et al. 2006. "Distraction Osteogenesis in the Irradiated Rabbit Mandible." *J. Plast Reconstr Aesthet Surg.* 59 (2): 181-7.

[12] Clark, C. L., Strider, J., Hall, C., et al. 2006. "Distraction Osteogenesis in Irradiated Rabbit Mandibles with Adjunctive Hyperbaric Oxygen Therapy." *J. Oral. Maxillofac. Surg.* 64 (4): 589-93.

[13] Eguchi, Y., Wakitani, S., Naka, Y., et al. 2011. "An Injectable Composite Material Containing Bone Morphogenetic Protein-2 Shortens the Period of Distraction Osteogenesis *in vivo*." *J. Orthop Res.* 29 (3): 452-6.

[14] Mizumoto, Y., Moseley, T., Drews, M., et al. 2003. "Acceleration of Regenerate Ossification during Distraction Osteogenesis with Recombinant Human Bone Morphogenetic Protein-7." *J. Bone Joint Surg. Am.* 85-A (Suppl 3): 124-30.

[15] Abudusaimi, A., AIhemaitijiang, Y., Wang, Y. H., et al. 2011. "Adipose-Derived Stem Cells Enhance Bone Regeneration in Vascular Necrosis of the Femoral Head in the Rabbit." *J. Int. Med. Res.* 39 (5): 1852-60.

[16] Moioli, E. K., Clark, P. A., Chen, M., et al. 2008. "Synergistic Actions of Hematopoietic and Mesenchymal Stem/Progenitor Cells in Vascularizing Bioengineered Tissues." *PLoS One* 3 (12): 3922-8.

[17] Peptan, I. A., Hong, L., and Mao, J. J. 2006. "Comparison of Osteogenic Potentials of Visceral and Subcutaneous Adipose-Derived Cells of Rabbits." *Plast Reconstr Surg.* 117 (5): 1462-70.

[18] Zimmermann, A., Pelegrine, A. A., Peruso, D., et al. 2015. "Adipose Mesenchymal Stem Cell Associated with Xenograf in a Guide Regeneration Bone Model: A Histomorphometric Study in Rabbit Calvaria." *Int. J. Oral. Maxillofac. Implants* 30: 1415-22.

[19] Fujio, M., Yamamoto, A., Ando, Y., et al. 2011. "Stromal Cell-Derived Factor-1 Enhances Distraction Osteogenesis-Mediated Skeletal Tissue Regeneration through the Recruitment of endothelial Precursors." *Bone* 49 (4): 693-700.

[20] El Hadidi, Y. N., El Kassaby, M., El Fatah Ahmed, S. A., et al. 2016. "Effect of Mesenchymal Stem Cell Application on the Distracted Bone Microstructure: An

Experimental Model." *J. Oral. Maxillofac. Surg.* 74 (7): 1463.e1-11.

[21] Hwang, Y. J., and Choi, J. Y. 2010. "Addition of Mesenchymal Stem Cells to the Scaffold of Platelet-Rich Plasma Is Beneficial for the Reduction of the Consolidation Period in Mandibular Distraction Osteogenesis." *J. Oral. Maxillofac. Surg.* 68 (5): 1112-24.

[22] Tee, B. C., and Sun, Z. 2015. "Mandibular Distraction Ostegenesis Assisted by Cell-Based Tissue Engineering: A Systematic Review." *Orthod Craniofac Res.* 18: 39-49.

[23] McCarthy, J. G., Schreiber, J., Karp, N., et al. 1992. "Lengthening the human mandible by gradual distraction." *Plast Reconstr. Surg.* 89 (1): 1-8.

[24] Deshpande, S. S., Monson, L. A., Cavaliere, C. M., et al. 2013. "Distraction Osteogenesis Following Low-Dose Hyperfractionated Irradiation in the Rat Mandible." *J. Oral. Maxillofac. Surg.* 71 (8): 1465-70.

[25] Deshpande, S. S., Gallagher, K. K., Donneys, A., et al. 2015. "Stem Cells Rejuvenate Radiation-Impaired Vasculogenesis in Murine Distraction Osteogenesis." *Plast Reconstr Surg.* 135: 799-806.

[26] Price, D. L., Moore, E. J., Friedman, O., et al. 2008. "Effect of Radiation on Segmental Distraction Osteogenesis in Rabbits." *Arch. Facial Plast Surg.* 10 (3): 159-63.

[27] Tsuchiya, H., Uehar, A. K., Sakurakichi, K., et al. 2005. "Distraction Osteogenesis after Irradiation in a Rabbit Model." *J. Orthop Sci.* 10 (6): 627-33.

[28] Lee, S. J., Kim, B. J., Kim, Y. I., et al. 2016. "Effect of Recombinant Human Bone Morphogenetic Protein-2 and Adipose Tissue-Derived Stem Cell on New Bone Formation in High-Speed Distraction Osteogenesis." *Cleft Palate Craniofac J.* 53: 84-92.

[29] Donneys, A., Tchanque-Fossuo, C. N., Farberg, A. S., et al. 2012. "Bone Regeneration in Distraction Osteogenesis Demonstrates Significantly Increased Vascularity in Comparison to fractures Repair in the Mandible." *J. Craniofac Surg.* 23: 328-32.

[30] Zhang, W. B., Zheng, W. L., Chua, T. T. D., et al. 2011. "Expression of Bone Morphogenetic Protein, Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor on Irradiated Mandibles during Distraction Osteogenesis." *J. Oral. Maxillofac. Surg.* 69: 2860-71.

[31] Felice, P. A., Ahsan, S., Donneys, A., et al. 2013. "Deferoxamine Administration Delivers Translational Optimization of Distraction Osteogenesis in the Irradiated Mandible." *Plast Reconstr. Surg.* 132 (4): 542-8.

[32] Chandra, A., Lin, T., Zhu, J., et al. 2015. "PTH1-34 Blocks Radiation-Induced Osteoblast Apoptosis by Enhancing DNA Repair Through Canonical Wnt Pathway." *J. Biol. Chem.* 290 (1): 157-67.

[33] Dongyang, M. A., Ren, L., Yao, H., et al. 2013. "Locally Injection of Cell Sheet Fragments Enhances New Bone Formation in Mandibular Distraction Osteogenesis: A Rabbit Model." *J. Orthopaedic Res.* 54: 1082-8.

[34] Hong, P., Boyd, D., Beyea, S. D., et al. 2013. "Enhance of Bone Consolidation in Mandibular Distraction Osteogenesis: A Contemporary Review of Experimental Studies Involving Adjuvant Therapies." *J. Plast Reconstr. Surg.* 66: 883-95.

[35] Recreo, P., Vera, J. L. C., Hristov, T. G., et al. 2015. "Adipose-Derived Stem Cells and Platelet-Rich Plasma for Preventive Treatment of Bisphosphonate-Related Osteonecrosis of the Jaw in a Murine Model." *J. Craniomaxillofac Surg.* 43 (7): 1161-8.

[36] Zhang, W. B., Zheng, L. W., Chua, T. T. D., et al. 2012. "Treatment of Irradiated Mandibles with Mesenquimal Stem Cell Tranfected with Bone Morphogenetic Protein 2/7." *J. Oral. Maxillofac. Surg.* 70: 1711-6.

[37] Campisi, P., Handy, R. C., Lauzier, D., et al. 2003. "Expression of Bone Morphogenetic Proteins during Mandibular Distraction Osteogenesis." *Plast Reconstr. Surg.* 111 (1): 201-8.

[38] Moioli, E. K., Clark, P. A., Chen, M., et al. 2008. "Synergistic Actions of Hematopoietic and Mesenchymal Stem/Progenitor Cells in Vascularizing Bioengineered Tissues." *PLoS One* 3 (12): 3922-8.

[39] Nomura, I., Watanabe, K., Matsubara, H., et al. 2014. "Uncultured Autogenous Adipose-Derived Regenerative Cells Promote Bone Formation during Distraction Osteogenesis in Rats." *Clin. Orthop Relat. Res.* 472 (12): 3798-806.

[40] Wu, G., Zhou, B., Chunbing, H., et al. 2015. "Gene Expression of Osteogenic Factors Following Gene Therapy in Mandibular Lengthening." *J. Craniofac. Surg.* 26: 1-4.

[41] Djasim, U. M., Wolvius, E. B., van Neck, J. W., et al. 2007. "Recommendations for Optimal Distraction Protocols for Various Animal Models on the Basis of a Systematic Review of the o" *Int. J. Oral. Maxillofac. Surg.* 36: 877-83.

[42] Domicini, M., Le Blank, K., Mueller, I., et al. 2006. "Minimal Criteria for Defining Multipotent Mesenchymal Stromal Cells. The International Society for Cellular Therapy Position Statement." *Cytotherapy* 8 (4): 315-7.

[43] Mazzeti, M. P. V., Oliveira, I. S., Ferreira, R. M., et al. 2010. "Qualitative Abs Quantitative Analysis of Rabbit'S Fat Mesenchymal Stem Cells." *Acta. Cir. Bras.* 25 (1): 24-7.