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# DIGITAL RADIOGRAPHY AND VITAL STAINING AS DIAGNOSTIC TOOLS TO ASSESS BONE REGENERATION: IN VIVO STUDY

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## ABSTRACT
**Background /aim:** The field of tissue engineering has developed over the past decade to recreate functional, healthy tissues and organs in order to replace diseased, dying or dead tissues**.** Digital radiography is a reliable and versatile technology that expands the diagnostic and image referral possibilities of radiology. The objective of this study is to evaluate the efficacy of DIGORA and double tetracycline labeling test as diagnostic aids in bone regeneration assessment. **Methods and Material:** Seven dogs were used for the study. Three critical sized calvarial defects were created in each dog. One defect was filled with autologous stem cells seeded on chitosan scaffold and soaked in osteogenic media, the second was filled with stem cells seeded on chitosan scaffold and soaked with Alendronate 10mg/ml and the third one is filled with stem cells seeded on chitosan scaffold. The rate of bone formation was tested using Digora and tetracycline double labelling test after eight weeks in each defect. **Results:** Both techniques showed enhanced bone formation in groups (A) & (B) compared to group (C). This was consistent with the H & E results published by our group in a previous study. **Conclusions:** DIGORA as a digital radiography tool and vital staining with double injection of tetracycline are reliable diagnostic and prognostic methods for assessing new bone formation.

**KEYWORDS:** Digora, digital radiography, double tetracycline labeling test, bone regeneration, stem cells, chitosan, scaffold.

## INTRODUCTION
The ideal strategy for bone formation would be to combine a biomaterial scaffold with competent cellular elements and molecular and environmental cues. Current efforts are directed towards investigating each of these components.[1]

Bluteau et al.[2] have defined tissue engineering as combining different life sciences and engineering principles aiming to describe structure - function relationships in mammalian tissues and developing biological alternatives to restore, maintain or improve tissue function and patient welfare. The regeneration of bone, and the osteogenic potential in osseous defects resulting from maxillary alveolar clefts are of great importance in terms of the restoration of jaw morphology and function.[3]

Bony defects represent a headache both at the socioeconomic and biomedical levels. Skeletal defects may result from traumatic accidents, tumors resection or surgical correction of congenital defects.[4] These defects are nowadays treated by a complexed, multistep reconstructive surgical procedures. Autogenous, allogenic and prosthetic materials are currently all the options available for the reconstruction of these challenging osseous defects.

Radiography is the major non -invasive, non- surgical method for detecting bone formation in a healing osseous defect. Thus, it is useful in clinical situations due to its speed, continuity of measurements and non - invasiveness. Bone healing is expressed radiographically by an increase in radiopacity, resulting in a higher optical density of the bone image.[5]

Different radiographic methods could be used for assessingt bone healing and this includes; computed tomography (CT), cone beam computed tomography (CBCT) and digital radiography (DIGORA).[5] It was reported that, digital radiography is a versatile and reliable technology expanding the diagnostic and image referral possibilities of radiology in dentistry.

Polychrome sequential labeling with fluorochromes is a standard technique for the investigation of bone formation and regeneration processes in vivo. Due to their high affinity for calcium the tetracyclines are incorporated at the site of active mineralization of hydroxyapatite. To generate parallel fluorochrome bands, the same tetracycline derivate is injected twice with a 10-day interval between injections, allowing morphometric measurements.[7]

Tetracycline markers viewed under fluorescent light reveal luminescent yellow-green bands within the bone. The separate dual bands indicate active mineralization of the new bone.[7]

A number of different types of tetracycline compounds can be used, and each will fluoresce a different color. Tetracycline and demeclocycline are the most commonly used agents. The intensity of the label depends on the medication dosage.[7]

If dynamic data on the new bone formation, including bone turnover, bone formation rates and mineralization defects are to be analyzed using histomorphometry, then administration of time-spaced tetracycline markers is necessary prior to the bone biopsy.[6,7]

This study aimed to study the reliability of Digora and double tetracycline labelling tests in assessing bone regeneration potential in critical sized bone defect in dogs using adipose derived stem cells in presence and absence of locally delivered alendronate.

## MATERIAL AND METHODS

### I - Stem cells isolation and culture

**Stem cells** used in this study were isolated from fat tissue (ADSCs) excised from the inguinal pad of fat of dogs, resuspended at density of 10.000 cells/$cm^2$ in complete culture media and incubated in a $CO_2$ incubator at 37°C in a humidified atmosphere of 5% $CO_2$ ( Sigma - Aldrich).[1]

### II- Surgical procedure

Seven healthy adult mongrel dogs aging from 12 to 24 months at an average weight of 20 kg were included in this study. Dogs were carefully chosen to have small sized head average 10-15 cm in the greatest diameter.[1]

Dogs were anaesthetized by a mixture of xylazine HCL (1mg/kg weight) and ketamine HCL 5mg/kg body weight. Anaesthesia was maintained through the operative time by venous drip of a mixture of 0.5 gm thiopental sodium and 500 ml dextrose 5% with a drip rate ranged from 28- 40 drops/minutes. A curvilinear sagittal incision (2.5 cm) was made in the cutaneous and subcutaneous tissues in the parietal, nasal and frontal bones. The underlying periosteum was similarly incised and the flaps were elevated to expose the calvarial bones. A standard critical sized surgically created defect was performed using a trephine bur 1cm in diameter. Three full thickness bone defects with a size of 1$cm^2$ in diameter were created at the parietal, nasal and temporal bones of each dog and the periosteum of 1 cm surrounding the defect was removed. The parietal defects were repaired with the autologous ASCs/chitosan+alendronate scaffolds, while the defects at the nasal defects (control) were treated with ASCs scaffold alone. The temporal defects were repaired with the autologous ASCs/chitosan+osteogenic media. The wounds were then routinely closed in layers using vicryl material size 0.[1] (figs. 1, 2).

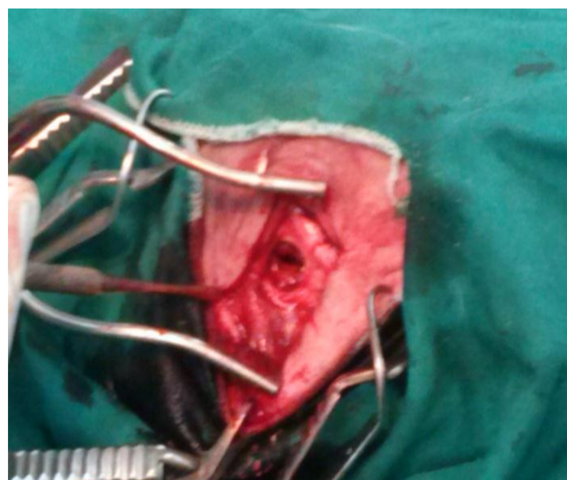

**Figure (1): Photograph showing full thickness bone defects with a size of 1cm in diameter.**

**Figure (2): Photograph showing bony defect after filling**

### Numerical evaluation of bone growth

### 1. Double tetracycline labeling test

Tetracycline (TC) labeling is used to determine the amount of bone growth within a certain period of time

(approx. 10-14 days period). Due to their high affinity for calcium the TCs are incorporated at the site of active mineralization of hydroxyapatite. Osteoid is new bone that has been laid down by the osteoblasts and is non-mineralized. The label is incorporated into the mineralized bone at the osteoid interface and fluoresces under UV light microscopy. Usually TC is given at two time points with a ten days gap in between the dosing periods. It is recommended that the biopsy not be taken until at least 3-5 days after the last label. Dosing of the TC is dependent on the model (human, rat, rabbit, canine etc.). To generate parallel fluorochrome bands, the same TC derivate is injected twice with a 10-day interval between injections, allowing morphometric measurements.

The bone must not be decalcified in order to view the labels. Decalcification of the bone removes the labels.

Bone formation rate (BFR) is the mean width of new bone formed per day, calculated by dividing the distance between two opposing points from the middle of two consecutive bands by the number of days elapsed between them.

The fluorochrome used in this work was TC, administered as an intramuscular injection in a dose of 20 mg/kg body weight at day thirty five following the surgery. A second dose was injected after a fourteen day interval. After sacrificing the dogs, ground sections were prepared and examined under fluorescent light microscope. The distance between the two fluorescent bands indicated the rate of bone formation.

**2. Assessment of the bone formation rate (BFR) using DIGORA**

DIGORA system (Soredex, Finland) used stored radiographs on a characteristic sensor (phosphorous board) similar to a periapical radiographic film. The radiographs were taken by placing the specimen, sectioned bone, on the sensor and using an X-ray machine with 50 kVp and 10 mA.

After exposure, the sensor was placed in the DIGORA scanner for reading and processing. Twenty-four seconds later, the image appeared on the monitor and can be used for measurments.

The system presented the maximum and minimum density of this image, as well as the mean density in pixels, the latter being used as the result.

Each digital radiograph was analyzed using the Direct Digital Radiography. The relative bone density of the pixels of the bone defects was measured 3 times for each point to diminish errors, then the mean values for bone density for each defect were recorded. The observations were then averaged.

The collected data were tabulated using Microsoft Excel (Microsoft Office 2007). Analysis of Variance was used to test the significance of difference between different groups. Tukey test was done to test the significane of difference between alendronate medium group and osteogenic medium group and between each group and control group. All statistical analyses were performed using SPSS for windows ver.10.1. The level of α was 0.05.

**Fig. (3): Photograph showing the measurement of bone density with DIGORA.**

## RESULTS

### 1. Double tetracycline labeling test interpretation

Bone formation rate (BFR) is the distance between the midpoints or between the corresponding edge of two consecutive labels, divided by the time between the two labeling periods.

Fluorescence microscopy showed linear double tetracycline labeling, indicative of improved bone formation and mineralization in all cases of group A and group B (fig. 4). Absence of tetracycline labeling in group C indicated that no mineralized bone matrix was formed during the experimental period.

### Statistical Results

Unpaired t-test for the mean BFR measurement in different groups using double tetracycline labeling test revealed a significant difference between the two groups (A and B) ($p=0.0024$) indicating that in group B more BFR was promoted than in group A (table 3, fig. 17). This means that the osteogenic medium allowed enhanced bone regeneration than aln. medium.

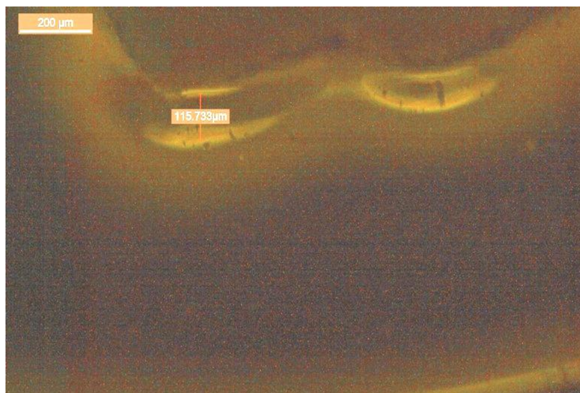


**Fig. (4): Microscopic image of regenerated tissue fluorescence showing BFR measurement using double tetracycline labeling test. Fluorescent TCH bands corresponding to the double injections. The middle of the most dense part of each label was the reference point.**

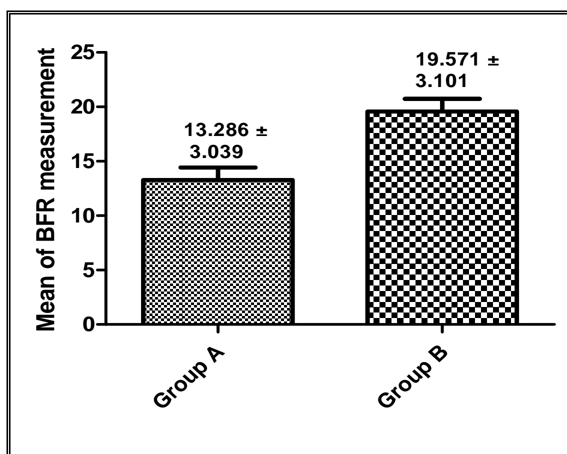


**Fig. (5): Histogram showing the mean BFR measurements in different groups using double tetracycline labeling test.**

### 2. DIGORA

ANOVA test for the mean BFR using DIGORA between different groups revealed a significant increase in bone density found in both groups A and B ($p<0.0001$). Tukey test comparing BFR of group A and group B with group C and comparing both experimental groups together using DIGORA revealed a significant increase in bone density of group B compared to group A defects ($p<0.01$).

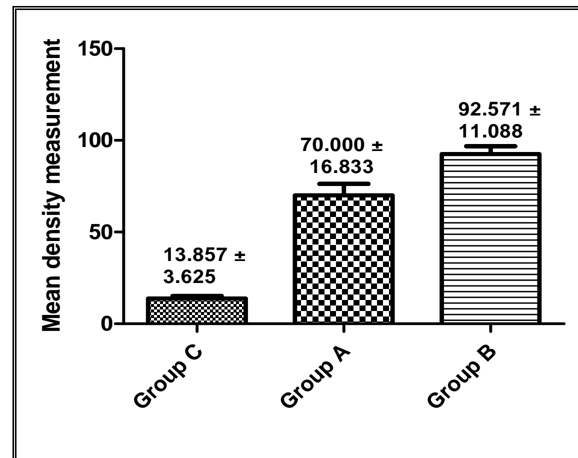


**Fig. (6): Histogram showing the mean BFR measurements in different groups using DIGORA.**

## DISCUSSION

In comparison with CT and CBCT, which are also available for assessment of bone grafting and regeneration, DIGORA was used in this study as it is the cheapest method of assessment, delivers the lowest radiation dose during exposure, provides a proper tool for diagnosis and three-dimensional viewing and analyses trabecular bone pattern for early detection of systemic disease as well as measuring of bone density. DIGORA, also, provides increased sensitivity to radiation and improved resolution.[8] Sensors used in this study were made the size of periapical films. Digora system was used for evaluating changes in bone density in all groups.

The present results demonstrated that DIGORA use for density analysis was efficient in order to provide numeric values about bone density alterations. A significant increase in bone density (gray scale) was found in group A and B. A significant increase in BFR of group B compared to group A was also found in the present work proving that the osteogenic medium has a more stimulating effect on bone formation over Aln medium.

In the present work, DIGORA results were consistent with the histological findings which mean that the DIGORA system is valuable and effective in assessment of bone healing and regeneration. This is in accordance with Thais et al.[9] who used DIGORA to evaluate radiographically the healing of rat calvarial CSDs using a xenomaterial for grafting. They proved that DIGORA results were consistent with the H & E specimens results

which means that DIGORA is accurate enough for bone healing monitoring.

Polychrome sequential labeling with fluorochromes is a standard technique for the investigation of bone formation and regeneration processes in vivo. However, for human application, only tetracycline (TC) and its derivates are approved as fluorochromes.[7]

TC labeling is dominant in reflecting the process of osteogenesis and in analysis of bone repair. TC itself exhibited the brightest fluorescence of all the derivates. Tetracycline Hydrochloride (TCH) among TC preparations, is considered one of the most intense labeling markers. TCH in comparison to all other in vivo markers has the least side effects regarding all variables. In the present study, the animals were sacrificed 48 hours after the last TCH injection. It has been documented that if the animal was sacrificed before 48 hours, this would cause the unfixed TC to elute out in solution and cause artifactual fluorescence.[10]

In this study, fluorescence microscopy showed linear double TC labeling, indicative of bone formation in all cases of alendronate and osteogenic media use. Absence of TC labeling in control defects indicated that no mineralized bone matrix was formed during the experimental period. In accordance to the obtained results, Pautke et al.[7] reported finding the fluorescent compound in areas of new bone proliferation after the administration of TC as an antibiotic. They reported that TC is fixed in the bone in the process of mineralization by its linkage to calcium, and once adhered, TC apparently remains until the marked bone is substituted during physiologic remodeling.

This study showed a notable increase in BFR in the osteogenic medium group compared to the Aln medium group which means that the osteogenic medium promoted an enhanced bone formation during the same interval of time.

Pautke et al.[7] recommended TC when intravenous or subcutaneous administration is planned. For oral administration, Sakellari et al.[10] showed that the good bioavailability of doxycycline (i.e. twice as high as that of TC) has to be considered. They also proved that oral administration of minocycline exhibited no detectable in vivo fluorescence. A similar result has been reported previously by Rabie et al.[11] Nevertheless, a study by Gerlach et al.[12] reported the successful use of minocycline for histomorphometric bone labeling.

A possible explanation for this discrepancy might be that a higher concentration was used and that the minocycline preparation and adminstration were performed differently. Gerlach et al.[12] suspended minocycline in carboxymethylcellulose while Pautke et al.[7] administered minocycline in aqueous solution using a nutrition tube at the same dosage as that for subcutaneous administration.

The present results of Digora and vital staining were consistent with the results of H & E stained sections previously published by our group.[1] Results of light microscopic evaluation of this work showed that healing progression was from outside towards the central part of the defect in all sections. This was obvious in both groups of defects filled with ADSCs seeded scaffolds.

Defects of the control group showed healing with minimal regeneration and no evidence of newly formed bone-like tissue was noted.[1]

These results proved that Digora and vital staining with double tetracycline injections are reliable tools for screening bone regeneration in accordance with the histological results.

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