EFFECT OF AUTOLOGOUS PLATELET-RICH PLASMA ON DISTRACTION OSTEOGENESIS IN THE MANDIBLE OF RABBITS: A MORPHOLOGIC AND MORPHOMETRIC APPROACH

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Distraction osteogenesis of the jaws is a common surgical practice in the treatment of pediatric craniofacial deformities. Autologous platelet rich plasma (PRP) has been used to increase the healing potential of bones in humans during distraction osteogenesis. This article aims to study the morphometric and morphologic parameters resulting from the effect of PRP on bone healing after mandibular distraction in rabbits. Right mandibular distraction was performed in 12 rabbits divided equally into 2 groups. PRP and physiological saline were injected, according to a defined protocol, in the callus following distraction of the experimental and control groups respectively. The rabbits were sacrificed after a consolidation period of 45 days and the mandibles were surgically removed. Bone mineral density, radiographic analysis, mechanical properties and histological features of the lengthened bones were assessed using radiographic examination, dual X-ray absorptiometry, biomechanical testing and histology. Results showed that the regenerate bone density, the amount of trabeculation in addition to the bone mineral density and mineral content, as measured by absorptiometry, were better with PRP but not significantly different between groups. Two radiographs revealed a more consistent healing in the experimental mandibles compared with erratic outcomes in corresponding controls. Two of the latter could not be subjected to any mechanical testing because the mandibular parts, connected with fibrous tissue, were separated. Consequently, the biomechanical test depicted greater maximal loads in the experimental group. The histological studies exhibited more ossification and less connective tissue fibers in the experimental group. PRP accelerated healing of mandibles in rabbits following distraction and improved their biomechanical properties. These findings have significant clinical implications on reducing the period of consolidation of the mandibles which may not be immobilized like other bones for long periods of time.

Distraction osteogenesis on the jaw is currently used by maxillofacial surgeons for treating various

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pediatric deformities such as mandibular and maxillary retrognathia (1, 2), lengthening of a short mandibular ramus and widening of a narrow mandible (3). Because of the long consolidation period, the main problem encountered following distraction is bone fracture with an incidence that varies from 3-39% (4). That led researchers to test the effect of some substances on the bone remodeling process.

Accelerating bone healing was mainly tested in tibia and femur, only rare studies addressed the craniofacial complex. The effect of adjuvant on bone healing in the consolidation period can be summarized as acceleration using parathyroid hormone (PTH) (5, 6), growth hormone (GH) (7, 8) and stem cells (9, 10). However, more experiments are needed with Calcitonin (11, 12), Alendronate (12) and Platelet Rich Plasma (PRP) before making final conclusions.

PRP serves as a reservoir of critical growth factors that contribute to bone healing including platelet derived growth factor (PDGF), transforming growth factor (TGF), insulin like growth factor 1 (IGF 1) (13-15). PRP improves the healing potential of femur, tibia and overlying soft tissue and shortens the consolidation period (16-18). In addition, it increases bone density and radiographic maturation rate (19). It is also believed that it produces an advanced state of osteogenic differentiation (20) and PRP improves wound healing in patients with multiple myeloma who developed osteonecrosis of the jaw (21). Furthermore PRP has similar effects on skeletal muscle lesions, it improves muscle regeneration and neovascularization (22).

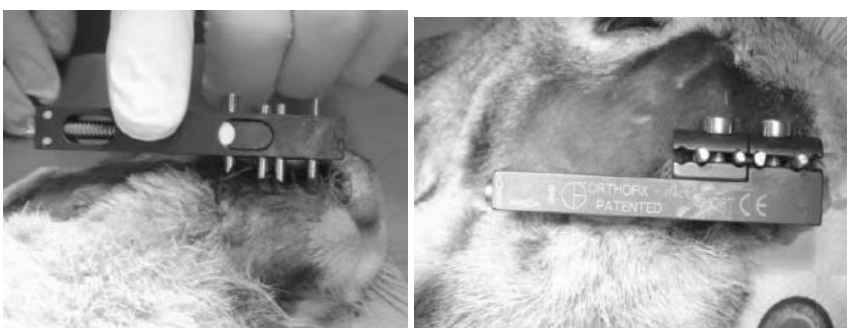
The possible effect of PRP on the quality and healing potential of a distracted mandible needs further studies. Our objective is to assess the morphometric and morphologic effects of autologous PRP on bone remodeling in the jaws of rabbits. While there is published data on bone formation rate in the appendicular skeleton, there is little such information on the jaws of rabbits.

MATERIALS AND METHODS

Twelve young white New Zealand rabbits weighing 2 kg each and divided into 2 groups of 6, were included in this study. Twelve fixators (M100- Orthofix USA) were inserted in the right mandibles of the rabbits with four 3mm half pins. Group one was injected with physiologic saline in the callus following distraction, while group 2 was injected with autologous PRP. All the rabbits were acquired and maintained in the animal house of the American University of Beirut (AUB) and treated according to guidelines set by the Institutional Animal Care and Use Committee of the American University of Beirut.

Surgical procedure

Following anesthesia by Ketamine (35 mg/kg), Xylazine (10 mg/kg) and Midazolane (2 mg/kg), a longitudinal skin incision was made along the inferior border of the mandible. The underlying muscles and periosteum were reflected to expose the mandibular body and nerve. The mental nerve served as a landmark during the surgery since two of the pins were placed directly anterior to the nerve and the remaining two posterior to it while the corticotomy was performed directly posterior to it.



(a) (b)

Fig. 1. ***A****) Inferior view of the distractor fixation on the pins.* ***B****) Lateral view of the distractor fixation on the pins.*

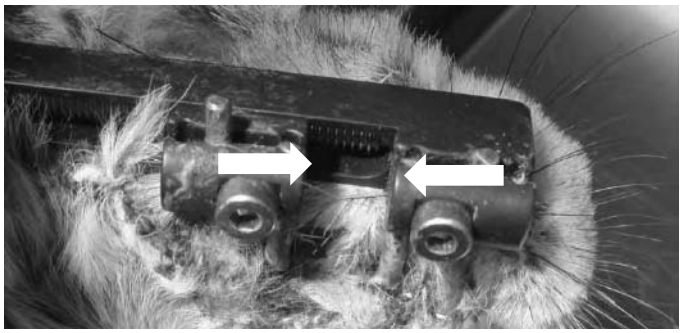


Fig. 2. *Lateral view of the rabbit's mandible showing 10 mm of mandibular distraction (between the white arrows).*

Fig. 3. *Anterior view of the rabbit's teeth showing a 10 mm shift between the maxillary and mandibular midlines upon occlusion as a result of mandibular distraction.*

The pins were checked at the end of the surgery for their parallelism. After insertion of the pins, the corticotomy was performed using an abrasive disc engaged in a rotary instrument. The corticotomy was performed between the mental nerve and the pin posterior to it. Penicillin was locally injected and then the wound sutured over the pins. Finally, the distractor was fixed on the pins with screws (Fig. 1).

Injection of antibiotics (streptomycin/penicillin 0.1 ml/Kg) and analgesics (tramal 10 mg/kg) were performed every 8 h for 3 days following surgery. The recovery period (latency period) was set for 5 days following distractor placement. During this period, 2 rabbits died in the first 2 days of the latency period due, most probably, to a severe bleeding during the surgery. They were replaced by 2 new rabbits on which new surgeries were performed.

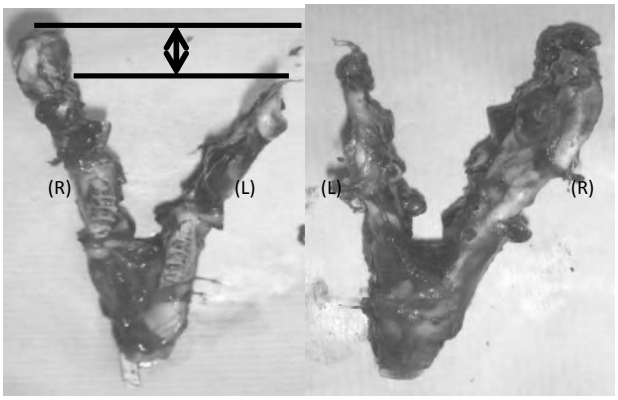
Protocol of distraction

After the recovery period, the distraction phase started at a rate of 1 mm per day for a total of 10 days, corresponding to 10 mm of bone distraction and callus formation (Fig. 2).

Checking the amount of distraction was performed by observing the occlusion between the maxillary and mandibular incisors. Normally, the incisors should be overlapping and the maxillary midline is aligned with the mandibular midline, however, following distraction we could clearly see the amount of shift between midlines (Fig. 3).

PRP preparation

Following the distraction phase, PRP was prepared according to the method described by Gimeno et al (23). Under general anesthesia, 12 ml of whole blood was collected from each rabbit then centrifuged for 15 m at 4°C with a 70g speed. The resulting 5 ml supernatant plasma was further centrifuged at 4°C for 5 m at 1010 g, resulting into two parts; the upper 4.7 ml were poor-platelet plasma (PPP) and the lower pellet was of 0.3 ml of platelet-rich plasma (PRP). Then, 4 drops of a 5% calcium chloride



(a) Upper view (b) Lower view

Fig 4. *Removed mandibles of both experimental and control groups after the consolidation period (day 60). Note the 10 mm length difference between right distracted mandibular part (R) and left non distracted mandibular part (L) (Black arrow).*

(CaCl2) solution were added to the PRP that became to transform into a gel like solution.

Injection of PRP and Physiologic Saline

Following blood withdrawal, 0.3 ml of PRP+CaCl2 or 0.3 ml of physiologic saline were injected (D15) into the rabbit's distracted mandibular areas of the experimental and control groups, respectively.

Consolidation period

The rabbits were kept in separate cages for 45 days and supplied with the appropriate food. Weight was measured every 2 weeks for all rabbits, only one rabbit from the experimental group showed a significant weight loss which led to its death in the 30th day of the consolidation period. The other rabbits showed a non significant ±200 g variation in their weight. The lost rabbit was not replaced. At the end of the consolidation period 5 rabbits remained in the experimental group and 6 in the control group.

Rabbits sacrificed

Following the consolidation period, the rabbits were sacrificed. The mandibles were removed. The extracted mandibles showed a 10 mm difference in length between the right and left mandibles which reflected the amount of distraction performed on the right mandible (Fig. 4).

The amount of distraction was measured on each removed mandible using calipers. One rabbit from the control group (N 5) was also after its removal excluded from the study for technical reasons.

Tests performed

Radiographic examination. The bone density and the amount of trabeculation within the regenerate area of each removed mandible were descriptively assessed on the radiographs and compared between both groups.

Regional dual X ray absorptiometry. Measurement of bone mineral density (BMD) and content of distracted areas was achieved using dual X-ray energy absorptiometry machine (Hologic Discovery, USA). Bone mineral content (BMC) and BMD for each rabbit were computed (4).

Mechanical test. The distracted mandibles were loaded in three-point bending test at the Faculty of Engineering, AUB. A force generated hits the middle area of the regenerate and causes its flexure and eventually leading into its breakage (Fig. 5).

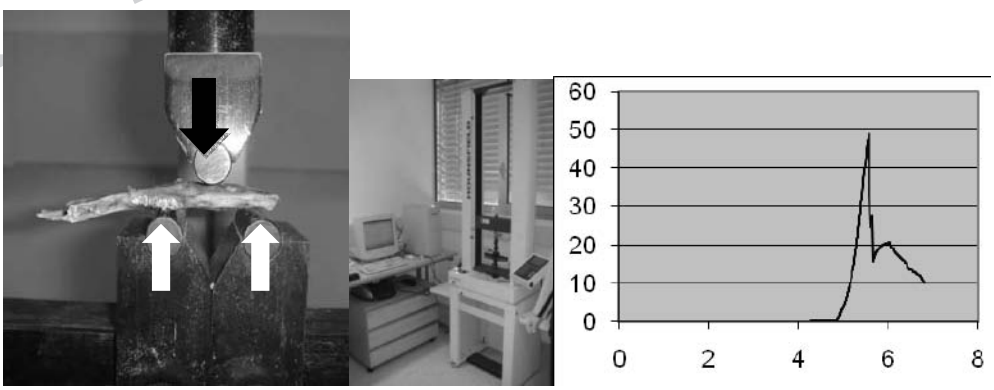


Fig. 5. ***A)*** *Mandible subjected to the three point bending test. Mandible seated on 2 points (white arrows) and a third point (black arrow) going down and hitting the regenerate middle area.* ***B)*** *Three point bending machine linked to a computer which represents the maximal flexural strength of the mandible on a graph.* ***C)*** *Maximal load of 49 N supported by the tested mandible.*

The generating force system was attached to a computer which represented the maximal flexural strength of the bone on a graph. The fracture strength was determined as the maximum load supported by each mandible before breakage; it was represented by the peak of the graph shown following the test. The X axis represented the flexion of the bone in mm and the Y axis represented the force corresponding to the flexure in Newton (N) (Fig. 5).

Histological test. Following the mechanical test, the regenerate area was sectioned. The first cutting area was medial to the pin previously placed medial to the mental nerve and the second cutting area distal to the pin; that is, placed distal to the mental nerve. The sectioned part contained the regenerate and the mandibular normal bone on both medial and distal sides. Following decalcification with a 0.5 mol/L ethylenediaminetetraacetic acid solution for 3 days, the bones were processed for routine microscopy according to established procedures. Five micrometer serial sections were cut from each block. Some were stained with Hematoxylin & Eosin (H&E) and some others with Masson Trichrome stain according to standard procedure for detection of collagen fibers within the tissue of the regenerate area.

RESULTS

The results were based on the treated mandibles of 10 rabbits. The control group consisted of 5 rabbits (3, 4, 7, 11 and 14) and the experimental group consisted also of 5 rabbits (2, 8, 10, 12 and 13). Rabbit 5 was excluded from the control group due to a lack of optimal distraction (7 mm) and rabbit 9 of the experimental group died on day 30th of the

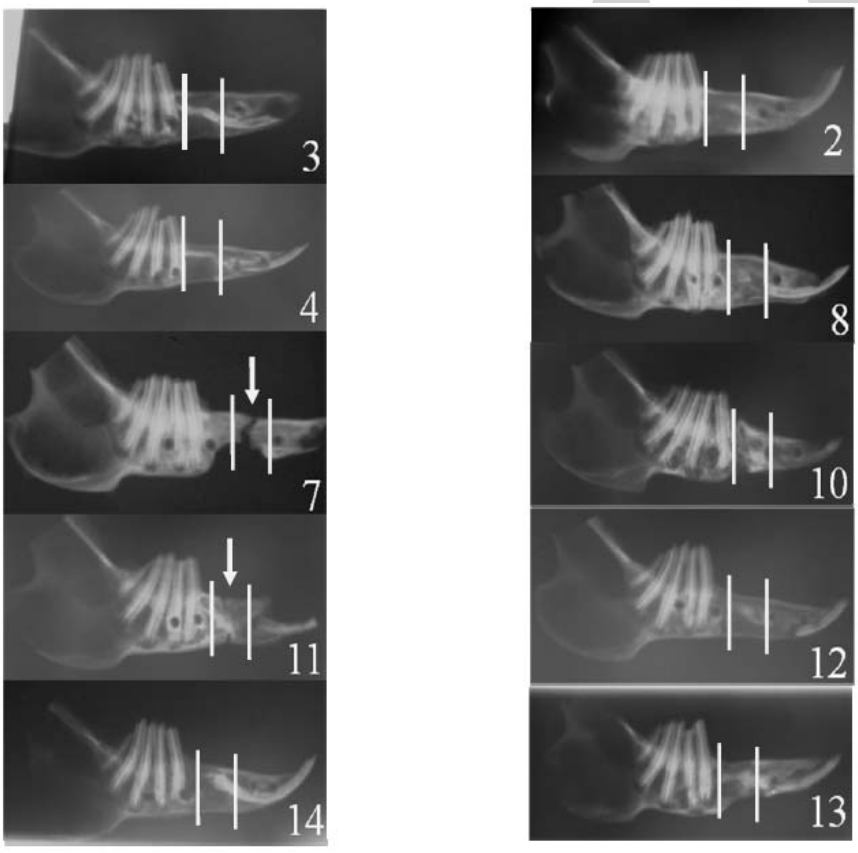


Fig. 6. *X-ray photos of the removed mandibles of both groups (Day 60). X-rays 3, 4, 7, 11 and 14 represent those of the control group (**A**) whereas 2, 8, 10, 12 and 13 represent the X-rays of the experimental group (**B**). Note the presence of gaps in 7 and 11 (White arrows) while no gaps existed in the experimental mandibles. The difference in trabeculaton and bone density between both groups was not significant. Note more predictable response in all experimental samples and more variations in the control group samples.*

Table I. *Regional dual x-ray absorptiometry (control group).*

Rabbits	Area (cm^2)	BMC (gm)	BMD (gm/cm^2)
N 3	1.21	0.27	0.224
N 4	1.21	0.18	0.152
N 7	0.92	0.14	0.150
N 11	1.19	0.28	0.24
N 14	1.03	0.16	0.156
Averages	**1.112**	**0.206**	**0.1844**
SEM	**0.101**	**0.086**	**0.080**
SD	**0.131**	**0.064**	**0.043**

BMC: Bone mineral content; BMD: Bone mineral density

Table II. *Regional dual x-ray absorptiometry (experimental group).*

Rabbits	Area (cm^2)	BMC (gm)	BMD (gm/cm^2)
N 2	1.32	0.21	0.162
N 8	1.16	0.17	0.147
N 10	1.09	0.35	0.32
N 12	1.13	0.12	0.111
N 13	1.06	0.19	0.176
Averages	**1.152**	**0.208**	**0.1832**
SEM	**0.101**	**0.086**	**0.080**
SD	**0.101**	**0.086**	**0.081**

consolidation period.

Macroscopic examination of the regenerate

A significant difference was noted between both groups concerning the amount of soft tissue in the middle area of the regenerate. In the experimental group, the regenerate area showed less soft tissue. In all control animals injected with saline, the relative amount of soft tissue was relatively more than controls. However, in 3 to5 animals of the control group; the soft tissue was significantly more abundant at the interface resulting in breakage of 2 of them at that site during manipulation of the mandibles.

Radiographic analysis

As shown in Fig. 6, all 5 mandibles of the experimental group showed complete ossification while 2 in the control mandibles showed clear gaps in the center of the regenerate areas. On the other hand, bone density and trabeculations did not show any significant difference.

Regional dual x-ray absorptiometry analysis

Although ossification was faster in the experimental group, no statistically significant difference has been noted, at this advanced stage of 45 days, between both control and experimental groups in the parameters tested, namely, BMC and BMD (Tables I and II).

Biomechanical testing

Mandibles of samples 7 and 11 from the control group were broken during manipulation. The experimental group samples showed a greater statistically significant strength as compared to control group samples. It might pertinent to note that, if the 2 outliers in both control and experimental

Table III. *Biomechanical test results.*

Experimental group	Maximal load (N)	Control group	Maximal load (N)
N 2	56.3	N 3	119.5
N 8	92	N 4	74.2
N 10	64	N 7	0
N 12	67.4	N 11	0
N 13	48.8	N 14	10.9
Averages	**65.7**		**40.9**
SD	**16.36**		**53.7**

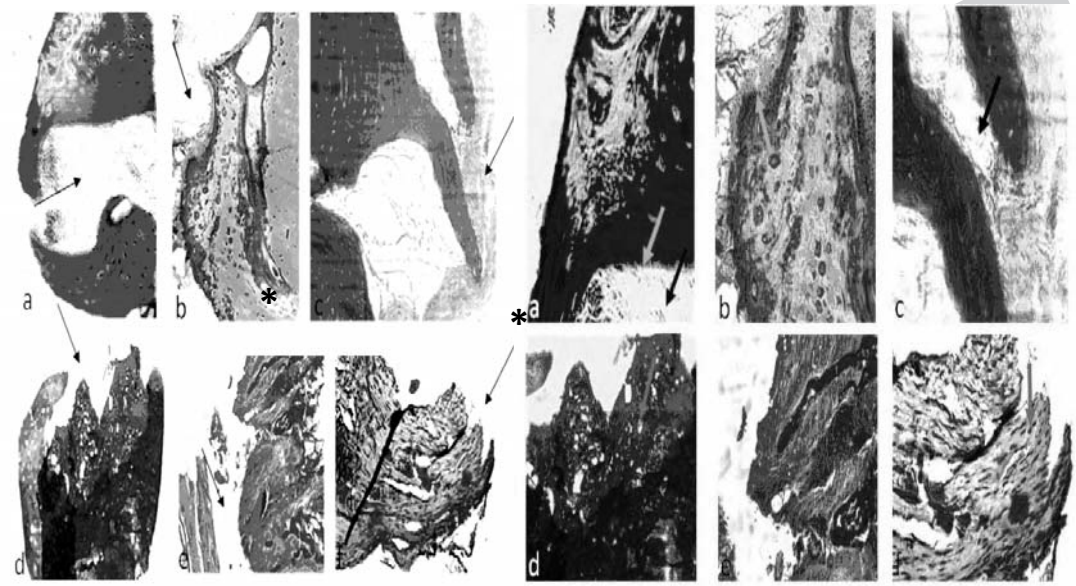


Fig. 7. *(Right panel) Photos of the histological cuts of the regenerate areas at 100 X magnification of the 3 samples of the control group (**a, b** and **c**) and 3 samples of the experimental group (**d, e** and **f**). Black arrows indicate the interface areas. The slides **a**, **b** and **c** clearly showed a great amount of green stained areas (black star) which corresponded to soft tissue and collagen fibers presence, reflecting a lack, or low level of calcification and bone maturation. The slides d and e were highly eosinophilic and did not show any green stained areas but more osteoid tissue and trabeculations which can be explained by the maturation of the woven non-calcified bone into a mature cortical bone. The slide f showed, still at the level of the interface, minor areas of green stain indicating incomplete ossification, however, much less than the control group. (Left panel) Photos of the histological sections at 200 magnifications of the regenerate areas of the 3 samples of the control group (a, b and c) and 3 samples of the experimental group (**d, e** and **f**). Black arrows represent the blood vessels in the interface area of the sections of the control groups. Yellow arrows represent the osteoblasts lining the interface area of the sections of the control group. Blue arrows represent the embedded osteocytes shown in the sections of the experimental group.*

groups were excluded (N3 and N8); one would detect a more significant difference between the experimental 59.12 Newton (N)±8.3 N versus 21.3N±35.65 N (Table III).

Histological study

The comparison between control and experimental groups was performed with respect to the amount of trabeculation, osteoid bone formation, and collagen

fibers presence. At low magnification (100 X) (Fig. 7, right), the regenerate areas of 3 animals from of the control group (a, b and c) and 3 samples of the experimental group (d, e & f) exhibited significant differences. The control group samples showed, at the level of the interface, a greater area of green stain for connective tissue than the experimental group. They indicated the presence of more collagen fibers and soft tissue at the interface level, thus, creating a weak point in the bone that corresponded to the area of least resistance.

Moreover, significant histological differences were noted between the sections from both study groups upon comparison at a higher magnification (200 X) (Fig. 7 left). First, blood vessels (black arrows) in large marrow cavities were noted in the interface areas of the control group sections (a and c) reflecting a bone regeneration activity going on in contrast to the experimental group sections (d, e, f) whereby the marrow spaces were already filled with bones trabeculae and osteoid bones.

Second, a large amount of soft tissue stained areas were noted in all sections of the control group (a, b and c), whereas, only one section of the experimental group showed some small green stained areas (f).

Third, the control group sections (a and b) showed at the interface border elongated cells, high cuboidal to columnar, lining its border (yellow arrows) which are bone forming cells (osteoblasts) generating new bone and replacing woven bone into mature cortical bone. Furthermore, the experimental group samples (D and F) showed osteocytes with their lacunae (blue arrows) embedded between the trabeculations of the mature cortical bone.

The fourth difference was related to the amount of trabeculation. The trabeculae were denser and greater in number in the experimental group sections.

DISCUSSION

Distraction osteogenesis is gaining more use as the treatment of choice for the surgical correction of congenital craniofacial skeletal anomalies. However, a long duration for bone consolidation is usually required. Platelet-rich plasma (PRP) can be easily prepared from autologous blood and is considered as a reservoir for growth factors that can stimulate osteoblastic activity and, therefore, speed up the regeneration process (24, 25).

In this study, we injected autologous PRP, locally in the distraction gap, following the distraction phase because, at that point, the colonization of the formed callus by stem cells is at its peak and that PRP has a great role in enhancing the differentiation of stem cells into osteoblasts and osteoclasts, thus, accelerating bone remodeling and regeneration (26).

The results in this study showed that PRP yielded a more predictable healing as shown in Fig. 6 where the radiographs of all experimental samples are alike, while more variations were encountered throughout the control samples. Two samples (7 and 11) from the control group showed soft tissue in the middle area of the regenerates reflecting the presence of immature woven bone at the interface, which caused their breakage during manipulation.

The unpredictable nonconsistent healing in the control group samples was noted in both the mechanical and the histological tests. As shown in Table III, a wide variation in the results of the control group samples (10.9N to 119.5N) was noted compared to results with less variation (48.8N to 92N) in the experimental group samples. A large amount of soft tissue was present in samples b and c of the control group, in addition to less green stained collagen reflecting a variation in the amount of immature woven bone still present (Fig. 7 right and left).

On the other hand, the samples d, e and f of the experimental group showed a mature bone and well defined trabeculations with minor amounts of soft tissue. These results indicate that PRP played a major role in decreasing variability between samples.

The macroscopic examination of the mandibles showed a great difference between both experimental and control groups concerning the presence of soft tissue at the middle area of the regenerate. In the experimental group, the regenerate area showed a well calcified area with little presence of soft tissue, thus indicating an accelerated rate of ossification.

Furthermore, in 3 to 5 samples of the control group there was a higher amount of soft tissue at the interface level and not enough ossification resulting into breakage of 2 of them at that site during manipulation and the breakage of the third sample at a very low load of 10.9N during the mechanical test. Hence, the middle area of the regenerate was

the last to calcify. Our macroscopic observations could be explained by the fact that during 45 days of consolidation period, 3 to 5 samples of the control group did not have enough time to fully calcify and, therefore, they showed relatively abundant soft tissue in the middle area and were easily broken at that site. On the other hand, all the experimental samples were fully calcified and, therefore, PRP, due to probably multiple growth factors present, accelerated bone remodeling at the regenerate area and helped newly formed bone to gain maturity in a faster timeframe.

In addition, the data emanating from the dual X-ray absorptiometry assessing the bone mineral content and density showed a trend of grossly stronger experimental mandibles treated with PRP without any gaps left at interfaces. However, the number of animals per group was small with wide variation in values leading to no significant statistical differences between groups. Such results could be explained by the fact that after 45 days, the proximal areas of the regenerates were ossified in both groups to different extents and leaving gaps in the controls filled with connective tissue elements. An intermediate time point of 30 days could have been very useful. So, for that reason, data in Tables I and II showed no significant differences between both groups. To detect such differences, a dual x-ray absorptiometry test should be restricted on the interface area of 0.2 mm and not on all the regenerate; this couldn't be achieved with the conventional dual x-ray absorptiometry machines present in our current facilities.

Our results are in line with the study Swennem et al. (25). The nonsignificant difference found between the control and experimental groups concerning the bone mineral density of the regenerate was concomitant with our findings (Tables I, II). The main difference between the 2 studies consisted of an increase in the bone density at the proximal areas of the regenerate in the experimental group where PRP was injected directly before the distraction phase as compared to the control group. It is important to note that the main difference was found at the interface area, the middle area of the regenerate, whereby, 3 samples of our control group showed, upon the macroscopic examination, a large amount of soft tissue in that area. Two of these samples were broken during harvesting at that site and one of them showed a very low flexural strength during the mechanical test (Table III). In addition, two of them clearly showed great amount of trichrome stained collagen fibers at the interface area as compared to the experimental group which did not show soft tissue.

However, in the experimental PRP treated group, after 45 days of consolidation period, it was shown that the proximal areas were completely calcified and the middle areas were the least calcified and consequently the last areas to complete ossification (26). These findings could explain our results indicating the presence of immature bone at the interface level in the control group as reflected by the extra time needed by this group to complete its ossification compared to the experimental group, receiving PRP, who had completed its full ossification during the same time frame.

The results in Table III showed a significant statistical difference between both groups. If we exclude 2 outliers from the control and experimental groups, the results showed a greater strength of the experimental samples as compared to the control group samples. This can be explained by the fact that the presence of soft tissue at the interface area in samples of the control group reflected a lack of ossification in these samples that compromised their flexural strength as compared to the experimental group samples which did not show soft tissue at the interface level reflecting their complete ossification. In conclusion, PRP, added to the experimental samples offered a greater strength for these samples.

Moreover, the histological study of the regenerate areas of both groups showed the presence of a relatively large amount of soft tissue at the interface level in the samples of the control group while there was little amount of soft tissue noted in the experimental sections. This finding reflected less ossification of the interface area in the control group samples and consequently the positive role of PRP in speeding bone remodeling in the experimental group.

Mandibular distraction osteogenesis proved its efficacy in treating various pediatric craniofacial deformities but with the disadvantage of requiring a long consolidation period which corresponds to a long immobilization period with the subsequent adverse events including mainly bone fractures and feeding disorders. As shown in this study, the use

of platelet-rich plasma played an important role in accelerating the ossification following distraction, especially at the interface, which is the last area to gain maturity; thus reducing this long consolidation period and accelerating bone remodeling and regeneration. After 45 days, full maturity was gained in the experimental samples injected with PRP as opposed to less maturation in the control samples. A larger sample size and more time points are needed for statistical analysis to ascertain the specific role of PRP.

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