Alveolar Sockets Preservation Using Hydroxyapatite / Beta tricalcium Phosphate with Hyaluronic Acid (Histomorphometric study)

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Abstract: Alveolar atrophy following tooth extraction remains a challenge for future dental implant placement. Immediate implant placement and postextraction alveolar preservation are two methods that are used to prevent significant postextraction bone loss. The purpose of this study is to investigate the usefulness of hydroxyapatite / beta tricalcium phosphate (HA/BTCP) with hyaluronic acid (HY) for alveolar sockets preservation. Thirty-two New Zealand white rabbits were subjected to the lower left incisor extraction. The rabbits were equally divided into three groups. The extracted sockets (n = 12/group) were filled with : HA/BTCP, HA/BTCP + HY and blood clot (control). All rabbits were sacrificed for histological and histomorphometric evaluation after 4 and 8 week healing periods. The results demonstrated that all sites examined in this study displayed evidence of new bone formation. A statistically significant difference in the amount of new bone formation were found between sites that healed for an average of 8 only. The results demonstrating approximately 78%,68 % and 63% of new vital bone formation for groups grafted with HA/BTCP +HY , HA/BTCP and control group respectively after 8 weeks postoperatively. In conclusion these results exhibited that the use of hydroxyapatite / beta tricalcium phosphate with hyaluronic acid appears to be more efficient in osteoconduction when compared with of hydroxyapatite / beta tricalcium phosphate alone and could be promising strategy for preservation of alveolar sockets.
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Keywords: hydroxyapatite / beta tricalcium phosphate, hyaluronic acid, alveolar sockets preservation, alloplastic material, allograft.

1. Introduction

The residual alveolar ridge generally after tooth extraction provides limited bone volume because of ongoing progressive bone resorption (Tallgren 2003). Healing events within postextraction sockets reduce the dimensions of the socket over time (Araujo, Sukekava et al. 2005). A reduction of about 50% in both horizontal and vertical directions has been observed over 12 months after extraction, with two-thirds of the reduction occurring in the first 3 months (Chen, Wilson et al. 2004).

The rate and pattern of bone resorption may be altered if pathologic and traumatic processes have damaged one or more of the bony walls of the socket. In these circumstances, fibrous tissue will likely occupy part of the socket, preventing normal healing and osseous regeneration. These morphologic changes may affect the successful placement and osseointegration of dental implants (Chen, Wilson et al. 2004).

The treatment of bone defects and socket preservation include autografting, xenografting and allografting cancellous bone. The increasing number of grafting procedures and the disadvantages of current autograft and allograft treatments (e.g. limited graft quantity, risk of disease transmission) drive the need for alternative methods to treat bone defects (Giannoudis, Dinopoulos et al. 2005).

The use of synthetic bioactive bone substitute materials is of increasing importance in modern dentistry as alternatives to autogenous bone grafts (Burg, Porter et al. 2000). Various alloplastic bone substitution materials of different origin, chemical composition, and structural properties have been investigated in the last years. The materials commonly used in all approaches are ceramics, polymers or composites. These alloplastic materials are either absorbable or non-absorbable, as well as naturally derived or synthetically manufactured (Burg, Porter et al. 2000).

Various types of biomaterials (minerals and non-mineral based materials as well as natural and artificial polymers) with different characteristics have been used for studying ossification and bone formation. For example, calcium phosphate ceramics include a variety of ceramics such as hydroxyapatite, tricalcium phosphate, calcium phosphate cement, etc. These mentioned ceramics have excellent biocompatibility and bone bonding or bone regeneration properties (Zhao, Pinholt et al. 2000).

A biphasic hydroxyapatite / beta tricalcium phosphate(HA/BTCP) is a bone-graft substitute produced by a single process to prevent clustering and to establish a new homogeneous molecule. HA/BTCP offers an interconnected porosity of 90% to support cellular penetration. Rapid attraction of newly formed

bone has been shown when HA/BTCP-coated implants were examined (Burr, Mori et al. 1993; Lee, Wang et al. 2001; Stewart, Welter et al. 2004). Thus, HA/BTCP is recognized as an osteoconductive and bioactive material. The main advantage is that it is an excellent cell carrier, i.e., mesenchymal stem cells to promote bone formation (De Kok, Peter et al. 2003; Fleckenstein, Cuenin et al. 2006; Mankani, Kuznetsov et al. 2006; Trojani, Boukhechba et al. 2006).

Mankani, Kuznetsov et al. 2001 and Mankani, Kuznetsov et al. 2006 observed in vivo studies that the best results in tissue engineering, i.e., greatest osteogenesis, were obtained when bone marrow stromal cells were combined with HA/ TCP particles. Furthermore, this ceramic alone was sufficient to induce cell differentiation and actually harbored an intrinsic osteoinductive property (Tan, Wang et al. 2007).

Hyaluronic acid (HY), a widely distributed polysaccharide component of the extracellular matrix of connective tissues and bone marrow, has been reported to play an important role in tissue repair and regeneration (Chen and Abatangelo 1999; Caplan 2000).

It plays a vital role in the functioning of extracellular matrices, including those of mineralized and non-mineralized tissues. It is a critical component of extracellular matrix and contributes significant tissue hydrodynamics, cell migration and proliferation hyalorenate is also produced by fibroblasts in the presence of endotoxin; it plays an important anti-inflammatory role through the inhibition of tissue destruction and facilitates healing,(Moseley, Waddington et al. 2002).

It also accelerates the regeneration by means of chemotaxis, proliferation and successive differentiation of mesenchymal cells, and shares bone induction characteristics with osteogenic substances such as bone morphogenetic protein-2 and osteopontin (Mendes, Silva et al. 2008).

The aim of this experiment was to examine the histology of bone formation pattern at 4 and 8 weeks following tooth extraction, and to evaluate morphometrically, the amount of newly formed bone, when using hydroxyapatite / beta tricalcium with hyaluronic acid.

2. Materials and Methods

A-Experimental model

This experiment was conducted on a total of thirty two New Zealand white mature male rabbits weighting 2.5- 4 kg. The animals were housed in separate cages in temperature - controlled rooms and were fed on standard food and had free access to tap water. The animals were cared for according to the guidelines of the local Ethics Committee of the Animal Research at the Faculty of Medicine, Cairo University, which approved the project before the beginning of the experiments.

B-Surgical procedure

Rabbits were anaesthetized intramuscularly with a mixture of Xylazine (Chanazine, Chanelle Pharmacuetical , Ireland)5mg/kg body weight and ketamine hydrochloride (Ketamine, Pharmazeutische Pröparate, Germany) 30 mg/kg body weight. The rabbits were then subjected to the lower left incisor extraction using lower anterior extraction forceps and the socket was excavated and cleaned of any remnants. The coaptation of the mucosa after grafting of the socket was performed by mattress technique using coated Vicryl 4/0 (Ethicon, Edinburgh,UK).

The extracted sockets (n=12 for group) were grafted according to prescribed conditions for each of the three groups: group I ,0.25 cc of HA/BTCP(Osteon , Genoss ,Korea) was moistened with 0.5 ml sterile saline, group II, 0.25 cc of HA/BTCP was moistened with 0.5 ml hyaluronic acid (Hyadent,BioScience,Germany) and group III filled with blood clot(control).The rabbits were monitored closely every day up to 2 weeks postoperatively and placed on a soft ration diet to reduce the possibility of local trauma to the site of operation.

C-Animal scarification

Six rabbits from each groups were sacrificed at 4 and 8 weeks for histological using an intramuscular injection of 60mg/ml/kg body weight sodium phenobarbitone (Phenobarbitone, Fawns & McAllan Pty Ltd, Melbourne, Victoria).

D-Histology

The specimens from the operated region were obtained at sacrifice and were fixed in 10% buffered natural formaldehyde for 24 hours. They were then decalcified in ethylene diamine tetra acetic acid (EDTA) for 60 days. As decalcification was completed, the specimens were washed for 24 hours in running water to remove all traces of EDTA. The specimens were gradually dehydrated in ascending concentrations of ethyl alcohol (50%, 70% and90%) and two to three changes of absolute alcohol to ensure replacement of water by alcohol. The specimens were passed from alcohol through two changes of xylene.

The specimens were infiltrated with paraffin into a constant temperature furnace, at about 60°C, until the xylene in the tissues was replaced by paraffin. Serial sections of 5 μm were cut through the middle of the explant in the transverse direction in the mesio-distal plane representing most of the induced defect area. The specimens were mounted on the positively charged slides (opti plus), stained with Hematoxylin

and Eosin (H&E) stain and then examined under a light microscope.

E-Measuring of the area percent of bone trabeculae

The area percent of bone trabeculae was estimated using Leica Quin 500 analyzer computer system, (Leica Microsystems, Swizerland). The cursor was used to outline the areas of bone trabeculae, which were then masked by a blue binary colour that could be measured by the computer. The image analyzer is calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. The area percent of bone trabeculae was estimated in 5 different fields in each slide using magnification (x100), (Fig.1). Mean values and standard deviation were calculated for each group.

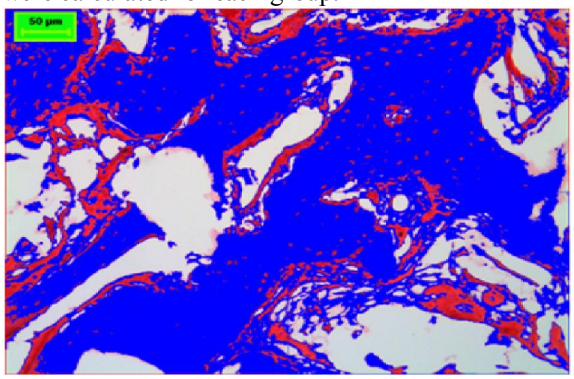

Figure1. Copy of the display seen on the monitor of the image analyzer revealing areas of bone trabeculae covered by a blue binary colour that could be measured by the computer system.

Statistical analysis:

The data obtained from computer image analysis were presented as mean and standard deviation (SD), tabulated and statistically analyzed. ANOVA and Post-Hoc were used for statistical analysis of the difference between groups. P value $\leq$ 0.05 was considered statistically significant**.**

Quantitative histomorphometric image analysis

The image analysis was performed using a computer system Leicca Quin 500, Image analyzer unit. Oral pathology department, Faculty of oral and dental medicine, Cairo University, in order to measure the thickness of newly formed bone trabeculae.

3. Results

1-Histologic Observations

A. Four weeks post operatively

Microscopic examination of control group 4 weeks postoperatively revealed the presence of thin irregularly arranged bone trabeculae demonstrating entrapped osteocytes. The bone trabeculae were separated by fibrous tissue composed of loosely arranged collagen fibers, fibroblasts and dilated blood vessels (Fig. 2).

In HA/BTCP group, irregularly arranged bone trabeculae of variable thickness were demonstrated. Bone vitality was evidenced by the presence of entrapped osteocytes within the trabeculae. The bone trabeculae were separated by fibrous tissue. Collagen fibers appeared parallel and properly organized in certain spots, but exhibited a loosely arranged irregular pattern in other areas.Dilated blood vessels were detected within the fibrous tissue (Fig. 3-4).

In HA/BTCP+HY , the histological appearance was similar to that of the HA/BTCP group. Irregular vital bone trabeculae were dispersed in a fibrous tissue supplied by dilated blood vessels (Fig. 5).

B. Eight weeks postoperatively

Microscopic examination of control group 8 weeks postoperatively showed bone trabeculae of almost regular thickness. Numerous osteocytes, denoting bone vitality, were seen entrapped in lacunae. Reversal lines revealing bone remodeling were detected within the trabecule. Mature fibrous tissue composed of fibroblasts, parallel compact collagen fibers, as well as dilated blood vessels were observed between the trabeculae (Fig.6).

In HA/BTCP group, regular bone trabeculae of variable thickness were demonstrated. Bone vitality was evidenced by the presence of entrapped osteocytes within the trabeculae. The bone trabeculae were separated by fibrous tissue. Collagen fibers appeared parallel and compact in most areas. Loose but regularly arranged collagen fibers were seen in other spots. Dilated blood vessels were detected within the fibrous tissue (Fig. 7-8).

In HA/BTCP+HY group**,** thick regular bone trabeculae were demonstrated. Osteocytes were seen within the trabeculae, while osteoblasts rimmed their borders. Parallel compact collagen fibers separated the trabeculae. Dilated blood vessels were detected within the fibrous tissue (Fig. 9).

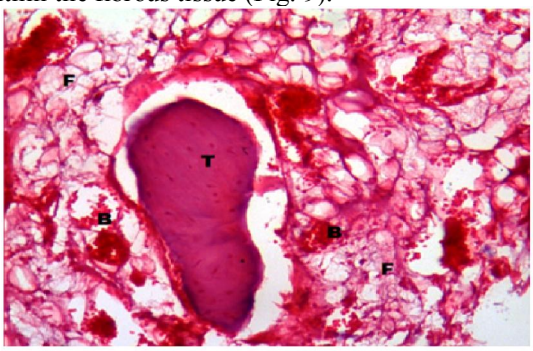

Figure 2. Photomicrograph of control group 4 weeks postoperatively revealing thin irregular bone trabeculae (T), loosely-arranged collagen fibers (F) and dilated bold vessels (B), (H&E x200).

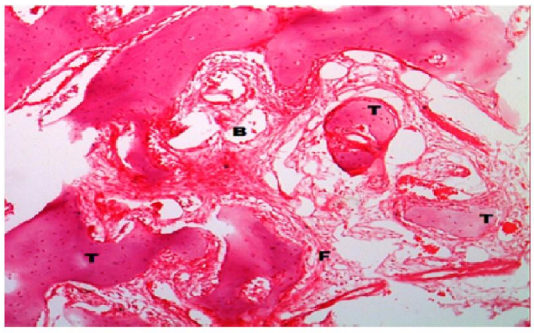

Figure 3. Photomicrograph of HA/BTCP group 4 weeks postoperatively revealing bone trabeculae (T) of variable thickness. Fibrous tissue (F) of variable compactness and dilated blood vessels are seen between the bone trabeculae (H&E x100).

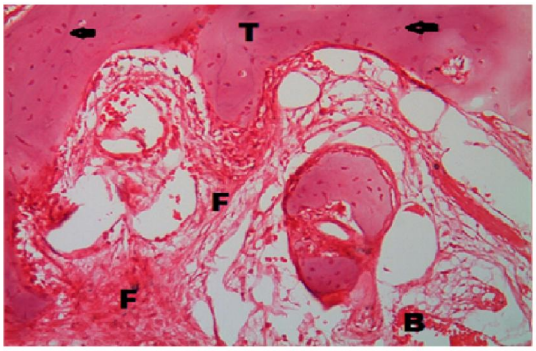

Figure 4. Higher magnification of the previous photomicrograph of HA/BTCP group 4 weeks postoperatively revealing bone trabeculae (T) of variable thickness. Osteocytes are seen entrapped in lacunae within the trabeculae (arrows). Fibrous tissue (F) and blood vessels (B) are seen between the bone trabeculae (H&E x200).

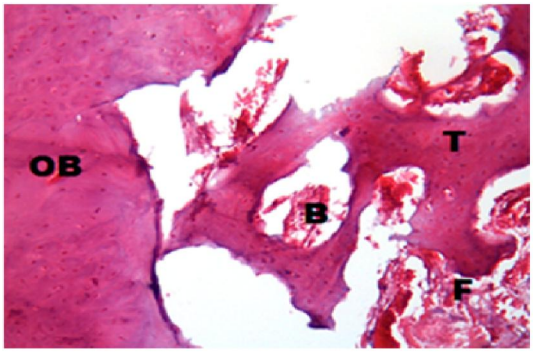

Figure 5. Photomicrograph of HA/BTCP+HY group 4 weeks. postoperatively revealing bone trabeculae (T) of variable thickness. Fibrous tissue (F) and dilated blood vessels are seen between the bone trabeculae. Original bone (OB) is seen at the periphery (H&E x200).

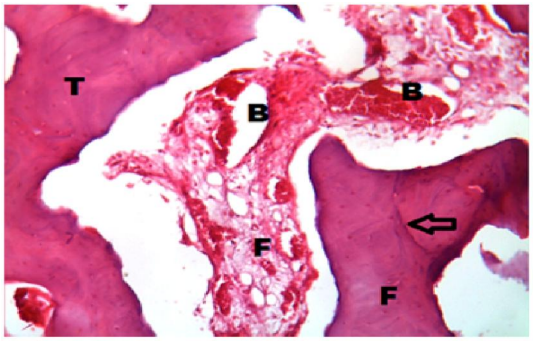

Figure 6. Photomicrograph of control group 8 weeks postoperatively revealing bone trabeculae (T) in uniform thickness. Reversal lines denoting bone remodeling are seen within the trabeculae (arrow). Parallel collagen fibers (F) and dilated blood vessels (B) are seen between the bone trabeculae(H&E x200).

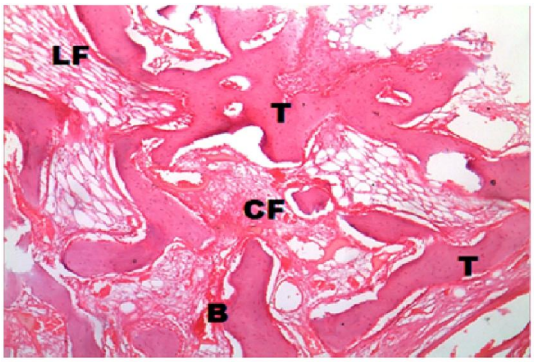

Figure 7. Photomicrograph of HA/BTCP group 8 weeks post-operatively revealing bone trabeculae of variable thickness (T). Compact (CF) or loosely arranged (LF) regular collagen fibers are demonstrated. Dilated blood vessels are seen within the fibrous tissue (H&Ex100).

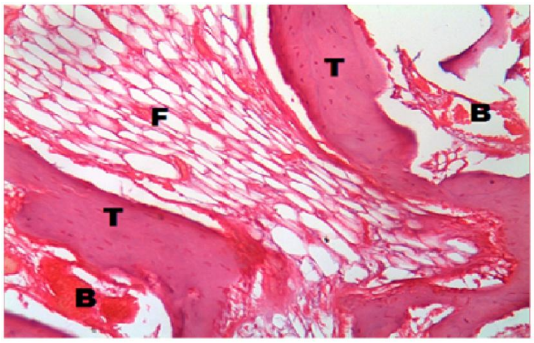

Figure 8. Higher magnification of the previous photomicrograph of HA/BTCP group 8 weeks post-operatively revealing bone trabeculae (T) enclosing viable osteocytes. Loosely arranged regular (F) collagen fibers and dilated blood vessels are demonstrated within the fibrous tissue (H&Ex200).

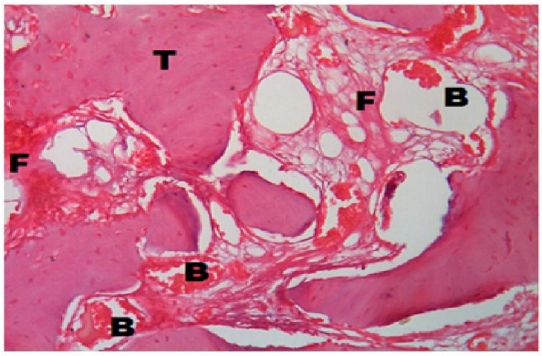

Figure 9. Photomicrograpg of HA/BTCP+HY group group 8 weeks post-operatively revealing thick regular bone trabeculae (T). Parallel compact collagen fibers (F) and dilated blood vessels are seen within the fibrous tissue (H&Ex200).

2-Histomorphometric Analysis

Histomorphometric estimation of the mean area percent filled by bone trabeculae 4 weeks postoperatively was slightly greater in the HA/BTCP group (56.801 ± 7.461) compared to control group (54.690 ± 15.871) and the HA/BTCP+HY group (54.284 ± 10).ANOVA test revealed that the difference was not statistically significant (p=0.875) (Table 1).

Eight weeks post-operatively, the area percent of bone trabeculae was greater in the HA/BTCP+HY group (78.080 ± 7.750) compared to the HA/BTCP group (68.345 ± 15.121) and control group (63.689 ± 1.937). ANOVA test revealed that the difference was statistically significant (p=0.010) (Table 2, Fig. 10). Post-Hoc (Turkey HSD) test revealed statistically significant between HA/BTCP+HY and control group (Table 3).

Table 1. Values of area % filled by bone trabeculae among the three groups at 4 weeks postoperative

	Vital New Bone (% ± SD)	P
Control	54.690 ± 15.871	0.134 (0.875)
HA/BTCP	56.801 ± 7.461	
HA/BTCP+HY	54.284 ± 10	

No significant differences between groups (P>0.05)

Table 2. Values of area % filled by bone trabeculae among the three groups at 8 weeks postoperative

	Vital New Bone (% ± SD)	P
Control	63.689 ± 1.937	5.532 (p=0.010)*
HA/BTCP	68.345 ± 15.121	
HA/BTCP+HY	78.080 ± 7.750	

* statistically significant at p<0.05

Table 3. Post-Hoc (Turkey HSD) to compare between the 3 groups at 8 weeks postoperative

	Control	HA/BTCP	HA/BTCP+HY
P1(Control)		0.550	0.008*
P2(HA/BTCP)			0.088

* statistically significant at p<0.05

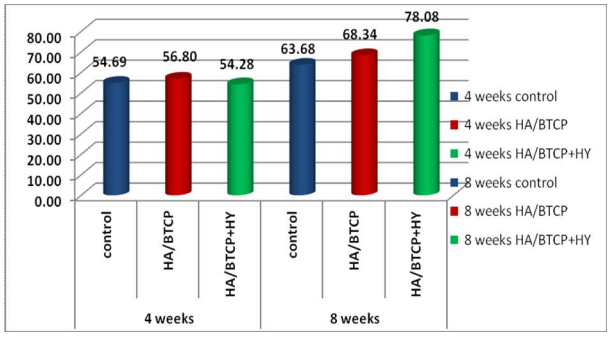

Figure 10. Values of area % filled by bone trabeculae among the three groups at 4 and 8 weeks postoperative.

4. Discussions

The primary aim of this study is to histologically evaluate new bone formation using HA/BTCP with or without HY at two different time points after tooth extraction and socket grafting. All sites examined in this study displayed evidence of new bone formation. A statistically significant difference in the amount of new bone formation were found between sites that healed for an average of 8 only ,while at 4 weeks no statistically significant difference.

Our results demonstrating approximately 54% new vital bone formation for group grafted with HA/BTCP +HY,57 % new vital bone formation for group grafted with HA/BTCP and 55% new vital bone formation for control group after 4weeks while they were 78%,68% and 63% in HA/BTCP +HY , HA/BTCP and control group respectively after 8weeks postoperative.

These findings are consistent with studies evaluating new bone formation using allograft when used for the purpose of alveolar socket and ridge preservation.(De Kok, Drapeau et al. 2005; Froum, Wallace et al. 2006; Fotek, Neiva et al. 2009). (De Kok, Drapeau et al. 2005) reported evidence of steady increase in bone formation in the alveolar socket sites in dogs following implantation of mesenchymal stem cells adherent to HA/ BTCP.

The use of synthetic bioactive bone substitute materials and biological mediators are of increasing importance in modern dentistry as alternatives to autogenous bone grafts (Burg, Porter et al. 2000).

Osteon is one of the alloplastic materials composed of hydroxyapatite70% and beta-tricalcium phosphate 30% which are closest to the major mineral components of human bone, and have an interconnected porosity structure (scaffolding) which is similar to that of human cancellous bone (Kim, Yun et al. 2008).

The combination of bone graft materials with biological mediators and collagen were reported in many studies to reinforce mechanical strength and decrease resorption rate (Brkovic, Prasad et al. 2008; Araujo and Lindhe 2009).

(Fickl, Zuhr et al. 2008) grafted the composite of Bio-Oss(Geistlich) and collagen into the dog extraction socket and observed limited resorption of buccal bone after extraction, (Araujo, Linder et al. 2009) reported delayed healing at an early stage , (Brkovic, Prasad et al. 2008) attempted preservation of the human alveolar ridge by using a composite of HA/ BTCP and collagen, and succeeded in inhibiting alveolar bone resorption was observed.

HY is an ideal vector for the bone morphogenic proteins (BMP), the only growth factors universally recognized to be able to stimulate the formation of new bone tissue (Caplan 2000; Hunt, Jovanovic et al. 2001; Kim and Valentini 2002; Lisignoli, Fini et al. 2002). HY of appropriate molecular weight alone in optimal concentration induce osteoblast differentiation and bone formation (Pilloni and Bernard 1998).

HY has a molecular weight-specific and dose-specific mode of action that may enhance the osteogenic and osteoinductive properties of bone graft materials and substitutes due to its stimulatory effects on osteoblasts (Huang, Cheng et al. 2003).

(Im, Ahn et al. 2010) demonstrated the use of HA/BTCP in combining with hyaluronic acid and atelocallagen had a superior rate for repairing osteochondral defects in a large animal model than treated with HA/BTCP.

Although there was no statically significant difference between all groups after 4 weeks postoperative , the group grafted with HA/BTCP+HY showed higher percentage of new vital bone formation than the other two groups after 8 weeks postoperative.

In addition of an optimum balance of the stable phase of HA and the soluble phase of BTCP could increase new bone formation, as it releases calcium and phosphate ions into the biological medium of extracted socket, (Daculsi, Laboux et al. 2003) HY may increase bone formation through: its morphogenetic effects due to its ability to act as a template for assembly of a multi-component, pericellular matrix as well as to its physical properties (Toole BP 1991; Stern, Asari et al. 2006).This matrix would provide a hydrated environment in which cells are separated from structural barriers to morphogenetic changes and receive signals from HY itself and from associated factors (Turley EA 1989; Toole BP 1991).

Another factor in increasing new vital bone formation for group grafted with HA/BTCP+HY, HY has a hygroscopic, rheological and viscoelastic properties, which can influence the cell function that modify the surrounding micro and macro environment as a result of complex interactions with the cells and other extracellular matrix components (Yoneda M 2001).

The presence of HY is well evidenced in the cytoplasm and nuclei of the cells in a number of tissues in vivo to modulate the cell behavior by interacting with specific cell surface receptor-hyaluronic acid binding proteins (HABPs) known as Hyaladherins. This phenomenon also plays an important role in maintaining and stabilizing the structural integrity of extracellular matrices (Sutherland 1998; Evanko and Wight 1999; Toole BP 2000).

Considering that no previous report exist reporting on the use of HA/BTCP combined with HY in treatment alveolar sockets preservation, our histological results are encouraging the use of hydroxyapatite and beta/ tricalcium phosphate with hyaluronic acid as promising strategy for preservation of alveolar sockets.

5. Conclusion

These results exhibited that the use of hydroxyapatite /beta tricalcium phosphate with

hyaluronic acid appears to be more efficient in osteoconduction when compared with of hydroxyapatite /beta tricalcium phosphate alone and could be a promising strategy for preservation of alveolar sockets.

Recommendations

The present study indicates that there are no statistically significant differences in the amount of new vital bone formation 8 weeks after extraction in the two groups grafted with HA/BTCP either with or without HY, Therefore, waiting an extended period of time after extraction to allow more time for bone formation and using of other allograft, xenograft, or alloplast materials in combined with hyaluronic acid may be recommended in further studies.

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