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FOURIER TRANSFORM INFRARED SPECTROSCOPIC ANALYSIS FOR HEALING SOCKETS TREATED WITH A MIXTURE OF GROWTH HORMONE AND BIPHASIC CALCIUM PHOSPHATE: EXPERIMENTAL STUDY ON DOGS

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ABSTRACT

The primary goal of alveolar ridge preservation procedures is volume preservation, the volume preserved by encouraging new bone formation in healing sockets. About 90% of patients referred for implant therapy must perform these procedures after extraction that employs the placement of grafting materials.

Objective: Evaluate and characterize the healing responses of Growth hormone, and bicalcium phosphate mixture in tooth extraction sockets of a canine model.

Materials and Methods: Six mongrel dogs were included in this study; they were divided into 2 groups according to the time of sacrifice (4 and 8 weeks). Each of these groups includes three animals. Animals were subjected to extraction of the third premolar bilaterally, The right side was treated with a mixture of 4 IU (1.6mg) Growth hormone and easy-graft ™ (a composite of hydroxyapatite and bèta-tricalcium phosphate – crystal) 1 x 0.15 ml (450-650 μm) in a granular form before wound closure while the left side was left as a control. The healing extraction sockets were evaluated by Fourier Transform Infrared spectroscopic analysis.

Results: All sockets healed without complications. Growth hormone and biphasic calcium phosphate groups showed new bone formation comparable to that of native bone.

Conclusion: The grafted sockets demonstrated acceptable biodegradability at 4 and 8 weeks postoperatively. The new bone formation was not delayed because of the graft.

KEYWORDS: Spectroscopic analysis, ridge preservation, tricalcium phosphate, hydroxyapatite and dogs.

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INTRODUCTION

Alveolar bone is a highly malleable tissue that undergoes remodeling. Irreversible resorption of the alveolar ridge is a normal phenomenon following tooth extraction. This leads to decrease in height and width with subsequent ridge collapse (1,2,3). The degree of alveolar ridge resorption closely related to the time. The main loss of tissue contour occurs during the first three months after tooth extraction up till a year. The loss in width is up to 50% during the first year, while the height loss might range between 0.5 to 0.9 mm during the same period. Ashman reported that loss of bone height and width approximately 40% to 60% respectively within 2 to 3 years. There are three main steps in the procedure of socket preservation on a single-rooted tooth: Extraction of the tooth, placement of grafting materials, and primary tissue closure (4,5,6,7).

Different approaches have been tried to improve the ridge contour following tooth removal. These are necessary for either dental implants placement or conventional prostheses. As both procedures need an adequate amount of bone in both vertical and horizontal dimensions to obtain ideal functional and esthetic reconstruction(8-15). Numerous biomaterials have tried as bone substitutes to maintain dental alveolar bone after extraction such as allografts, xenografts, synthetic biomaterials which showed a positive effect reducing the loss of volume compared to untreated sockets. While the greatest success in bone grafting has been achieved with autogenous bones, however, it was faced by its limited supply and donor site morbidity (16,17,18). Synthetic bone graft substitutes were preferable than other bone grafts due to their availability, reasonable price, the absence of risk for transmission of pathogens and decreased allergenic potentiality. Most of them based on hydroxyapatite or other calcium phosphate minerals, which in many cases are similar to the natural mineral found in human bone (19,20). Calcium phosphate-based materials have been used for porous scaffolds formation whose Ca/P ratios have been ranged from calcium-tetraphosphate to hydroxyapatite or tricalcium phosphate. A variation in biodegradability exists as those with a low Ca/P ratio resorb more rapidly affecting the mechanical strength of the material, while those with greater Ca/P ratios degrade at a slower rate but showing less bone formation. A combination of different calcium phosphates materials used to reach an optimum resorption rate for the graft material resulting in better stability in the horizontal dimension (14,20).

Easy-graft™ CRYSTAL is a totally resorbable material composed of a porous biphasic calcium phosphate (BCP) containing 40% tricalcium phosphate (TCP) and 60% hydroxyapatite of synthetic origin. This biomaterial was used in orthopedics for 20 years and characterized by good biocompatibility and osteoconductivity. However, some research suggested osteoinductive capacities of (BCP) (21).

Growth hormone (GH) or Somatotropin is one of the growth factors that were considered to play a promising role in bone remodeling and bone growth. Local administration of growth hormone has rarely been applied during surgery, but now a well established working hypothesis is that local administration of GH in a single dose at time of surgery could accelerate the osseointegration process (22).

The availability of modern computers had enabled the rapid and powerful FTIR data processing and conversion. The FTIR spectroscopy is a measurement of wavelength and intensity. It provides information on all tissue components such as proteins and minerals. Therefore, the IR spectroscopy is an effective analytical method to investigate biosamples (23).

MATERIALS AND METHODS

The experimental study was performed at the Department of Surgery, Anesthesiology, and Radiology, Faculty of Veterinary Medicine, Suez Canal University. Six adult healthy male mongrel dogs with an average weight of 10-20 kg, comparable age (about 1year were included in this study. All animals were examined to rule out the presence of any disease. Animals were kept under supervision for a week before the experiment (acclimatization). Feeding was kept standardized as much as possible. Animals were housed individually. Animals were divided into 2 groups according to the time of sacrifice (4 and 8 weeks). Each of these groups includes three animals.

All surgical procedures were performed using injectable general anesthesia, each dog was premedicated with an intramuscular injection of Valpam (*product of AMOUN Pharmaceutical Industries co (APIC) S.A.E El Salam city, Cairo, Egypt)* in a dose of 0.5mg / KG body weight 10-15 min. prior to induction anesthesia. Cannulation and injection of prophylactic antibiotic were carried out using Flumox (*Egyptian International Pharmaceutical Industries co (EIPICO) 1O TH of Ramadan city, EGYPT.)* 500mg, 2ml IV every 24 hours for 5 days, followed by Fortecortin (*product of GALAXCO Pharmaceutical Industries co .El Salam city, CAIRO, EGYPT)* 2ml IM. Induction anesthesia was carried out using IV administration of Sodium thiopental (*Egyptian International Pharmaceutical Industries co (EIPICO) 10 th of Ramadan city, EGYPT*) 2.5% solution 20-30 mg/kg body weight. The main reflexes were abolished. Maintenance anesthesia was achieved using IV thiopental sodium 20 mg / kg body weight in dilution of 2.5 % saline solution.

Animals were subjected to extraction of the third premolar bilaterally. The crown was hemisected vertically with tungsten carbide fissure bur **(Fig. 1 A&B)**. Elevators were used gently to extract roots without trauma to the alveolar bone. The right side was treated with a mixture of 4 IU (1.6mg) Growth hormone (*SOMATROPIN 4 IU SEDICO Pharmaceutical Industries co, 6th October city.EGYPT)* and easy-graft ™ crystal *(Wagistrasse 23-8952 Schlieren/Zurich-Switzerland*) 1 x 0.15 ml (450-650 μm) in a granular form before wound closure while the left side was left as a control **(Fig 2 A and b)**. Releasing incisions followed for tension-free wound closure using 4.0 vicryl sutures **(Fig. 3 A)**. Postoperative antibiotic therapy and analgesics (*CATAFLAM ®NOVARTIS PHARMA Pharmaceutical industries co, CAIRO, EGYPT.*) 75mg / 2ml IM was administrated once a day for 3 days. Plaque control with chlorohexidine (*ANTISEPTOL KAHIRA, Cairo, Egypt)* mouth lavage was used.

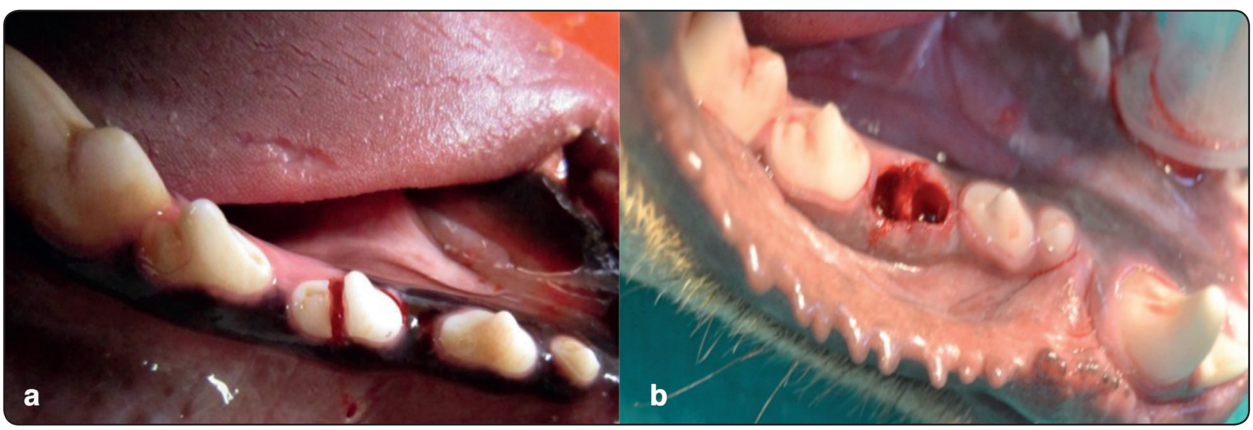

Fig. (1) A) A photograph showing vertical crown hemi-sectioning of the 3rd mandibular premolar tooth. B) A photograph showing socket after tooth extraction.

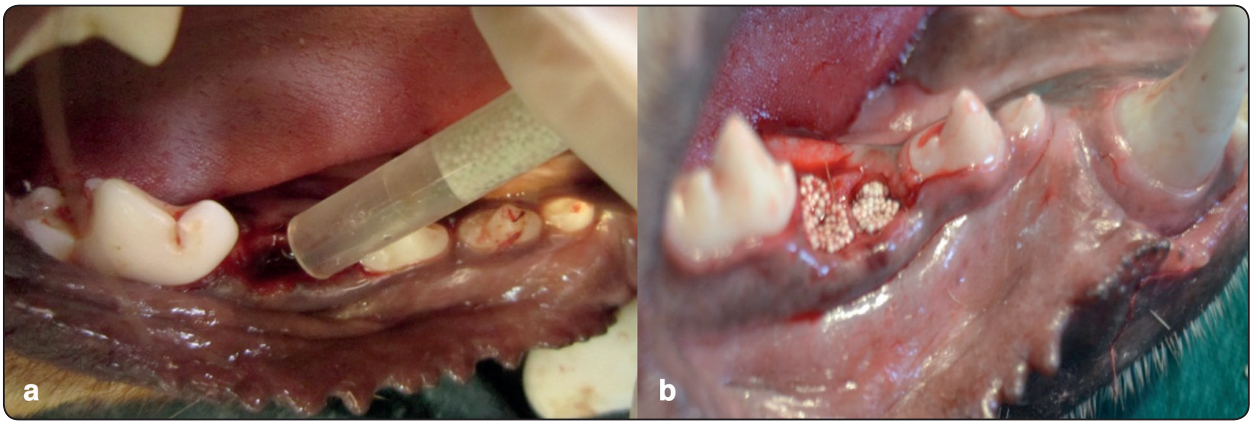

Fig. (2) A) A photograph showing easy graft application. B) A photograph showing granules in the socket.

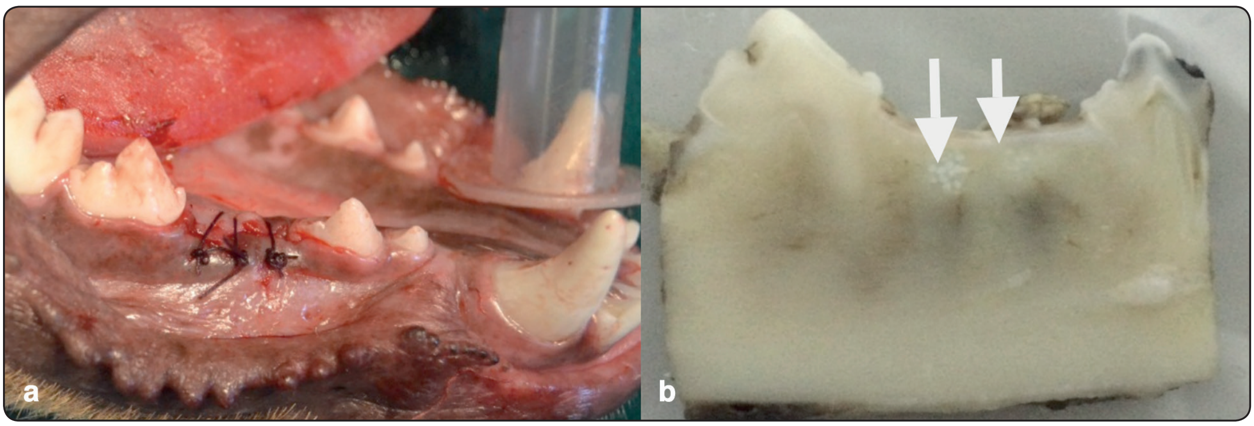

Fig. (3) A) A photograph showing interrupted suturing for primary closure. B) A photograph showing a section of dissected mandible the graft remnants is apparent at the cervical area (white arrow).

Clinical follow-up for animal wound healing was registered (visual signs of inflammation, mobility, and dehiscence). Animal sacrifice was performed by rapid IV injection of 1 mg 5% thiopental sodium. The mandibles were dissected, sectioned and put in neutral formalin 10% **(Fig. 3 B)**.

Chemical qualitative and quantitative analysis

The studied samples were prepared and analyzed spectroscopically. Bone blocks were removed from formalin and gently washed before analysis under distilled water, then dehydrated to avoid water spectrum observation, The assessment was done for the new bone formed inside the socket and the bone proper for each sample which were analyzed qualitatively and quantitatively by using Fourier Transform Infra-Red spectrometer (FT/IR-6100, Jasco, Japan). All spectra were recorded in the range (2000–400 cm^{-1}), the number of scans was 32, and the resolution was 4 cm^{-1} and scan speed 2 mm/s. Spectroscopic parameters that are evaluated in the infrared spectra of bone include; degree of hydroxyapatite crystal maturity CO_3/ PO_4 and mineral to matrix ratio to suggest degree of calcification PO_4/ Amide I. The data were analyzed by using base line technique to analyze the spectral vibration infrared according to ***Numata*** et al, 2008. **Table 1** shows the selected parameters to assess bone quality[24].

TABLE (1) Spectroscopic parameters analyzed for bone quality assessment.

Parameter	*IR peaks assignment*	*Comments*
CO_3/ PO_4	Relative area of sub-band at (873 cm^{-1}/ 1030 $cm^{-1)}$	Degree of HA crystal maturity or indicates the amount of carbonate substitution for phosphate in the mineral crystals
PO_4^{-3}/ Amide I	Relative area of v_1, v_3 (900-1200 cm^{-1}) phosphate/amide I (1575-1720 cm^{-1})	Degree of calcification

Statistical analysis

Statistical analysis was performed with IBM® SPSS® (SPSS Inc., IBM Corporation, NY, USA) Statistics Version 24 for Windows. Data presented as Mean, standard deviation (SD), Median, Minimum, and Maximum. Data explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests. Data for Chemical analysis showed nonparametric distribution, so Mann-Whitney test used to compare Different Groups. Wilcoxon Signed rank test used to compare between different follow-up periods. The significance level was set at $P \leq 0.05$.

RESULTS

Hemostasis was obtained when the graft applied into the extraction socket. The material easily compressed into the socket. The graft had good handling characteristics.

Using FTIR (Fourier transform infrared), both bone quality and quantity were assessed with the help of a special spectral analysis software**.**

Analysis of the molecular structure of bone revealed the presence of well-defined band at 1032 cm^{-1} which are characteristic for asymmetric stretching vibration of PO_4^{-3} (υ3), while the symmetric stretching vibration band for CO_3^{-2} group were located at 873 cm^{-1} . The symmetric stretching vibration bands for Amide I group was located at 1654 cm^{-1} .

Bone spectra of all study groups were compared together at 4 weeks and 8 weeks study periods. It was noticed that at 4 weeks the intensity of amide I and PO_4 had higher than the control group, while the CO_3 was higher and shifted for the test group. On the other hand, at 8 weeks the intensity of PO_4 and CO_3 were higher for test group while Amide I showed corresponding results for test and native bone **(fig 5).**

Upon comparing the difference in the recorded infrared (IR) means in each of the studied groups in comparison with native bone along the follow-up periods, a non-significant difference was noted between all periods in both studied groups **(Table2 & 3) (fig. 5).**

Fig. (4) A photograph showing the healing of extraction site after 4 weeks

TABLE (2) Mean, standard deviation (SD), Median, Minimum and Maximum of the degree of calcification PO4/Amide I for different groups.

		Difference between normal bone and control site					Difference between normal bone and test site					p-value
		Mean	SD	Median	Maximum	Minimum	Mean	SD	Median	Maximum	Minimum	
PO4/ Amide I	4 W	0.17	0.74	0.25	0.95	-0.76	0.78	0.81	0.77	1.78	-0.19	0.486 NS
	8 W	0.9139	0.7144	0.7293	1.9079	0.2891	-0.0360	0.4929	-0.2647	0.7001	-0.3147	0.114 NS
p-value		0.465 NS					0.068 NS					

= Significant, NS=Non-significant

TABLE (3) Mean, standard deviation (SD), Median, Minimum and Maximum of hydroxyapatite crystal maturity CO_3/PO_4 for different groups.

		Difference between normal bone and control site					Difference between normal bone and test site					p-value
		Mean	SD	Median	Maximum	Minimum	Mean	SD	Median	Maximum	Minimum	
CO_3/PO4	4 W	0.0127	0.0221	0.0171	0.0335	-0.0169	0.0303	0.0859	0.0127	0.1494	-0.0538	0.886 NS
	8 W	0.0215	0.0259	0.0195	0.0536	-0.0067	-0.0033	0.0410	0.0013	0.0404	-0.0562	0.486 NS
p-value		0.465 NS					0.465 NS					

**= Significant, NS=Non-significant*

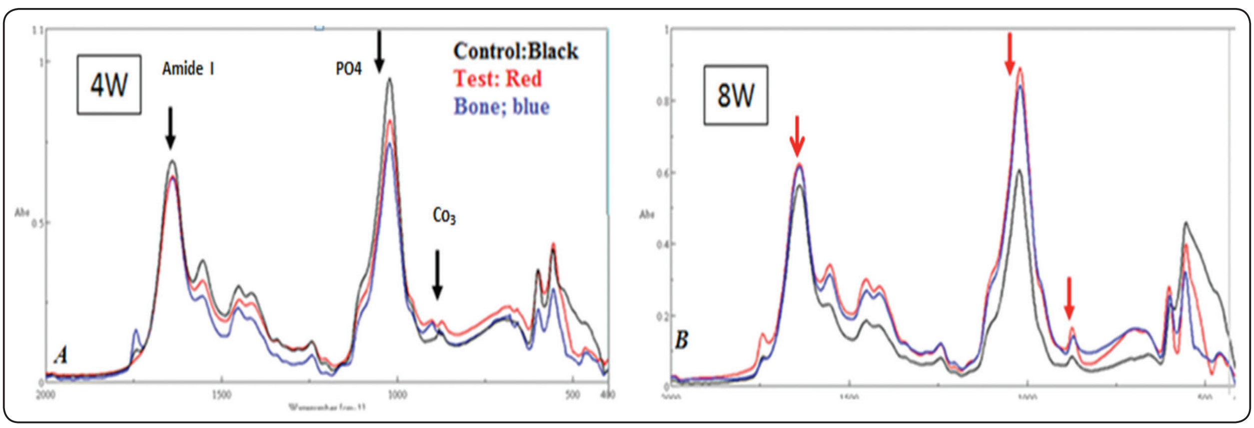

Fig. (5) Bone spectra of control, test and native bone groups in different study periods (A) 4 weeks (B) 8 weeks. The arrows point to Amide I, PO4 and carbonate peaks.

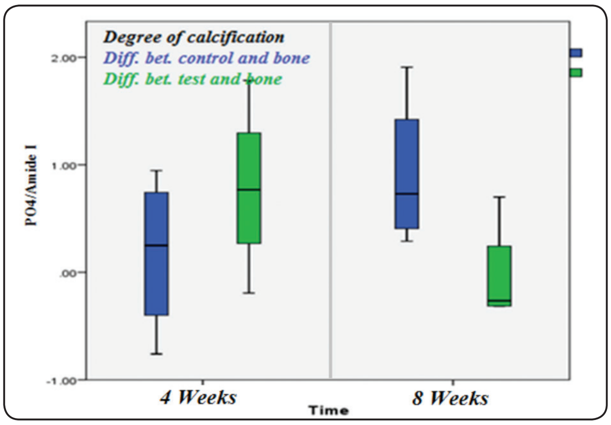

Fig. (6) Schematic representation for the degree of calcification (PO_4/Amide I).

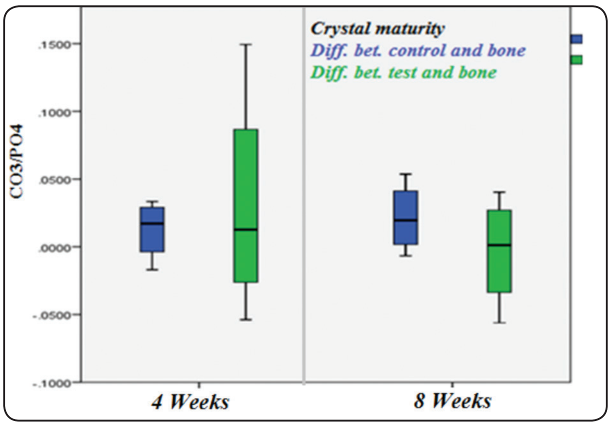

Fig. (7) Schematic representation for hydroxyapatite crystal maturity (CO_3/PO_4).

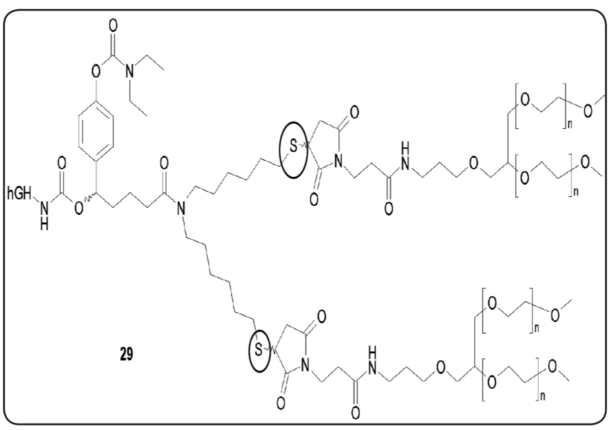

Fig. (8) Recombinant human growth hormone chemical structure showing sulphate.

DISCUSSION

Various bone graft substitutes have been used as an effective mean for maintaining alveolar ridge dimensions after tooth extraction. However, most of these grafts acted as scaffold for bone remodeling without causing any enhancement for new bone formation [(16)]. Alloplastic grafts considered being biocompatible materials causing only minimal fibrotic reaction during remodeling process but the main disadvantage existed in their late resorption and again limited new bone formation. Beta tricalcium phosphate β-TCP as an alloplastic material characterized by its mechanical strength, chemical stability and bioresorption properties. However, other studies considered β-TCP as a rapidly resorbed material so it may loses its space-maintaining capacity [(26,27,28)].

Biphasic calcium phosphate BCP belong to calcium phosphate ceramics, consisting of a homogenous 60/40 mixture of hydroxyapatite (HA) and β-TCP (the same chemical composition of bone). It has been formed to overcome the rapid resorption properties of β-TCP. While the β-TCP dissolved rapidly giving a space for new bone formation, the HA by its slow resorption rate acted as a scaffold maintaining the space dimensions. Moreover, BCP is also used as a carrier for therapeutic agents (bone morphogenetic proteins, human growth hormone) the so called "Engineered Osteoinductivity". It was concluded that HA/β-TCP ratio optimization in BCP was important for gene expression for bone cell adhesion, which can affect the early healing phase [(29)].

Surface modification of bone graft substitutes was a new interesting field in bone engineering,. Growth hormone GH acted as bone stimulators by increasing osteoblastic lineage differentiation and proliferation, matrix production and potentiating mineral deposition. GH assumed to be a type of "Engineered Osteoinductivity"[(30)]. Hong supported the use of Local (BCP) ceramic application into the fresh extraction sockets of dog and stated that" GH release from BCP implants loaded with 1 microg of GH was rapid during the first 48 h and then sustained release for a total of 9 days [31]. It could exert an impulse effect in the first hours of the process of healing by accelerating the recruitment of preosteoblasts. Moreover, Hedners and colleagues observed that GH local administration to the mandibles of rats significantly stimulated local bone formation [31].

Phosphate and Calcium was the skeletal framework of the bone apatite structure. Minimum replacement occurs specially for CO_3 ions in collagen and apatite for 7% in the ideal crystal structure. Therefore, hydroxyapatite included in the bone express PO_4, CO_3 and Amide influencing the crystallization predominating collagen type I (amide I). FTIR spectroscopy provided useful information for chemical analysis of BCP products; location and intensity of phosphate, carbonate and amide peaks [32,33].

The healing sequence for a dog model starts by granulation tissue formation at the 1st week followed by woven bone formation at the second week. However, at the 3rd week the trabeculae started to follow the anastomosing capillaries forming a 3-dimensional lattice, then trabecular bone formation was completed by the 4th week. Finally ended by mineralized dense lamellar bone that takes place at the 6th week.[34]. Regarding the time of sacrifice, the specimens were analyzed after 4w and 8w postoperatively. Thus an observation period of 8w was considered appropriate for control and test groups. At 4 weeks and 8 weeks, there were no statistically significant differences between the test and control groups when compared with native bone. This was a proof that the graft was comparable to native bone.

The middle ⅓ of socket was preferred for FTIR analysis as the coronal and apical parts might result in errors attributed to the definite crestal resorption and early revascularization respectively.

Iibuchi et al 2010, studied the effect of insertion of scaffold constructed of synthetic octa calcium phosphate (OCP) and porcine collagen sponge (OCP/Col) on bone regeneration in extracted sockets in dogs. Histological examination showed that the OCP/Col-treated group had more formation of new bone than the untreated control with gradual resorption of OCP and replacement by newly formed bone. At the same time, spectroscopic analysis spectrum of the implanted OCP/Col after 3months showed a similar pattern to dog native bone. This was observed with a peak around 1078 cm^{-1} that indicates the conversion of OCP in OCP/Col to Hydroxyapatite. CO_3^{-2} were observed at bands around 1410–1450 cm^{-1} in the spectra of the implanted OCP/Col [34].

These results were consistent with our results as the mixture of bicalcium phosphate and GH (most of its components were collagen) BCP/GH showed enhanced new bone formation as regard maturity. This was observed with a peak around 1072 cm^{-1} that indicates the conversion of BCP in BCP/GH to Hydroxyapatite. While CO_3^{-2} were observed at bands around 872 cm^{-1} in the spectra of the implanted BCP/GH.

Robert et al 2012, in 4-month case series, studied on 40 patients the effect of enhancing extraction socket therapy with biphasic calcium sulfate. Clinical results showed adequate maintenance of sockets volume and density when drilled for implants placement. At the same time, histological results appeared with complete resorption of the biphasic calcium sulfate graft and replacement with vital bone [35]. Also Mayer et al 2016, evaluated (clinically, histologically, and histo-morphometrically) the use of composite Biphasic calcium sulphate and biphasic calcium phosphate for socket preservation. The results showed more connective tissue at the control sites compared with the test sites. They conclude that the use of a composite alloplastic material in socket preservation decreased post-extraction bone resorption and allowed new bone formation [15].

The chemical structure of our GH contains sulfate (FIG 8) and phosphate which help to incorporate both elements in the graft so, the biphasic calcium phosphate and growth hormone had sulphate that may be the case of comparable results to Robert et al 2012 and Mayer et al 2016 [15,35].

Within the limitation of this study, the results were supporting the use of growth hormone and bicalcium phosphate BCP/GH in socket preservation to maintain bone quality and quantity within the normal ranges present in native bone. This will be suitable for further dental implants placement or conventional prostheses.

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