

EFFECT OF ABSORBABLE GELATIN SPONGE ON OSTEOGENIC ACTIVITY IN ATROPHIED POSTERIOR MAXILLA. (HISTOPATHOLOGICAL AND GENE EXPRESSION STUDIES)

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ABSTRACT

Objective: Clinical trials studied different materials and techniques for atrophied ridge augmentation regarding function implant stability and bone level. Microanalysis of the bone reaction to augmentation is still under investigation. The present study aims to examine the histological changes in augmented areas as well as the gene expression levels of one of the genes involved in bone regeneration.

Methods: 9 core biopsies were collected from subjects during implant placement after having their posterior maxillary region augmented with absorbable gelatin sponge six months earlier. The cores were studied by routine histology, histomorphometry and osteoprotegerin OPG gene expression level.

Results: Histological examination revealed active bone formation criteria in all specimens with variable presentation. Histomorphometry demonstrated that the new bone area percentage comprised about 30% (new bone 24.13 ± 5.01 %) & (mature bone 54.83 ± 12.13 %) of the total bone area percentage detected by Hematoxylin and Eosin and Masson's trichrome stain. Gene expression level of OPG revealed significant elevation ($P = 0.017$) when compared to normal standard control levels.

Conclusions: Based on histology, histomorphometry and gene expression findings posterior maxillary augmentation with absorbable gelatin sponge showed an elevated bone activity. However, it is still needed to verify if such effect resulted from the incorporation of gelatin sponge or the sinus membrane lifting only.

KEY WORDS: absorbable gelatin sponge, atrophic maxilla, osteoprotegerin, augmentation.

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INTRODUCTION

The posterior maxilla is often a critical area for insertion of dental implants especially with long-standing edentulous states as maxillary sinus pneumatization progresses with age, positive air pressure and teeth extraction.¹

Within the thin residual ridge, the osteointegration around dental implants is considered an aching problem to the clinician. Post extraction changes that occur in the posterior alveolar ridge, as well as pneumatization of maxillary sinus, may lead to implant perforation into the antrum.²

Although a variety of grafting materials have been utilized for sinus floor augmentation^{3,4}. Sometimes, the creation and maintenance of space via stabilization of the elevated sinus membrane are quiet enough to stimulate bone regeneration in the area even without the need for graft material.⁵ Sohn et al 2009⁶ used absorbable gelatin sponge for sinus augmentation and demonstrated radiographic evidence of elevation of the bone level. It was suggested that the placement of a dental implant in the maxillary sinus with a gelatin sponge can be a suitable procedure for sinus augmentation.⁷ Other studies reported that sinus membrane itself obtains an osteoinductive potential.⁸

In experimental studies⁹, no differences were found between membrane-elevated and grafted sites regarding implant stability and the bone area within and outside implant threads. The amount of augmented bone tissue in the maxillary sinus after sinus membrane elevation with or without bone grafts does not differ after 6 months of healing. Although the ability of bone to regenerate in a sub-sinus model is well documented both in vivo and clinically, the early steps of bone formation and bone dynamics over time remain under investigation.¹⁰⁻¹²

Osteoprotegerin gene (OPG) is a member of the TNF receptor superfamily that is considered as an important regulator of bone remodeling.¹³

Elevated expression of OPG indicates the decrease in osteoclastic differentiation and maturation which decreases in turn bone resorption for having such a controlling role on osteoclast.¹⁴ The level of Osteoprotegerin (OPG) gives a clue about the remodeling process¹⁵.

Remodeling activity can be estimated in different ways either clinically, radiographically histologically or through detection of different growth factors' expression involved in the remodeling process. Therefore, it was the purpose of the present study to assess the quality and quantity of new bone formation in the maxillary sinus grafted with absorbable gelatin histologically and through OPG expression assessment.

MATERIALS AND METHODS

Ten core biopsies were collected from systemically healthy patients, from outpatient clinics of the oral and maxillofacial department, faculty of dentistry Cairo University, during dental implant fixation procedure in the posterior maxillary region. Ridge augmentation, with absorbable gelatin sponge, was performed six months earlier in the area of interest.

The subjects were females with age ranging from 23 to 56 years. They were non-smokers, non-alcoholics and free from systemic or local pathologic problems. Nine sinus elevation procedures were performed in two stages; in the first stage surgery, maxillary sinus floor elevation using lateral window technique was performed and the created sub-antral space was maintained with absorbable gelatin sponge and covered using collagen membrane. Six months later, in the second stage surgery, core biopsy specimens were harvested from the planned implant sites at the time of implant placement. The study protocol was approved by the research ethics committee of the faculty of dentistry, Cairo University.

Specimens' processing and examination:**Routine histological examination**

The harvested core biopsies were fixed in 10 % buffered formalin for 48 hours, washed and then *decalcified* in Ethylenediaminetetraacetic acid (EDTA). Then *dehydrated in ascending grades of alcohol, cleared in xylol then embedded in paraffin blocks*. Each specimen was oriented longitudinally to include both native and newly formed bone. 5 μ thick sections were prepared on regular glass slides for histological examination. Prepared sections were stained with Hematoxylin and Eosin stain according to the conventional method for routine histological examination, and Masson trichrome stain for differentiating mineralized bone & newly formed bone.

Histomorphometry analysis

Calculations of the total bone area percentage were made on the Hematoxylin and Eosin stained sections, while those for old and new bone area percentages were performed on the Masson trichrome stained sections.

The data of the area percentage of the newly formed bone was estimated using Leica Quin 500 analyzer computer system, (Leica Microsystems, Switzerland). The image analyzer consisted of a colored video camera, colored monitor, hard disc of IBM personal computer connected to the microscope, and controlled by Leica Qwin 500 software.

For measuring the area percent of osteoid newly formed uncalcified bone, the cursor was used to select the blue-green color of the Masson Trichrome stain denoting the newly formed bone trabeculae. The selected color was automatically masked by blue binary color. The image analyzer is calibrated to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. The area percent of newly formed bone was estimated in five different fields in each group using

magnification (x200). Mean values and standard deviation (SD) were calculated for each group.

Hematoxylin and Eosin stained sections were used to evaluate the total area percentage of bone, after selecting the hematoxylin color of the bone trabeculae (after erasing the stained areas of the bone marrow). The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research.¹⁶

Real-time PCR quantitative analysis for Osteoprotegerin (OPG) gene expression**Total RNA extraction**

Total RNA was extracted according to manufacturer instructions using the SV Total RNA isolation system. (Promega, Madison, WI, USA). The RNA concentrations and purity were measured with an ultraviolet spectrophotometer.

Complementary DNA (cDNA) synthesis

The cDNA was synthesized from 1 μ g RNA using SuperScript III First-Strand Synthesis System as described in the manufacturer's protocol (#K1621, Fermentas, Waltham, MA, USA).

Real-time quantitative PCR (RT-PCR)

RT-PCR amplification was done with Applied Biosystem with software version 3.1 (StepOne™, USA). The reaction includes SYBR Green Master Mix and gene-specific primer pairs (Table 1). It was designed by Gene Runner Software (Hasting Software, Inc., Hasting, NY) from RNA sequences of the gene bank. Every set of primers exhibited 60° annealing temperature. RT-PCR was done in 25- μ l reaction volume formed of 2X SYBR Green PCR Master Mix, 900 nM of every primer plus 2 μ l of complementary DNA. Amplification was applied under specific time and temperature conditions of several cycles of denaturation and annealing/extension. v1.7 sequence detection software from PE Biosystems (Foster City, CA) was used to calculate

the records of RT assays. Comparative Ct technique was used to determine the relative expression of the mRNA gene. Data were standardized to beta-actin which is the control housekeeping gene.¹⁷

TABLE (1): Showing the primer sequence of the studied gene

Primer sequence	
Osteoprotegrin (OPG)	F: 5-TGGCACACAGTGATGAATGCG-3 R: 5-GCTGGAAAGTTTGCTCTTGCG-3
Beta actin	F-5-GCGCTCGTCGTCGACAACGG-3 R-5-GATGGCTACGTACATGGCTG-3

Statistical analysis

Microstat7 for Windows statistical package was used for statistical analysis in this study. One sample “t” test was used. The difference was considered statistically significant at $p \leq 0.05$

RESULTS

Histological Results:

Histological features of the studied core biopsies revealed variable interpretation when compared to a normal reference sample used as control from a similar area. (fig.1). The control sample revealed an intact structure of interconnecting lamellar bone trabeculae that enclosed variable-sized bone marrow cavities with evidence of the red bone marrow inside some of the cavities. Higher magnification showed the bone resting and reversal lines in the studied field with the regular distribution of the osteocyte lacunae (fig.1)

In the study sections, bone appeared as separate lamellar trabeculae surrounded by the red bone marrow. Osteocytes were widely distributed within the trabeculae. In between, delicate fibrous tissue rich in fibroblasts was detected paralleling with the trabecular surfaces (fig.2).

The bone activity was evident by multiple reversal lines in many of the studied sections with the normal existence of bone cells. Delicate fibrous

tissue was detected around trabeculae (fig.3). The osteoblastic lining of the bone surface was spotted in some locations with marked condensation (fig.3, inset).

A mixed histological picture was seen in one of the cases, highly cellular fibrous tissue was seen patterning the future trabeculae. Alongside we could detect immature highly cellular bone trabeculae. More dense trabeculae were seen in some areas

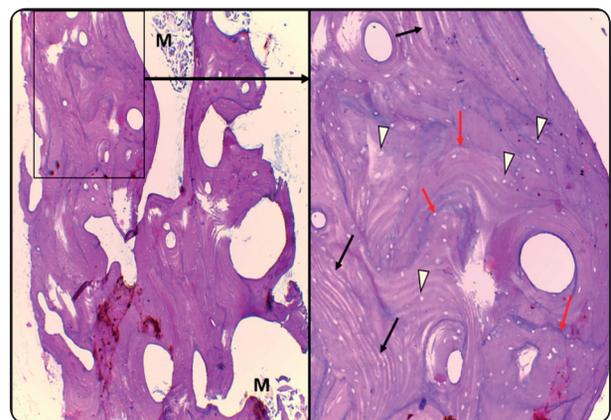


Fig. (1): A photomicrograph of the control reference sample showing: To the left, core biopsy of maxillary non-augmented bone with interconnecting trabeculae enclosing variable-sized bone marrow cavities and containing red bone marrow (M). To the right, higher magnification showing reversal lines (red arrows), resting lines between lamellae (black arrows) & osteocyte lacunae (white arrow heads) (H & E, X40 & X100)

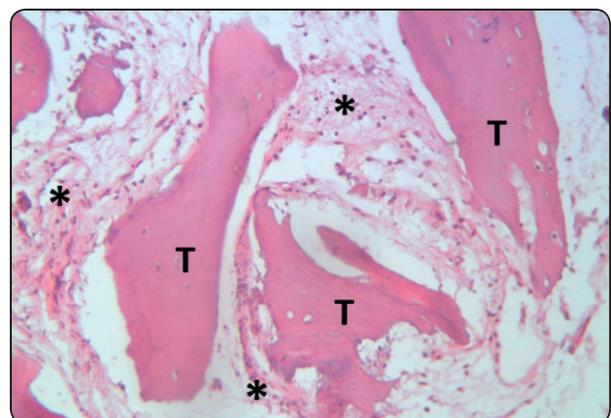


Fig. (2): A photomicrograph showing; bone trabeculae (T) and cellular fibrous tissue (asterisks) paralleling with the trabecular surfaces. (H & E, X200)

intervening between new bone and native bone. Osteoblasts were aligned around the forming bone. The original trabeculae were interlacing with different patterns (fig.4)

Another unique pattern was also detected among the studied specimens. That was exhibiting areas of lamellar bone surrounded by well-defined reversal lines separating larger areas of an atypical bone morphology in the form of continuous perforated sheets with very few or no osteocytes (fig. 5)

Sometimes, the newly formed bone was not identified in hematoxylin and eosin-stained sections,

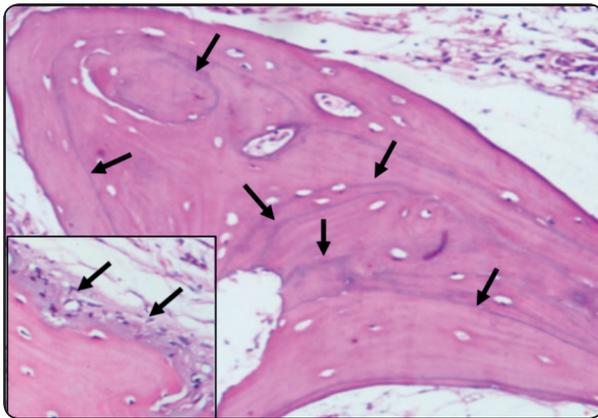


Fig. (3): A photomicrograph showing; multiple reversal lines denoting remodeling activity (black arrows) and osteoblasts condensed along the bone surface (inset black arrows) (H&E, X200)

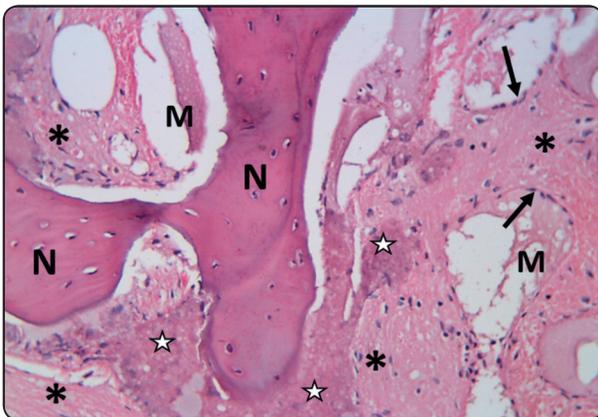


Fig. (4): A photomicrograph showing; native bone (N), newly formed highly cellular and highly fibrous bone trabeculae (asterisks), denser bone trabeculae (white stars) intervening between the native and newly formed bone, bone marrow cavities (M) and osteoblasts lining the newly formed trabeculae (arrows) (H&E, X200)

although it was evident in the Masson's trichrome stained sections (fig.6)

Histomorphometry:

Histomorphometric data are expressed in the form of a graphic bar diagram in figure 7. Image processing revealed that the mean values of the mature and immature bone percentages were $54.83 \pm 12.13 \%$ and $24.13 \pm 5.01 \%$ respectively. These mean values reveal that about 31% of the total bone area belonged to the newly formed bone trabeculae adding a quantitative presentation to the histological results.



Fig. (5): A photomicrograph showing; lamellar bone (white star), osteocytes in their lacunae (yellow arrow heads), well defined reversal line surrounding the lamellar bone (arrows) and atypical bone pattern with few or no cellular existence (black stars) (H&E, X200)

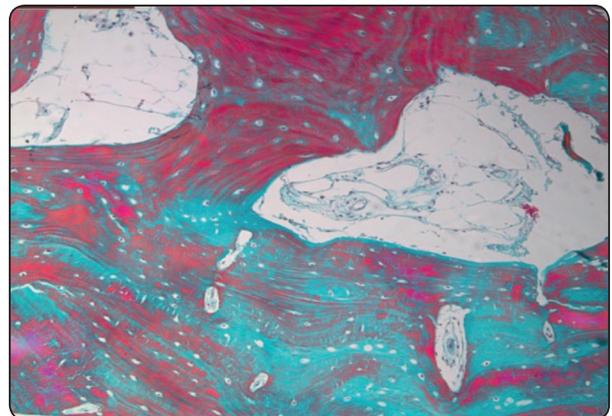


Fig. (6): A photomicrograph of the one of the cases demonstrating; native bone (red) infiltrated by newly formed immature bone (green) (Masson's Trichrome X200)

TABLE (2): One-sample “t” test comparing gene expression values to reference value.

Mean	St Dev	Reference Value	Difference	SEM	“t”	Probability
2.47	1.18	1.02	1.45	0.446	3.251	0.017*

*= Significant

Osteoprotegerin (OPG) Gene expression results

The mean gene expression value was statistically significantly higher (mean=2.47 & P=0.017) than the hypothesized reference value. The study expression values were compared to normal standard values of the gene in similar areas. (Table 2) (fig. 8)

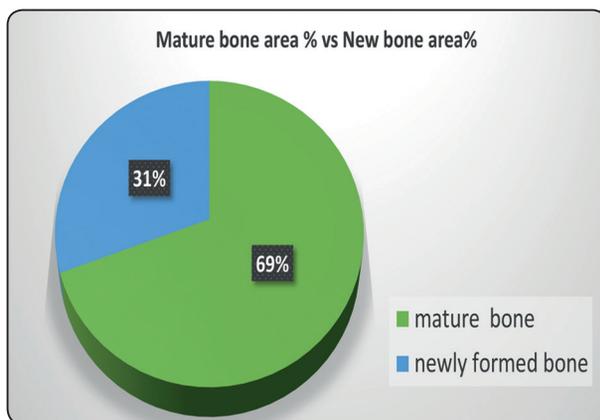


Fig. (7): Pie chart showing newly formed bone area percentage compared to mature bone area percentage.

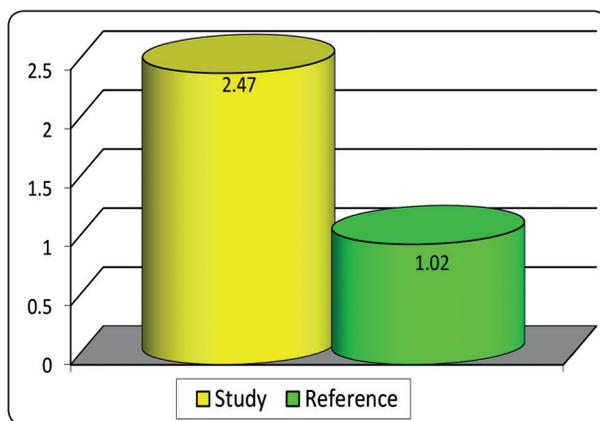


Fig (8): Mean values of OPG in the study group and the reference value.

DISCUSSION

In the present study, bone formation was assessed by histology, histomorphometry & quantitative gene expression analysis. Histological examination revealed highly cellular fibrous connective tissue interlacing with newly formed bone with variable degrees in the different studied cases. Reversal lines and condensation of bone-forming cells was also a common feature. Histomorphometric analysis revealed that the mean values of the mature and immature bone percentages were $54.83 \pm 12.13 \%$ and $24.13 \pm 5.01 \%$ respectively. These results agree with those of other studies of sinus elevation either with grafting or not ^{6,18}. This leads to the hypothesis that the key to bone reformation in the sinus may not be the grafting materials but actual space maintenance under the elevated sinus membrane.

The exact mechanism of bone formation in the maxillary sinus is always a point of investigation. Lundgren et al ⁹ claimed that alveolar bone regenerates as a reflex to space created from the elevation of the sinus membrane or from seeding bone fragments into the blood-filled sinus during surgery. Sohn et al ⁶ attributed that to the migration mesenchymal stem cells from the exposed sinus wall. The success of sinus augmentation was believed to depend on adequate angiogenesis and differentiation of osteogenic cells from the border of native bone ¹⁹.

Sonale et al 2018 suggested that absorbable gelatin sponge was effective in restoring bone height especially with immediate postoperative implant placement more than waiting five months postoperatively⁷.

Meanwhile, the current results may not agree with Sasayama et al²⁰ who proved that vacuum heated gelatin sponge scaffold combined with adipose-derived stem cells revealed better results than its single application in restoring congenital jaw clefts. The histological results also coincide with others regarding the histological and gene expression findings in response to the grafting procedures with recombinant bone morphogenic protein combined with absorbable collagen sponge. The authors detected similar histologic changes regarding the newly formed trabeculae and the highly cellular bone marrow. The osteoblasts were also detected lining the bone trabeculae²¹. These observations were similar to the current histological finding regarding the bone reaction towards grafting materials.

Our results also agree with Samieirad et al 2018²² who demonstrated that gelatin sponge was useful in the sinus floor augmentation.

Osteoprotegerin (OPG) is a member of the TNF receptor superfamily that regulates the process of bone remodeling via suppression of osteoclast differentiation and even induces their apoptosis which favors in turn bone formation.¹³

Upregulation, as well as, its downregulation was studied in research work demonstrate the rate of bone formation in maxillary sinus augmentation with different materials.^{23,24}

OPG is a gene linked to the process of bone remodeling in different researches usually become elevated with active bone formation. In the current research, it recorded a significantly elevated level among sinus augmentation procedures concerning normal standards. That agreed with other investigators who studied the gene expression and recorded a significant increase in its level after orthognathic surgery¹⁵. Although in other researches the gene exhibited an elevation in response to grafting, the authors didn't find it of statistical significance²⁵. Consequently, linking the significant elevation of the OPG gene in the present research to both histological and morphometric findings supports the role

of absorbable gelatin sponge in the process of osteogenesis in the herein research.

In conclusion, absorbable gelatin sponge augmentation succeeded to induce bone formation in the augmented area as confirmed by the investigation methods used in the current research. To be definite of the result further investigation is needed to compare gelatin sponge to sinus lifting only to verify or to disapprove the effect of the material.

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