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Bone healing in critically sized defects in rat calvaria, transplanted with chitosan alone, or associated with collagen and / or chondroitin sulfate:histological and histomorphometric pilot study

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Abstract

Background: Chitosan is a natural biopolymer that has gained a special interest in bone regeneration in recent years. **Objective**: The objective of this study is to show the bone formation obtained following a transplantation of sponges of chitosan alone, chitosan combined with chondroitin sulfate or chitosan combined with chondroitin sulfate and collagen, in rat critical calvarial bone defects. **Material and Methods**: 12 Wistar rats were divided into 4 groups of 3 rats each. Critically sized bone defects were made in calvaria, and grafted by sponges of:collagen / chitosan / chondroitin (group 1), chitosan (group 2), chitosan / chondroitin sulfate (group3). Bone defects of group 4 remained empty for control. The animals were sacrificed 12 weeks after the surgery. **Results**: Histological analysis showed the formation of lamellar bone in the chitosan group. In the chitosan / chondroitin sulfate group, formation of a less mature bone than that of the chitosan group was also observed. However, the least bone formation was observed in the collagen / chitosan / chondroitin sulfate group. Histological sections showed the presence of fibrous tissue. The results for this group are similar to the control group. In groups 2 and 3, the materials appear completely resorbed while in group 1 the resorption of the matrix was incomplete. **Conclusion**: Despite the size of the sample, this study has shown that chitosan alone or in combination with chondroitin sulfate promotes bone formation. On the other hand, the combination chitosan / chondroitin sulfate / collagen showed a negative result.

Keywords: Bone regeneration, Biodegradation, Chitosan, Chondroitin sulfate, Collagen.

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INTRODUCTION

Bone regeneration has become a frequent and essential practice in periodontal and implant surgery. One of the current trends is the use of biopolymers. These materials allow faster healing and are assumed, by their natural origin, to have a better biocompatibility [1].

Chitosan is an ionizable polysaccharide, obtained after deacetylation of chitin, a biopolymer of high molecular weight, non-toxic and biodegradable [2]. Chitosan and its derivatives, in various physical forms (gel, powder, membrane, etc.), have been shown to be effective in bone repair [3,4], periodontal healing and in tissue engineering [5-10].

The degree of N-acetylation and molecular weight are the most important parameters for characterizing chitosan. The degree of N-acetylation is a structural parameter that will influence charge density, crystallinity, solubility, and propensity for enzymatic degradation. The higher the degree of N-acetylation, the faster the biodegradation [11]. In addition, a low degree of N-acetylation would promote better cell adhesion (Chatelet *et al*, 2001, Prasitsilp *et al*, 2000) [12, 13].

The molecular weight of chitosan (50-2000 kDa) is also a very important factor affecting its physicochemical properties. It is also involved in biological properties such as biodegradation and biocompatibility [14, 15].

The similarity of the structure of chitosan with that of the extracellular matrix glycosaminoglycan contributes to its high tissue biocompatibility[16].

However, chitosan has poor physical properties [16] which may limit its use. Its physical and biological properties can be modified by associating it with other materials. Many products have been developed in which chitosan is associated with calcium phosphate, tricalcium phosphate, gelatin, collagen, hydroxyapatite [17-19]. It has also been associated with biological signaling molecules [20- 23].

The aim of this study is to compare the bone formation obtained after transplantation with chitosan alone, chitosan combined with chondroitin sulfate or chitosan combined with chondroitin sulfate and collagen, in critical size bone defects created on rat calvaria. Since the degradation of a bone substitute material is important and beneficial for osteoconduction and a key feature in tissue engineering, the secondary goal of this study is to describe the resorption pattern of chitosan.

MATERIAL AND METHODS

Sponges made of chitosan, collagen and chondroitin sulfate developed by Shahabeddin *et al*. In 1990 [24] were used (provided by the Lebanese Human Tissue Bank). These sponges are composed of type I and III collagen, chitosan (85%) and chondroitin sulfate (bovine cartilage extract), in a proportion of:72% collagen, 20% chitosan and 8% chondroitin sulfate at 1.25%. The authors used these sponges as a matrix for keratinocytes in dermal regeneration. They obtained an epidermis morphologically comparable to human skin tissue.

Based on this study, more than 75% chitosan chitin deacetylated (chitosan powder, medium molecular weight, Aldrich 419419) as well as a glycosaminoglycan, chondroitin 4 sulfate from the bovine trachea (Sigma 27042 chondroitin sulfate powder) have been used.

Solutions of 1% chitosan, chondroitin sulfate at 1.25% and a solution combining chitosan and chondroitin sulfate in a ratio of 3:1 were prepared.

These solutions were put in a wellbox, then frozen at -80 degrees for one night and then for a second night in a freeze-dryer at -50 degrees to obtain a sponge shape.

For this study, 12 Wistar rats (males of 300-350 g) were used. The animals were housed in individual metal cages, with an ambient temperature of 25 °C. Artificial lighting was put in place to maintain a biological rhythm of day and night (12 hours of day and 12 hours of night). They were fed ad libitum.

Anesthetic intramuscular injection of Ketamine and Xylazine (0.8ml Ketamine + 0.2ml Xylazine) was administered.

A vertical incision of approximately 1.5 cm in the anterior region of calvaria followed by detachment of a flap of total thickness was performed. A bone defect was created using a 8 mm diameter trephine drill.

The defects thus created will be grafted by the 3 products as follows (Fig 2)

Group 1:3 rats grafted by sponges:collagen / chitosan / chondroitin sulfate

Group 2:3 rats grafted by sponges: chitosan

Group 3:3 rats grafted by sponges: chitosan / chondroitin sulfate

Group 4: without filling (control group).

The flap was subsequently sutured by resorbable sutures.

Each animal received a postoperative intramuscular injection of antibiotics (24,000 IU Penicillin-Benzathine).

The rats were sacrificed at 12 weeks.

For histological preparation, the Exakt® technique (Microm France) was used.

Histomorphometric measurements were performed under an optical microscope (Olympus Bx 4500) connected to a digital camera (Nikon Coolpix 4500). Three sections per calvaria were analyzed manually using UTHSCA Image Tool 3.0 software. The bone area was calculated by adding the newly formed bone surfaces in each section.

Statistical Package Software for Social Science (SPSS for Windows, Version 18.0, Chicago, IL, USA) was adopted to perform statistical analysis of data. The significance level used corresponds to -p-value ≤ 0.05.

The primary endpoint of the study is the bone formation area expressed in mm^2. The statistical unit is the section. The bone formation surface was compared between the 4 groups:Chitosan; Chitosan / chondroitin sulfate; Chitosan / chondroitin sulfate / collagen; control.

This is a small sample where the numbers in the different groups are equal. A parametric test that is more powerful than a nonparametric test was used after verifying the normality of the distribution by the Kolmogorov-Smirnov test. A one-way analysis of variance was performed followed by multiple Tukey comparisons (HSD).

RESULTS

Histological sections are observed under an optical microscope (Olympus Bx 4500).

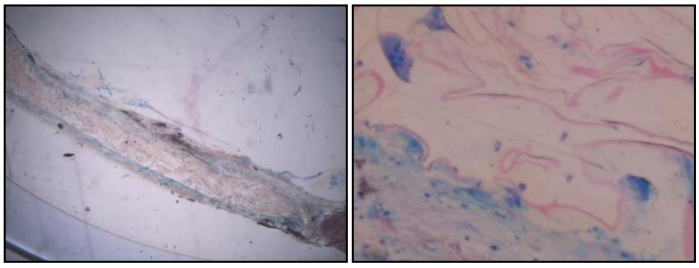
In the group collagen / chitosan / GAG combination, very minimal bone formation is observed at the edges of the defects. The presence of a fibrous tissue is especially noticeable (Fig. 1-a). On some sections at high magnification (x40) inflammatory cells and multinucleated giant cells, responsible for the resorption of the matrix with the presence of fibroblasts, are seen. It can also be noted the presence of non-resorbed matrix fibers (Fig. 1-b).

In the group chitosan alone at 1%, at low magnification (x10), the presence of lamellar mature bone is noted (Fig. 2-a). At high magnification (x40) the presence of osteocytes in osteoplasts can be seen throughout the bone lamellae (Fig. 2 b-c).

On sections of the second rat grafted with chitosan alone, a newly formed bone is also visible. This osseous formation appears centripetal, from the edges of the defect towards the center, with in the middle the presence of fibers (Fig. 3-a). At high magnification (x40), there is the presence of a "woven bone" with a large amount of bone cells arranged in an anarchic way. In the center of this fibrous zone fibroblast cells are noted (Fig. 3-b).

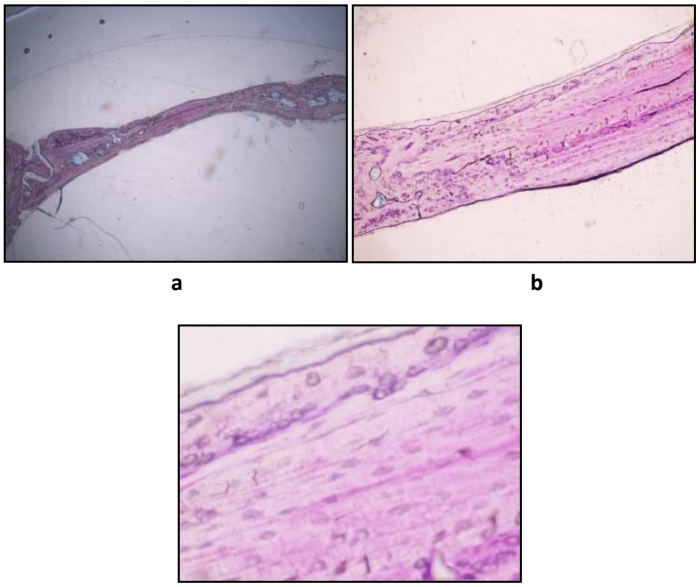
In some sections, a woven bone can be seen in the center of the defect (Fig. 4-a). At high magnification (x40) the osteoblastic cells are clearly visible (Fig. 4-b).

In the group chitosan / chondroitin sulfate combination, centripetal bone formation is observed at low magnification (x10) (Fig. 5-a). High magnification (x40) also shows the presence of fibroblasts with some osteoblasts in the center (Fig. 5-b).



a b

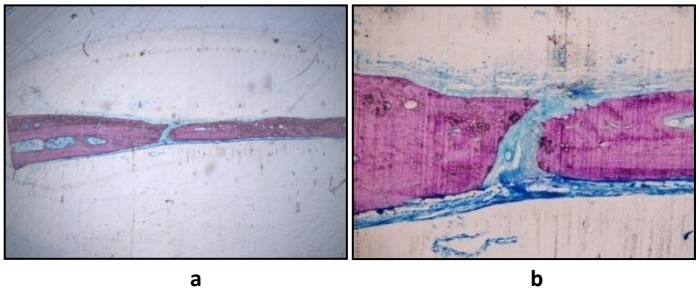
Figure 1: Histological sections, defects grated with collagen / chitosan / chondroitin sulfate a. Low magnification (x10), fibrous tissue. b. high magnification (x40) multinucleated giant cells, presence of matrix fibers.



a b

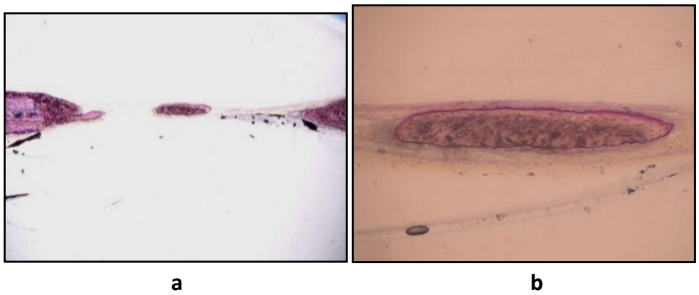
c

Figure 2: Defects grafted with Chitosan at 1%. a. Low magnification. Presence of a mature lamellar bone. b-c High magnification (x40) Presence of osteocytes throughout the bone lamellae.



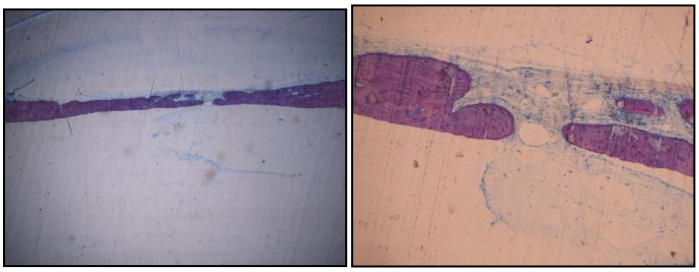
a b

Figure 3: Chitosan at 1% a. At low magnification (x10), centripetal bone formation. b. At high magnification (x40), presence of bone cells. In the center, fibroblastic cells are noted.



a b

Figure 4: a. At low magnification (x10) **b.** At high magnification (x40), presence of bone cells in woven bone.



a b

Figure 5: Bone defects grafted with chitosan / chondroitin sulfate. at. At low magnification (x10) there is a centripetal bone formation. b. Presence of fibroblasts and osteoblasts in the center.

This study revealed a statistically significant difference between the 4 groups (-p-value = 0.014, ANOVA).

Tukey multiple comparisons showed that the average bone formation area was significantly elevated with Chitosan, followed by Chitosan / Chondroitin sulfate. No statistically significant difference was found between the control group and the Chitosan / chondroitin sulfate / collagen group (-p-value = 1.000) at which bone formation was significantly the smallest.

DISCUSSION

Chitosan is a biopolymer of natural origin, which seems to offer several advantages concerning its use, alone or in combination with other materials in the bone regeneration, and this thanks to its numerous biological characteristics.

It is currently accepted that chitosan does not result in an immunological reaction and has a high biocompatibility [25]. Similarly, glycosaminoglycans are known for their immunosuppressive properties and their ability to reduce the reaction of tissue to foreign bodies [26]. Collagen is also non-toxic, biocompatible and well tolerated [27].

In the treated rats, we did not have any inflammatory reactions. The three rats that died during the study died three to four weeks after the intervention, which excludes the infectious cause.

The concentration of the components of a material can regulate the porosity of this material [28]. Pore size is important for cell growth, for vascularization and therefore for osteoconductivity. It must be between 100 and 150 µm to allow tissue growth [29]. When pore size is wide, this allows for better cell proliferation by facilitating revascularization and transport of oxygen and nutrients [30]. A size of 300 to 400 µm is considered optimal for bone regeneration in vitro because it allows rapid cell migration within structures [31, 32].

According to Tian (2001) [33], depending on the degree of biodegradation, the minimum pore size to allow osteoconduction should be 200 µm.

After testing different concentrations, Shahabeddin *et al* (1990) [24] opted for a concentration of 1.25% chondroitin sulfate to obtain 50-120 µm pores in their collagen / chitosan / chondroitin sulfate sponges. These sponges have been tested in the skin tissue. Therefore the porosity of the sponge obtained may not be sufficient to allow good induction at the bone level.

One of the most promising factors of chitosan is its ability to be transformed into porous structures. According to Arpornmaeklong *et al*. 2007 [34], the association between collagen and chitosan increases the pore size of the resulting matrix.

They also reported that chitosan sponges allowed better cell growth than other groups. The strong attraction between positive charges on

the surface of chitosan and negative charges on the cell surface improves the metabolic activity of cells [35]. These authors concluded that the chitosan / collagen combination improves the differentiation and proliferation of osteoblasts. However, the technique of manufacturing the size and pore structure of these matrices must be improved.

Pang EK *et al* in 2005 [18] evaluated the effect of chitosan on human fibroblasts of the periodontal ligament *in vitro*, and bone formation at bone defects in rat calvaria in vivo. After testing several concentrations, they found that cell growth was greatest with a concentration of 1% chitosan. Inhibition of cell growth was observed beyond this concentration. Following this, 1% of chitosan was considered the critical concentration by these authors. Which is why we chose this concentration in our study.

Machida *et al*, in 1986 [36], conducted a study on chitosan degradation in vitro using lysozymes and *in vivo* after implantation in rats. They reported that chitosan is a biocompatible and biodegradable material. In the results we obtained, no trace of sponge components was found in chitosan graft defects and chitosan / chondroitin sulfate 12 weeks after surgery. The degree of deacetylation plays a role in the resorption of chitosan. The higher the degree of deacetylation, the slower the degradation of the material will occur. According to Adekogbe and Ghanem in 2005 [37], a degree of deacetylation of 100% results in almost no resorption. These authors have shown that for chitosan matrices with a degree of deacetylation of 90%, the degradation time is 42 days if chitosan is used alone, and 63 days if it is associated with dimethyl 3-3 dithio bis propionimidate (DTBP).

The rapid degradation of chitosan can also limit its use. This is why it is most often associated with other components.

At the level of defects grafted by the collagen / chitosan / chondroitin sulfate association, matrix fibers with multinucleate giant cells are present after 12 weeks, which is a sign that the degradation of the material is in progress. As reported in the literature, crosslinking reduces the biodegradability of the material [38]. Similarly, several studies have shown that the incorporation of chitosan into a collagen matrix increases the mechanical strength of this matrix and reduces the rate of biodegradation against collagenase 39]. The crosslinking phenomenon confers resistance to the formed sponge and renders it insoluble in physiological fluids [40]. With regard to glycosaminoglycans, the fact that they can reduce the reaction to foreign bodies could lead to a delay in the degradation of the matrix [5].

It should also be noted that the chitosan used in these sponges is 85% deacetylated, whereas the chitosan used in chitosan sponges alone is 75% deacetylated. All these hypotheses could delay the biodegradation of these sponges.

A study by Hidaka *et al*, in 1999 [41], found that membranes made with chitin deacetylated at 65.70 and 80% favor osteogenesis in rat calvaria, whereas when the degree of deacetylation is 94%, osteogenesis was minimal. Therefore, according to this study, it can be concluded that the higher the degree of deacetylation, the lower the osteogenesis, thisbeing related to resorption. In our study, we used chitosan with a degree of deacetylation ≥ 75%, which may be one of the factors that contributed to the formation of new bone especially in sites grafted with chitosan alone or in combination with chondroitin sulfate. On the other hand, chitosan used in the manufacture of sponges associating chitosan with chondroitin sulfate and collagen has a degree of deacetylation of 85%, which could partly explain the absence of bone formation and the formation of a fibrous tissue.

It has already been reported that molecular weight plays an important role in cellular morphology and osteoblast activities *in vitro* [42]. Kung *et al*, in 2011 [43], evaluated different molecular weights of chitosan in a material composed of chitosan and collagen. The bone parameters obtained in the group with chitosan having a molecular weight of 750 kda were slightly higher than those of the group with a molecular weight of 450 Kda. However, these differences are not statistically significant. The chitosan used in our study was of medium molecular weight.

In our study, a better bone formation was obtained at 12 weeks in the chitosan group followed by the chitosan / chondroitin sulfate group and lastly in the collagen / chitosan / chondroitin sulfate group. The results obtained in this last group were very close to those obtained with the control group.

Our results are consistent with those of Lee *et al*. in 2002 [21], who reported new bone formation without the presence of fibrous tissue in defects in rat calvaria and chitosan grafting.

Ezoddini *et al* in 2012 [44], in their studies on rat tibias using chitosan powder reported that chitosan significantly accelerated the bone process when compared with untreated defects at 1, 2 and 4 weeks after surgery.

In contrast, Oktay *et al*. In 2010 [45], compared the effect of a chitosan sponge and platelet-rich plasma gel (PRP) alone or in combination with cranial defects in rabbits. They observed the results at 4 and 8 weeks. Defects filled with chitosan show very limited bone formation. Fibrous tissue was found between chitosan particles and bone. The chitosan used in this study had a deacetylation degree of 86%, and a concentration of 3%. In the chitosan group associated with PRP, better bone formation was observed. Indeed, PRP release growth factors that will activate macrophages. These macrophages will release more lysozomal enzymes that degrade chitosan and consequently promote osteogenesis [46]. Spin-Neto *et al* in 2010 [47] also reported negative results when using chitosan in critical size defects in rats calvaria.

CONCLUSION

This study showed the interest of chitosan in bone healing when used alone or in combination with a glycosaminoglycan, chondroitin sulfate. On the other hand, it showed a negative result concerning the collagen / chitosan / chondroitin sulfate combination.

Chitosan seems to have many interesting properties for use in tissue engineering for bone regeneration. It would be interesting to investigate more about chitosan, especially its shape, its porosity, its degree of deacetylation and its molecular weight. There is also a need for further research on its association with other biomaterials in order to find a balance between the physical stability and the resorption of the obtained material.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of the animals were followed. All procedures performed on animals were in accordance with the ethical standards of the research committee at Saint Joseph University.

Informed consent: This study does not involve human participants, consent not available.

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