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Evaluation of distraction osteogenesis of membranous onlay bone graft: An experimental animal study

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***Abstract*---**Purpose: This study aimed to assess the changes of membranous bone onlay grafting in the mandible of dogs after application of distraction osteogenesis. Materials and Methods: Twenty adult Mongrel dogs were divided into four groups; each with five dogs. A 3–4 cm zygomatic arch membrane onlay graft were secured on the lateral side of the mandible. Vertical osteotomy was performed after 1, 2, 3, and 4 weeks for Groups I, II, III, and IV, respectively. One week after osteotomy, distraction osteogenesis was initiated at a rate of 1 mm/day for ten days using an external apparatus. The apparatus remained for six weeks to stabilize the bone. Radiographs were taken at 2, 4, and 6 weeks post-distraction, and the dogs were euthanized six weeks after distraction for analysis. Results: The results showed new bone formation between the distracted mandibular segments in all dogs. Groups II, III, and IV showed new bone formation beneath the onlay graft, but this was not observed in Group I. In Group IV, the onlay graft partially reabsorbed, but the newly formed bone had a near-normal cortical structure. Histological analysis revealed active osteoblastic activity and bone formation in Groups II and III, with a significant amount of fibrous tissue. Group IV showed higher osteoblastic differentiation and bone resembling typical cortical morphology. Conclusion: These findings suggest that distraction should be performed at least four weeks after onlay grafting and demonstrate the feasibility of distraction osteogenesis in membranous bone onlay grafts.

***Keywords*---**Mandible, Dogs, Distraction, onlay graft.

Introduction

Distraction osteogenesis was proposed as an alternative method of reconstruction, through which the mandibular gaps could be replaced by new bone [1,2]. The application of distraction osteogenesis in the craniofacial skeleton was described by the using of divergence osteogenesis to increase mandibular length. Afterward, numerous trials had been performed for cranial complex rehabilitation. [3]. Distraction osteogenesis found to be effective in formerly irradiated bone and might be a straightforward technique for reconstructing the mandible after ablative surgical treatment of the head and neck [4,5]. It provides a major advance in the treatment of congenital mandibular deformities, maxillary and midface hypoplasia [6–8]. In addition to a successful surgical substitute for orthognathic surgery that treats transverse mandibular insufficiency and widens the jaw without removing teeth [9].

Bone is still the golden tissue for recreating the craniofacial skeleton's shape and function [10]. By membranous and endochondral calcification, mesenchymal tissue gives rise to bone in the embryo. Within clinical setting, bone grafting has become more necessary as a result of tumors, trauma, craniosynostosis, and simple cosmetic bone surgeries. [11] Although these methods were effective, but there are still several problems as possible facial nerve injury, inferior alveolar nerve injury, infection and loss of bone tissue bulk particularly in patients of mandibular hypoplasia [12,13].

Throughout a long time, many kinds of bone grafts were utilized to treat deficiencies in the craniofacial bone; however, autogenous grafts have numerous drawbacks, including restricted donor locations, donor morbidity, discomfort, growing imperfections and resorption [1]. Freestanding membranous bone grafting has been shown to generate fresh bone and has a good survivability percentage among the young people having mandibular abnormalities. Compared to endochondral bone, onlay membranous bone grafts had a higher rate of surviving, with earlier vascular development at three days, excellent vasculrization at seven days, and full vasculrization at fourteen days, according to numerous authors [11,14].

Different surgical bone grafts replacements in animal models were produced for mandibular lengthening with distraction osteogenesis. Distraction osteogenesis created fresh bones within the maxillofacial skeleton through direct mesenchymal stimulation[15,16]. In some cases the bulk of tissue available for distraction osteogenesis not enough, so the procedures was repeated to compensate the deficient bone[17,18]. Repeated the procedure lengths the treatment period, so it is proposed that addition of membranous onlay graft to the mandible may aid in having good bulk of tissue necessary for distraction osteogenesis [19].

Accordingly, the application of free membranous bone onlay grafting, for understanding how bones develop while distracted when treating extreme mandibular hypoplasia that necessitates both a bone transplant and distraction, osteogenesis of the mandible might aid to reduce the course of therapy. The scarce amount of the available literature have indicated the need for further research to evaluate experimentally distraction osteogenesis after membranous bone graft in animals.[20] And therefore the present study was conducted to answer the following questions: 1) Is it feasible for bone to develop throughout a free membranous bone onlay graft's distracted osteogenesis? 2) Are distraction-induced osteogenesis sites in a free membranous bone onlay graft and typical bone histological different histologically? 3) When is distraction feasible following membrane bone onlay grafting?

Materials and Methods

Twenty adult Mongrel dogs were utilized for the present research. All the dogs were examined to ensure normal health and ability to survive the surgical procedure. All the animals were operated at the department of surgery, faculty of veterinary medicine, Zagazig University.

During two weeks prior to surgery, the animals had been undergoing clinical care and were given meat that had been cooked, bread, milk, as well as water. Each animal was kept in a different cage, at the experimental surgical unit, faculty, of veterinary medicine Zagazig, University, provided with meals and a pail of water, and given the best possible living circumstances. This was to follow the selected dogs clinically and to get rid of internal parasites such as ankylistoma, taenia solium and taenia saginata which was determined by urine and fecal analysis.

External parasites such as scabies were eliminated by injecting all dogs with Ivomac[1] subcutaneously (200mg/kg body weight). All dogs were vaccinated against Rabies[2], Distemper Hepatitis[3] and Leptospirosis[4] by the Tribue vaccine[5'].The animals were divided into four equal groups, 5 dogs each, according to the time of osteotomy after bone graft fixation.

Animal groups:
Group I Include five dogs and the osteotomy performed 1 week post distraction.
Group II Include five dogs and the osteotomy performed 2 weeks post distraction.
Group III Include five dogs and the osteotomy performed 3 weeks post distraction.
Group IV Include five dogs and the osteotomy performed 4 week post distraction.

Preparation and premedication:

One day before the operation, the animals fasted for food intake for and water intake for 4 hours 12 hours prior to the operation in order to get them ready for anesthesia. Dogs were weighted to determine the medicine dosage used and to obtain a first report of the dog's overall health. A course of systemic broad spectrum antibiotic for animals[1] began one day prior to operation and sustained before 5 days following the operation the operated animals were premedicated by atropine sulphate[2] 0.5mg and combelen[3] 0.2mg/kg body weight. Both drugs were administrated intramuscularly 20 minutes before induction of general anesthesia.

Induction of general anesthesia:

All animals were anaesthetized using intravenous injection of sodium thiopental in a dose of 30mg/kg B.W. in cephalic vein. In fifteen seconds, a third of the prescribed dosage was injected; after regaining of respiration, the remainder of the dose was administrated slowly until loss pedal and pupil narrowing, regular breathing development, and ocular responses. The maintenance of anesthesia was induced by another dose of thiopental Na[5] according to the signs of surgical plane of general anesthesia.

Operative technique:

1- Preparation of the operative seats
The hair was shaved at the zygomatic region and the lower jaw. The shaved area was washed with water and soap, then sterilized with ethyl alcohol and betadine

solution 7.5% then the animal were draped with sterile drape except the operative areas.

2- Preparation of the graft:

Using no. 10 Bard parker blade, a longitudinal skin incision was made over the middle area of the zygomatic arch. The subcauteneous tissues were dissected and the zygomatic arch was exposed. A full severed thickness 3-4cm segment of the zygomatic arch was harvested by using saw wire and the wound at the zygomatic arch was sutured in a classical manner. The periosteal covering of the zygomatic arch was longitudinal incised and dissected Fig. (1). The graft was kept in a container of saline solution (0.9 % sodium chloride) until making the recipient site.

3- Preparation of the mandibular wound:

A submandibular 5-6cm skin incision was performed at 0.5mm below and parallel to the lower border of the mandible. The periosteoum was sharply divided and elevated to expose the lower border and the buccal surface of the mandibular body in the molar region. A great care was taken to preserve the periosteum intact during its elevation that is considered a fundamental principal of the Ilizarov technique. The periosteal elevation was continued around the lower border of the mandible to expose the lower half of the medial surface with a small tunneling medially against the planned osteotomy site. Utilizing screws, a subperiosteal chamber having solid bone-to-bone connection was created for the membranous bone transplant. The wound was flushed by normal saline and closed in layers.

Fig (1): Photograph showing the membranous a full- thickness 3-4 cm segment of zygomatic arch bone graft was positioned in a subperiosteal pocket having solid bone-to-bone contact utilizing screws

Osteotomy application:

The mandibular body underneath the bone graft and the osteotomy that was created after one week in group I, two weeks in group 1, three weeks in group III and four weeks in group IV. To avoid compromising the blood supply, a vertical incision above osteotomy was created in the skin covering the mandibular body and bone graft, and a small amount of soft tissue dissection was performed over the graft. Using a surgical bur, a vertical osteotomy was created in the middle of the bone graft and the external cortex of the mandibular body below. An osteotome was used to make a greenstick fracture of the interior cancellous part while preserving the integrity of the inferior alveolar neurovascular bundle. The wound was flushed by normal saline and closed in layers as in the usual manner. All dogs were given a 5 days course of using amoxicillin (Ibiamox) 500mg during surgery and every 12 hours until removal of skin sutures. 3 days fluid therapy (dextrose 5%, ringer lactate and 0.9% Na Cl solution intravenously) and daily cleaning of the wound around with application of gentamycin antibiotic ointment, soft diet was provided until3 days postoperatively, then removal of the skin sutures after 7 days of the operation was performed.

The Distraction Device:

Fixation clamps are outwardly coupled to percutaneous pins that secure the extraoral devices* to the mandible. In consequently, a linear distraction bar (distractor) connects the fixation clamps so that, once actuated, the bar actively pulls the clamps and the associated bone sections away. Simply the device comprises of a telescopic rod which is triggered by a screw system holding 2 double-pin fixation bulbs in place. By rotating one clamp and moving the second clamp across the telescopic distractor axis, the appliance may be adjusted to the anatomical size and form of the mandible according to Hoffiman Mini Lengthener.

1- External distractor device fixation:
The external distractor device was fixed by tow bicortical pins, 3mm in width, which were inserted subcutaneously at a distance of around 20 mm from one another and one at each side of osteotomy line (Fig. 2).

Fig (2): Photograph showing the external distractor device with its components was fixed by tow biocritical pins, 3mm in diameter percutaneously and one at each side of osteotomy line

2- Distraction regimen:
After seven-day delay time, distraction was initiated at a daily rate of 1 mm for 10 successive days. The distraction appliance was designed so as to give one millimeter of distraction with each complete turn of its screw. The distraction was tested for any signs of instability during the daily distraction and also after that.

The appliance had remained in position for six weeks after the distraction was finished to permit bone consolidation. The animals had been euthanized by giving an overdose of thiopental Na.

Radiographic evaluation:

Radiographs were done at 2, 4, and 6 weeks post-distraction performed standardized contact x-ray of the skull dorso-ventral and latral views were using Tur x-ray machine (GDR)* equipped with a cylindrical (PID), 60k.v. the exposure factors were, 8ma/s and 40cm focal film distance X-ray cassette was loaded with 16x 24cm fuji medical x-ray film. All radiographs were taken with the same apparatus at the same position and the same exposure factors.

Gray scale analysis:

The radiographs were scanned using artec scanrom 4 E 3* scanner. The scanner is a fully functional, portable scanner. It allows for scanning negative films as well as photographs without requiring additional tools. The features of this scanner arc: 24-bit true color and up to 4800DPI (dot per inch) resolution. In addition to scanning images, it is capable of scanning positive films without requiring additional tools. It was connected to an IBM compatible computer. The scanned images were analyzed by scion image 4** software in gray scale.The gray scale is a scale of achromatic colors having 256 graduation ranging from 0 for black to 255 for white.

Two lines were traced on each image to determine the region of interest (ROI).The first line is traced tangential the occlusal plane of last molar tooth, while the second line tangent the lower border of underlying mandible. The two lines tangential forming a center of the circle traced. The radius of circle resulting with diameter of 1.5 cm is the region of interest, and the gray value of the (ROI) was measured by software program Adobe photoshop 8CE. For each gray value the percentage of radio-opacity is calculated from the following equation:

$$\text{Percentage of radio} - \text{opacity} = \frac{\text{Gray valve of ROI}}{256\ (\mathit{Gray\ scale})} \times 100$$

All data were collected statistical analysis by software program SPSS version 12 at level of significant 5%.

Microscopic examination:

After euthanization and utilizing a saw, longitudinal samples of the extended portion of the mandible as well as the nearby typical bone were created. The samples were subjected to the decalcification within 10% nitric acid and 10% sodium citrate over two days after the excised mandible was fixed for one week in 10% neutral buffered formalin. Following decalcification, the distracted area was cut with a scalpel, cleaned to get rid of extra fixative, and then let to pass across progressively stronger ethyl alcohol solutions to dehydrate it. Hematoxylin and eosin were then applied to the slices for microscopic inspection.

Results

Gross Observations

Every dog had a full recovery and accepted the distraction tool. Throughout the expansion, the device was washed every day. In all animals, there was a fresh bone visible between the disoriented original mandibular sections.
In group I, the length increased by 8 mm. While it was 7mm in group II, 6mm in group III, and 8mm in group IV. In group II, the membranous bone-only graft was partly resorbed, and across the portions, no fresh bone grew. On the other hand, fewer fresh bones had grown across the distracted area's margins in groups II and III compared to in the mandibular section underneath.
Due to inflammation, group IV had partly resorption of the membrane bone-only graft within the vicinity of the pin fixation site. Within each segment, nevertheless, a large amount of fresh bone had grown that seemed almost typical on the cortical surface.

Histologic Results

Healthy osteoblastic differentiation was seen in the membranous bone-only graft, and the bone structure resembled that of the supporting mandible. There were 10 functional capillaries in the membranous bone alone graft and 11 in the underneath mandible at magnifications of 100 and 400, respectively. These results imply that the membranous bone-only graft's vascularization has reverted to a relatively typical state. Within the middle area of the distracted space in the underneath mandible, there was broad fibrosis and growth of numerous osteoblasts developing osteoid. Additionally, there were partly calcified, differently shaped weaved bone trabeculae and variable-sized fresh capillaries combined in with undeveloped weaved bone trabeculae and a tiny amount of fresh capillaries.

The membranous bone onlay graft in the animals belonging to group I did not exhibit any fresh bone development in the distracted region.in dogs of group II, The diverted area was nearly crossed via cross section of group I and II at six weeks after distraction showing direct bone development from the surface of the host bone margin toward the central interzone with collagen fibers arranged parallel to the direction of the distracted force.

Every animal had increasing calcification in the distraction region of the native mandibular section, as seen by sequential postoperative radiographs which composed of a core radiolucent zone sandwiched between proximal and distal sclerotic zones. Animals from groups II and III had tiny radio dense areas along the margin of the distraction zone of the membranous bone-only graft, while animals from group I did not have any radio dense regions in the distraction area of the membranous bone-only graft.

Animals in group IV had regions of calcification proximally and distally, along with a radiolucent middle zone, over the whole diameter of the distracted sits in both the membranous bone onlay graft and the underneath mandibular section. After six weeks, the newly formed bone expanded toward the distraction zone's core and developed more radio dense (Fig. 3-5).

The result of the present study including four groups of dogs to evaluate bone density expressed by gray scale analysis by means of pixels of ROI (region of interest). Table (1) showing that bone density by means of gray scale analysis for group I at one week post-distraction processes was 170.348+14.51454. Bone density by means of gray scale analysis for group II at two weeks post-distraction processes was 174.964+37.54466. Bone density by means of gray scale analysis for group III at three weeks post-distraction processes was 178.548+15.99878. Bone density by means of gray scale analysis for group IV at four weeks post-distraction processes was 224.676+5.935855

Statistical comparison between the between the main four groups by means of gray scale analysis Group I, Group II, Group III, Group VI at 1,2,3,4 weeks with One-way ANOVA test was found that there is a very highly significant difference F Value = 6.67194 P<0.01 within group analysis and significant difference between groups Table (2). Statistical comparison between two different means by independent t test the difference between mean values by pixels of Group I Vs Group II was (4.616) non-significant (P>0.5), the variation amongst mean scores by pixels of Group I Vs Group III was (-0.102) non-significant (P>0.5), the variation amongst mean scores by pixels of group I Vs group IV was (-54.382) very significant (P<0.001), the variation amongst mean scores by pixels of Group II Vs Group III was (-3.584) insignificant (p>0.05), the variation amongst mean scores by pixels of Group II Vs group IV was (-49.712) significant (p<0.05), the variation amongst mean scores by pixels of Group III Vs group IV was (-46.125) very significant (p<0.001).

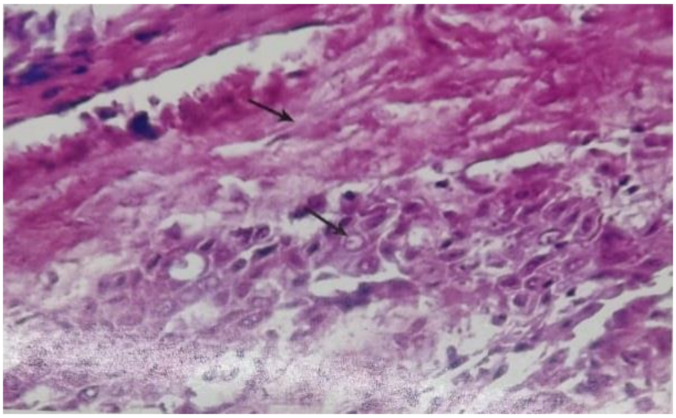

Fig. (3): Photomicrograph of the mandible and membranous bone onlay graft of group I at six weeks after distraction showing a wide unossified gap filled by a loosely textured fibrous tissue showing collagen fibers deposition in a parallel pattern as well as a few cartilage island and undeveloped weaved bones are observed (H&E Stain, mag.X400)

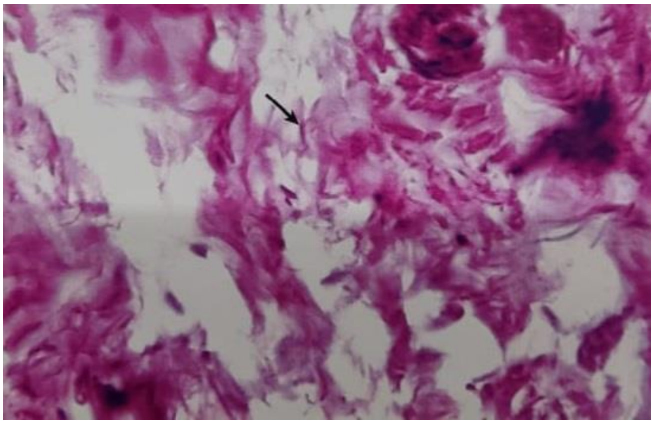

Fig. (4): Photomicrograph showing membranous bone onlay graft and underneath mandible in group II at six-weeks following the distracted zone almost bridged by new bone and cartilage island and undeveloped weaved bone. (H&E Stain, mag. X 400)

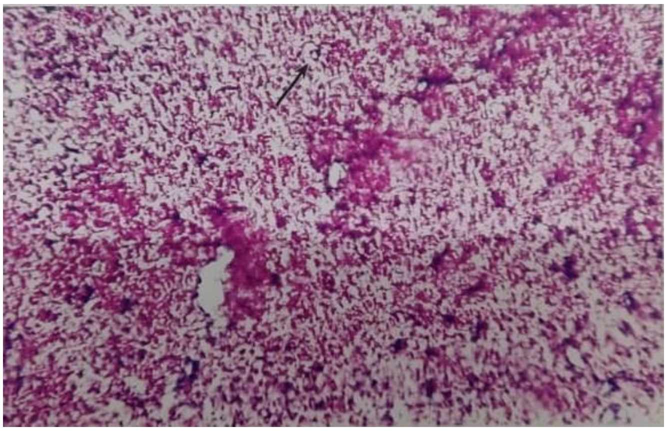

Fig. (5): Photomicrograph showing membranous bone onlay graft and underneath mandible in group IV the distraction gap was complet obliterated by osteogenic activity of bone spicules in the form of trabecular network enclosing several islands of fibrous tissue. (H&E Stain, mag. X 400).

Discussion

The microscopic observations in this study confirmed that most of the newly formed bones in the distracted zone of the normal jaw in a membranous bone-only graft developed through intramembranous bone formation, with only a small portion of the distraction space filled by cartilage. These outcomes could be due to the alignment of the distraction vector in the applied device, despite the variation in length gains from 6 to 10mm, with the device being activated to 10mm. Mehrara et al. demonstrated that osteocalcin gene expression coincides with calcification during mandibular distraction in rats and that transforming growth factor-B1 may play a key role in vasculogenesis and osteogenesis [21] . It is advised to use an intraoral device or four pins to accurately transfer the activation length to the distraction zone. The craniofacial skeleton can tolerate both a full osteotomy and a liberal lifting of the periosteum, improving survival rates of non-vascularized bone grafts with preserved periosteum [22]. The periosteum likely aids in revascularization and osteogenic potential, potentially preventing resorption and aiding early revascularization of membranous bone onlay grafts. [23,24]

Many studies have shown that in animal models, non-vascularized membranous bone grafts experience less resorption than endochondral bone grafts. Membranous bone grafts likely maintain their structure and show significant subperiosteal callus formation, making them a better option for craniofacial rehabilitation. High oxygen tension and rigid fixation can facilitate initial regeneration[25]. It was also reported that mobility due to inadequate fixation leads to bone resorption, highlighting the importance of fixation during distraction. [26] Membranous bone grafts, compared to endochondral bone grafts, demonstrate better volume preservation due to delayed or rapid revascularization, aiding graft survival [27]. Studies suggest that membranous bone outlasts endochondral bone and that cortical bone grafts outperform cancellous bone grafts in preserving volume, with cortical bone being considered superior for grafting. [28].

In the current study, the zygomatic arch was chosen as the donor site due to its ease of harvest and shorter operation time, though it is less commonly used for practical grafting.[29,30]. It was expected that new bone would form at the margins of the distraction zone in groups II and III, with nearly typical bone development in group IV and revascularization of the membranous bone graft from surrounding tissues. The study suggests that distraction should not be performed earlier than four weeks after bone grafting to allow new bone formation within the graft. The soft tissue dissection and osteotomy likely influenced revascularization, leading to limited osteoblastic activity following distraction.

Successful bilateral mandibular lengthening in patients with significantly hypoplastic jaws was also reported. CT imaging to assess mandibular volume following distraction osteogenesis, found an average increase of 27% in the distracted hemimandible and 25% in bilaterally distracted mandibles. However, this increase was sometimes insufficient to correct severe facial bone hypoplasia, despite increases in soft tissue and skeletal volume. For extreme craniofacial microsomia cases, combining free vascularized scapula bone grafts with mandibular distraction osteogenesis is recommended [31]. Distraction osteogenesis can be successfully performed on membranous onlay bone grafts, with excellent results when distraction is initiated at least 4 weeks post-graft [19]. Membranous bone grafting demonstrated superior survival and growth compared to endochondral grafting, maintaining their volume over time. It was found that at 5 months post-procedure, distraction osteogenesis produces bone with higher remodeling rates than onlay grafting, as evidenced by increased osteoblast and osteoclast activity [32]. The use of osteopromotive membranes enhances bone deposition in both inlay and onlay graft, with membranous graft showing better incorporation and less resorption. These findings highlight the biological differences of membranous bone graft and the potential benefits of combining grafting techniques with distraction strategies.

Conclusion

These findings suggest that distraction should be performed at least four weeks after onlay grafting and demonstrate the feasibility of distraction osteogenesis in membranous bone onlay grafts.

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Figure legends:

Fig. 1: Photograph showing the membranous a full- thickness 3-4 cm segment of zygomatic arch bone graft was positioned in a subperiosteal pocket having solid bone-to-bone contact utilizing screws.

Fig. 2: Photograph showing the external distractor device with its components was fixed by tow bicortical pins, 3mm in diameter.

Fig. 3: Photomicrograph of the mandible and membranous bone onlay graft of group I at six weeks after distraction showing a wide unossified gap filled by a loosely textured fibrous tissue showing collagen fibers deposition in a parallel pattern as well as a few cartilage island and undeveloped weaved bones are observed (H&E Stain, mag.X400)

Fig. 4: Photomicrograph showing membranous bone onlay graft and underneath mandible in group II at six-weeks following the distracted zone almost bridged by new bone and cartilage island and undeveloped weaved bone. (H&E Stain, mag. X 400)

Fig. 5: Photomicrograph showing membranous bone onlay graft and underneath mandible in group IV the distraction gap was complet obliterated by osteogenic activity of bone spicules in the form of trabecular network enclosing several islands of fibrous tissue. (H&E Stain, mag. X 400).

Every animal had increasing calcification in the distraction region of the native mandibular section, as seen by sequential postoperative radiographs.

Table (1): Descriptive analysis of gray -scale analysis of ROI in all groups by pixels

No. Of the Dog	group I (one week post distraction)			group II(Two weeks post distraction)			group III (Three weeks post distraction)			group IV- (four weeks post distraction)		
	Mean	**Minimum**	**Maximum**	**Mean**	**Minimum**	**Maximum**	**Mean**	**Minimum**	**Maximum**	**Mean**	**Minimum**	**Maximum**
1	158.68	157	56262	180.34	198	60932	198.32	382	67322	230.32	237	64332
2	165.72	198	56298	198.48	230	68234	156.45	398	69837	225.72	198	56298
3	158.79	178	53762	109.32	240	68374	180.32	287	68392	228.79	178	53987
4	193.32	187	72334	187.34	256	60923	170.42	309	72334	223.32	187	53762
5	175.23	142	52133	199.34	297	63422	187.23	443	37492	215.23	142	52133
Mean	165.720			187.340			180.320			224.676		
SD	14.514			37.544			16.998			5.935		
CV	8.75%			20.4%			8.78%			2.64		

Mean Arithmetic mean
SD Standard deviation
CV Coefficient of variation

Table (2): Statistical comparison between two different mean by independent t test

Difference between two means			
Group I Vs Group II	-4.616 0.3077^{Ns}		
Group I Vs Group III		-0.102 0.74021^{NS}	
Group I Vs Group IV			-54.382 6.5530***
Group II Vs Group III		-3.584 0.185083^{NS}	
Group II Vs Group IV			-49.712 2.71529*
Group III Vs Group IV			-46.125 6.1775***

NS Non Significant
* Significant Difference $p<0.05$
*** Very Highly Significant