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Jul 01, 2013

RE: EJOTO-D-13-00033R1, entitled "Effect Of Chitosan On Bone Restoration In Nasal Bone Defect (an experimental study) Mosaad Elsisy MD, Ashraf Elhamshary MD, Yasser Haroon MD & Samira Sallam MD* Departments of Otorhinolaryngology & Physics*, Faculties of Medicine & Sciences*, Benha University, Egypt"

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I am pleased to inform you that your work has now been accepted for publication in The Egyptian Journal of Otolaryngology. All manuscript materials will be forwarded immediately to the production staff for placement in an upcoming issue.

Thank you for submitting your interesting and important work to the journal.

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Dr. Tamer Youssef
Associate Editor
The Egyptian Journal of Otolaryngology
Effect Of Chitosan On Bone Restoration In Nasal Bone Defect
( an experimental study)

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Departments of Otorhinolaryngology & Physics*, Faculties of Medicine & Sciences*,
Benha University, Egypt

Abstract

Objective: The aim of this work was to study the effect of chitosan in restoration of bone defect (an experimental study).

Materials and methods: The study included 54 male guinea pigs. Nasal bone defect was done. The experimental animals were divided into a control group (A), calcium sulphate group (B) and chitosan coated calcium sulphate group (C). Three dimensional computed tomography (3D, C.T) and histological examination were done at intervals of 1, 2 and 3 months for measuring the change in the size of the bone defect and confirmation of bone formation respectively.

Results: The decrease in the size of the bone defect was significant in group (C) than groups (A & B). Also histological results showed formation of woven bone after one month in groups B & C and formation of lamellar bone in group C in the 2nd month while the lamellar bone was formed in group B in the 3rd month.

Conclusion: Radiological and histological studies showed that the new bone formation on defected nasal bone was more in group (C). These findings suggest that chitosan is very effective in early bone formation.

Key words: Chitosan, guinea pigs, bone formation

Introduction:

Autologous bone grafts, allografts, xenografts and bone graft substitutes are all supposed to stimulate early bone formation. As the autograft resorbs and revascularizes, osteoprogenitor cells differentiate into osteogenic cells. This osteogenic cell activity result in new bone generation and a healing of the bony defect. However, there is only a limited amount of autologous bone that can be harvested. Also, the secondary surgery at the harvested site adds an additional degree of morbidity

Calcium sulfate has been used in contained bone defects at sites without substantial compressive load (2&3). Unlike hydroxyl apatite or tricalcium phosphate, which is not completely absorbed and has a high residual rate, calcium sulfate is completely absorbed, and the rate of absorption and bone formation are relatively proportional(4).

Chitosan is a polysaccharide obtained by deacetylation chitin, which is the major constituent of exoskeleton of crustaceous water animals (5,6&7). It has been found to affect cellular migration and tissue organization during the wound- healing process; therefore, it may also enhance bone formation(5). Muzzarelli, et al.(8) and Klokkevold, et al.,(9) suggested that chitosan aids in the differentiation of osteoprogenitor cells and thus may also facilitate bone formation.

The aim of this work was to study the effect of chitosan on restoration of segmental bone defect.
**Materials and methods:**

**Experimental group:**

The study was done after obtaining approval from Benha University Ethical Review Committee and in accordance with the guidelines for animal experiments. This study was conducted on 54 male guinea pigs (weight 500 to 600 gm), that were divided into three groups: group A: (control group) nasal bone defect was done without placement of any materials, group B: nasal bone defect was closed by placement of calcium sulfate tablets & group C: nasal bone defect was closed by placement of calcium sulfate tablets coated by chitosan.

The animals were reared under similar conditions to exclude environmental and nutritional factors. The animals were males to obviate the impact of gender on speed and adequacy of bone formation and wound healing. The animals were of nearly similar age and weight. If any of animals died during follow-up it was replaced by a new one to optimize the number according to the statistical rules.

**Preparation of calcium sulfate tablets:**

Mixing calcium sulfate hemihydrate powder in distilled water (Fig.1). The water-calcium sulfate hemihydrate weight ratio was in the range from 0.27 to 0.30. Applying high pressure. Drying in 60°C convection oven for 24 hours (Fig.2).

**Preparation of calcium sulfate tablets coated with Chitosan:**

Medium molecular weight (300,000 g/mol), 90% deacetylated chitosan (Sigma-Aldrich Co, USA) was dissolved in 2% acetic acid solution to 3% (Chitosan 3g/ 2% acetic acid 100 ml). Calcium sulfate tablets were coated by chitosan twice through a machine. (Figure 4). The tablets were dried in a 40°C convection oven for 24 hours (Figure5).

**The operative procedure:**

Anesthesia with ketamin was given intramuscularly in a dose of 10 mg /kg. The hair on the maxilla, nose and calvarium was shaved (Figure 6). One ml of 1% lidocaine with adrenaline (1:100 000) was injected along the nasal dorsum subperiosteal. A vertical skin incision was made along the frontal calvarium and down the nasal dorsum just posterior to the nares and nasal tip (Figure 7). The peristeum was incised in the midline, elevated carefully and retracted laterally beyond the maxillo-nasalis suture lines. A diamond shaped design was drawn on the bone by a foil template with long diagonal equals 1.5 cm and short diagonal equals 1 cm. Under the operating microscope, a drill was used to carefully burr off the nasal bones down to the underlying mucosa, the depth of bone defect was about 0.2 cm (figure 8). After copious saline irrigation to remove bone dust and debris, the periosteum was then closed as tight as possible with a running 10-0 nylon suture: in group A without placement of any material (figure 9), in group B after placement of calcium sulfate tablet (figure 10) & in group C after placement of calcium sulfate tablet coated with chitosan (figure 11). The skin was then closed using 4-0 proline sutures (figure 12).
Radiographic and histological studies:

At intervals of 1, 2 and 3 months 6 animals of each group were taken and CT-imaging was done. The CT-imaging of the skull was performed using coronal images. For CT technique using GE prospeed spiral CT scanner, the animal was put in prone position with the head hyper-extended resting on the chin after anesthesia like in operative procedure. Axial images were obtained with spiral technique (supine position) and coronal reconstructions of axial images. Gantry angle, perpendicular to hard palate to obtain direct coronal images. The cuts were taken as contiguous 1 mm sections, 1 mm table feed, pitch 1 from the frontal bone down to involve the whole nose. Low exposure (KVp120, 250mA) can be used because of the bone algorithm technique photography at suitable windows to visualize both bone and soft tissues on the single set of images, 3D reconstruction was made using axial and coronal images for calculating residual defect size (surface area = [long diagonal x short diagonal]/2). Animals were then sacrificed and nasal bone specimens were taken and fixed in a 10% buffered formalin solution, decalcified with edetic acid, hydrochloric acid, cut in a coronal plane, embedded in paraffin sections and stained with hematoxylin and eosin for histological examination.

Statistical analysis:

The collected data were tabulated and analyzed using the suitable statistical methods. Paired t test was used to compare between 2 means and one way ANOVA test used to compare between more than 2 means and standard deviations of values of more than 2 groups. The SPSS program (version 11 in IBM compatible computer) was used. Levels of significance: P values > 0.05 were considered statistically non significant, P values <0.05 were considered statistically significant & P values <0.01 & P values <0.001 were considered statistically highly significant.

Results:

Bone growth and healing of the defect regarding to group A showed non significant decrease in the surface area at the 2nd and 3rd months compared to 1st month and also the 3rd month compared to 2nd month (table 1 & figure 13). While in group B & C the decrease in the surface area showed significant decrease at the 2nd and 3rd months compared to 1st month and also the 3rd month compared to 2nd month (tables 2 & 3 & figure 13).

The mean decrease of the surface area at 1st month regarding all groups showed non significant result in group B compared to group A (p = 0.067), significantly higher in group C compared to group A (p = 0.001) and significantly higher in group C compared to group B (p = 0.003) (table 4).

The mean decrease of the surface area at 2nd month regarding all groups showed significant higher result in group B compared to group A (p = 0.021), significantly higher in group C compared to group A (p = 0.001) and significantly higher in group C compared to group B (p = 0.013) (table 5).

The mean decrease of the surface area at 3rd month regarding all groups showed significant higher result in group B compared to group A (p < 0.001), significantly higher in group C compared to group A (p < 0.001) and significantly higher in group C compared to group B (p = 0.002) (table 6).

Figures: 14 - 18 showed an example of C.T results of study groups.
Histological results showed formation of woven bone formation after one month in groups B& C and formation of lamellar bone in group C in the 2nd month while the lamellar bone was formed in group B in the 3rd month (figures: 19-22).

**Discussion:**

Because of limitations in the value of biological grafts (infection, vascular necrosis, atrophy, resorption, limited amount of material supply, occurrence of immunological response due to genetic differences and the risk of induction of transmissible diseases), considerable attention has been directed to the use of synthetic materials (10-14). The aim of using such material is for the graft to promote adequate bone regeneration at the defective site by acting as a scaffold for osseous growth. Dense and porous hydroxyl apatite and tricalcium phosphate ceramics are the materials most widely utilized (15&16).

Peltier and Jones (17) proved that the bone graft substitute should be resorbed at a rate that was balanced with new bone growth. Ceramic substitutes such as tricalcium phosphate and hydroxyl apatite may inhibit bone formation and weaken the intensity of newly formed bones because they typically exhibit a biologic residence time greater than that required for new bone formation (3).

Calcium sulphate is an osteoconductive substance, which, unlike tricalcium phosphate and hydroxyl apatite, is completely absorbed. Calcium sulphate induces angiogenesis causes the transfer to the bone graft area of osteoprogenitor cells and facilitate new bone formation (1,18-19).

Chitosan is a carbohydrate biopolymer extracted from N-acetylated chitin, a structural ingredient in the skeletons of crustaceans (such as lobster, crab, crawfish) and the cell wall of fungi (20-22). Chitosan has biocompatibility and biodegradability as well as osteo-induction (9). Normally, chitosan is combined with a growth factor-like fibroblast growth factor, which is in the trabecular bone and which helps the mitosis of various kinds of matrix cells (6). Chitosan activates macrophages and mono nuclear cells and induces the production of fibroblast growth factor and platelet-derived growth factor (7). Malette, et al., (23) have reported that in an experiment with dogs, the injection of chitosan into the bone defect area causes the increase of bone regeneration.

Our results showed that mean decrease of the surface area was significant in groups B and C but significantly higher in group C than group B. Also the remodeling of woven bone into lamellar bone was early in group C than in group B. This means that bone formation occur in group B and C but growth rate of new bone is more with group C. In agreement with our study, the study of Cui et al., (4) who studied the effect of chitosan-coated pressed calcium sulfate pellets combined with recombinant human bone morphogenetic protein 2 on restoration of segmental bone defect of 12-mm radius in rabbits, they found that chitosan coated pressed calcium sulphate pellets showed relatively higher density and slightly slower resorption that closely coincides with growth rate of new bone. This made it possible to restore segmental bone defect and particularly when combined with recombinant human bone morphogenetic protein 2, that coated pellet would enhance its osteogenesis.

Halil, et al., (24) studied the effect of slow release of bone morphogenetic protein-2 and transforming growth factor-beta-2 in a chitosan gel matrix on cranial bone graft survival in experimental cranial critical size defect model. The bone formation becomes apparent at the time point of 8th postoperative week and still persists at 14th
postoperative week. This study goes hand to hand with our results as lamellar bone formation occurred in the 2nd month and persists at the end of 3rd month.

Kim, et al., (1) studied the role of bone morphogenic protein (BMP), transforming growth factor beta- induced gene h3 (ig- h3 ) and chitosan in early bone consolidation in distraction osteogenesis in a dog model. They found that new bone was generated in all groups. The amount of new bone generation in the distracted zone was in the order of the BMP group, the ig h3 group, the chitosan group and the control group. The difference between our results & their results due to the use of different materials in their study.

In our study there are no cases with extrusion of materials or infection. This is supported by the study of Bhaskara and Chandra (25). Who found that acute systemic toxicity tests in mice did not show any significant toxic effects of chitosan, eye irritation tests in rabbits and skin irritation tests in guinea pigs did not reveal any undesirable toxic effects of chitosan. Pyrogen free status could be noticed with chitosan films on animal pyrogen testing, samples retrieved after 3 and 7 days of intramuscular implantation did not reveal identifiable untoward changes.

In conclusion: The findings of this study indicates that chitosan coated pressed calcium sulfate tablets showed higher growth rate of new bone than calcium sulfate tablets. This indicate that chitosan enhances the process of osteogenesis.

References:


Fig (1): Calcium sulfate hemihydrate powder

Fig (2): Calcium sulfate tablets

Fig (3): Chitosan powder

Fig (4): spin XNG-m1 used for coating

Fig (5): Chitosan coated calcium sulfate tablets

Fig (6): Shaving

Fig (7): Skin incision

Fig (8): Burring the Defect

Fig (9): Diamond shaped defect in group A

Fig (10): Calcium sulfate tablet

Fig (11): Chitosan coated tablet in the defect in group B

Fig (12): Skin closure
Table (1) means ($\chi$-) ± standard deviations (SD) of surface area cm$^2$ among group (A) at different times

<table>
<thead>
<tr>
<th>Time</th>
<th>Surface area (cm$^2$)</th>
<th>($\chi$-) ± SD</th>
<th>$\chi$- of difference</th>
<th>Paired t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.75±0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>First month (M1)</td>
<td>0.54±0.05</td>
<td>0.21±0.26</td>
<td>t1=1.98</td>
<td>&gt;0.05</td>
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</tr>
<tr>
<td>Second month (M2)</td>
<td>0.53±0.04</td>
<td>0.22±0.27</td>
<td>t2=1.99</td>
<td>&gt;0.05</td>
<td></td>
</tr>
<tr>
<td>Third month (M3)</td>
<td>0.54±0.04</td>
<td>0.21±0.26</td>
<td>t3=1.98</td>
<td>&gt;0.05</td>
<td></td>
</tr>
</tbody>
</table>

$t_1 = 0$ versus $M_1$  $t_2 = 0$ versus $M_2$  $t_3 = 0$ versus $M_3$

Table (2) means ($\chi$-) ± standard deviations (SD) of surface area (cm$^2$) among group (B) at different times

<table>
<thead>
<tr>
<th>Time</th>
<th>Surface area (cm$^2$)</th>
<th>($\chi$-) ± SD</th>
<th>$\chi$- of difference</th>
<th>Paired t</th>
<th>P</th>
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<tr>
<td>0</td>
<td>0.75±0</td>
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<tr>
<td>M1</td>
<td>0.46±0.03</td>
<td>0.29±0.03</td>
<td>t1=22.87</td>
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<tr>
<td>M2</td>
<td>0.35±0.03</td>
<td>0.4±0.03</td>
<td>t2=32.67</td>
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<tr>
<td>M3</td>
<td>0.21±0.4</td>
<td>0.54±0.03</td>
<td>t3=44.1</td>
<td>0.000</td>
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</table>

$t_1 = 0$ versus $M_1$  $t_2 = 0$ versus $M_2$  $t_3 = 0$ versus $M_3$

Table (3) means ($\chi$-) ± standard deviations (SD) of surface area (cm$^2$) among group (C) at different times

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<th>Time</th>
<th>Surface area (cm$^2$)</th>
<th>($\chi$-) ± SD</th>
<th>$\chi$- of difference</th>
<th>Paired t</th>
<th>P</th>
</tr>
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<tr>
<td>M1</td>
<td>0.37±0.04</td>
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<td>t1=23.27</td>
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<td>M2</td>
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<td>0.5±0.03</td>
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<td>M3</td>
<td>0.16±0.03</td>
<td>0.59±0.03</td>
<td>t3=48.18</td>
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$t_1 = 0$ versus $M_1$  $t_2 = 0$ versus $M_2$  $t_3 = 0$ versus $M_3$

Table (4) Mean (±SD) decrease of wound surface area estimated at the end of the 1st month in relation to baseline surface area

<table>
<thead>
<tr>
<th>% of surface area decrease</th>
<th>Value</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
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</thead>
<tbody>
<tr>
<td>$P_1$ (A versus B)</td>
<td>29.6±7.4</td>
<td>38.2±4.6</td>
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<td>50.7±5.3</td>
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<tr>
<td>$P_2$ (A versus C)</td>
<td></td>
<td></td>
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<td>0.001</td>
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<tr>
<td>$P_3$ (B versus C)</td>
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<td>0.003</td>
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Table (5) Mean (±SD) decrease of wound surface area estimated at the end of the 2nd month

<table>
<thead>
<tr>
<th>% of surface area change</th>
<th>Value</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P₁(A versus B)</td>
<td>46.2±4.2</td>
<td>54±3.9</td>
<td>66.4±4.1</td>
</tr>
<tr>
<td></td>
<td>P₂(A versus C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P₃(B versus C)</td>
<td></td>
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</tr>
</tbody>
</table>

P₁ = 0.021
P₂ = 0.001
P₃ = 0.013

Table (6) Mean (±SD) decrease of wound surface area estimated at the end of the 3rd month

<table>
<thead>
<tr>
<th>% of surface area change</th>
<th>Value</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P₁(A versus B)</td>
<td>50.1±8.9</td>
<td>70.8±4.6</td>
<td>79.5±3.4</td>
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<tr>
<td></td>
<td>P₂(A versus C)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>P₃(B versus C)</td>
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P₁ < 0.001
P₂ < 0.001
P₃ = 0.002

Fig (13) Mean change of wound surface area estimated throughout the study period in the three groups
Fig (14): example of group A after one month.  

Fig (15): example of group B after one month.

Fig (16): example of group B after 3 months.

Fig (17): example of group C after 2 months.

Figure (18): Example of group C after 3 months.
**Fig (19):** Group B after 1\textsuperscript{st} month of implantation showing newly formed woven bone was seen at the periphery of the augmented space.

**Fig (20):** Group B after 3\textsuperscript{rd} month of implantation showing the augmented space deeply concave due to resorption of calcium sulfate.

**Fig (21):** Group C after 1\textsuperscript{st} month of implantation showing many trabeculae of woven bone embedded in cell-rich fibrovascular tissue

**Fig (22):** Group C after 2\textsuperscript{nd} month of implantation showing some newly formed lamellar bone seen embedded in fatty tissue.
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<th>منظمة التقييم في نفس التخصص</th>
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<td>تجهيز الماد المستعملة في البحث</td>
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**EFFECT OF CHITOSAN ON BONE RESTORATION IN NASAL BONE DEFECT (AN EXPERIMENTAL STUDY)**

تأثر الكينوزان على إعادة تكوين نظام الأذن (دراسة تجريبية)

المجلة المصرية للأذن والأنف والحنجرة

تقبل النشر: 1 يوليو 2013

المؤلفين:

- د. مصطفى السيد
- د. أشرف الهنawy
- د. ياسر هدين محمد
- د. سمير محمد سلام
المختصر العربي:
الهدف من البحث:
دراسة قدرة الكيتوزان على إعادة تكوين عظام الأنف.
طريقة البحث:
شملت هذه الدراسة 54 خنزير غني وقد تم حفر تجويف في عظام الأنف على شكل معين له قطرين الأطول يساوي 15 مم والأصغر 10 مم في جميع الخنازير. وقد قسمت إلى ثلاث مجموعات متساوية في العدد: المجموعة الأولى لم يتم وضع مادة محفزة في التجويف، والمجموعة الثانية تم وضع قرص على شكل التجويف من سلفات الكالسيوم، والمجموعة الثالثة تم وضع قرص على شكل التجويف من سلفات الكالسيوم المكسوة بمادة الكيتوزان. تمت متابعة إعادة تكوين عظام الأنف من خلال عمل أشعة مقطعية ثلاثية الأبعاد، وفحص نسيجي لعظام الأنف بعد 1 و2 و3 شهور.

النتائج:
مساحة السطح للجرح المحفور قد قل بنسبة كبيرة في المجموعة الثالثة عن المجموعة الثانية والثالثة. وقد بينت نتائج الفحص الخلوي لأنسيج عظام الأنف أن هناك تكوين نسيجي عظمي جديد في المجموعتين الثانية والثالثة ولكن كان أسرع في المجموعة الثالثة عن الثانية.

الاستنتاج:
من هذه الدراسة التجريبية وجدنا نتائج مشجعة على استخدام أقراص سالفات الكالسيوم المكسوة بالكيتوزان في تصحيح عيوب عظام الأنف نظراً لتأثير الكيتوزان الواضح في سرعة تكوين العظام.

 أهم كلية طب بنها
د. محمد محمد

المتقدم للترقيه

رئيس قسم الأنف و الأنف و الجصر

أ.د. محمد محمد سلمان

14/8/20