Original Article

Effect of Dietary Ascorbic Acid on Osteogenesis of Expanding Midpalatal Suture in Rats

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Abstract

Objectives: After maxillary expansion, a long period of retention is necessary to prevent early relapse. Therefore, it is beneficial to accelerate bone formation in the expanding midpalatal suture to reduce relapse. This study was designed to evaluate the effect of dietary vitamin C on osteogenesis of rat midpalatal suture during expansion.

Materials and Methods: Fifty-four male Wistar rats were randomly divided into three groups, each with a control and an experimental subgroup. An open-loop spring was bonded to maxillary incisors of each animal to expand the premaxillary suture. Experimental groups received dietary vitamin C in their water. The rats in the three groups were sacrificed at three, nine or 17-day intervals after bonding the spring. Then, the premaxilla was dissected and sections were made and stained with hematoxylin and eosin and osteopontin marker. Osteoblasts and osteoclasts were counted in the suture. Two-way ANOVA and the Mann-Whitney-U test were used for analyzing the data.

Results: After three days, the number of osteoblasts was significantly higher in the vitamin C group but after nine days it was significantly higher in the control group and after seventeen days there were no significant differences between the groups. Osteoclast counts were not significantly different between vitamin C and control groups.

Conclusion: Vitamin C had a positive effect on osteogenesis at the beginning of bone formation in the expanding suture, but after nine days it had a negative effect on suture osteogenesis in rats.

Key words: Osteogenesis; Osteoblast; Osteoclast; Ascorbic acid

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INTRODUCTION

Maxillary expansion is a common mechanotherapy in orthodontics, with an aim to increase constricted maxillary arch width. Expansive mechanical forces stretch the collagenous fibers, increase sutural remodeling and lead to new bone formation [1]. In clinical situations, it is known that a long period of retention is necessary to prevent early relapse of the expanded arch [2]. Therefore, it might be beneficial to accelerate bone formation in the expanding midpalatal suture to reduce relapse and shorten the retention period [1,2].

The effect of many substances has been evaluated on the bone formation and resorption during expansion of the suture. Sawada and Shimizu [1] found that application of transforming growth factor-β1 during the early

stages of expansion may induce rapid bone formation. Saito and Shimizu [2] reported that low-power laser irradiation had therapeutic benefits in inhibiting relapse of the expanded suture through its stimulatory effect on bone formation. Uysal et al. [3] found positive effect of dietary boron on early phase of bone regeneration during sutural expansion. Uysal et al, [4] who investigated t
he effect of ED-71, a new vitamin D, concluded that it increases bone formation of the expanded suture in rats. Lee et al. [5] showed that injection of bisphosphonate during retention time may produce more secure retention through inhibiting bone resorption by osteoclasts.

Oztürk et al. [6] reported that zoledronic acid, a bisphosphonate, increases the number of osteoblasts and bone formation in the expanding suture and decreases the relapse ratio.

They used computed tomography for evaluating bone density in another study and reported that zoledronic acid increased the density of the bone formed at the expanded suture [7].

Ascorbic acid or vitamin C, as anessential factor in osteogenesis, stimulates procollagen and alkaline phosphatase activity [8].

Vitamin C induces cultured stem cells to differentiate into osteoblasts [9,10] and osteoclasts [11,12]. It promotes synthesis of type I collagen, interaction with integrins, activation of protein kinase pathway and phosphorylation of osteoblast-specific transcription factor [13].

Vitamin C is essential for fracture healing in vivo [14]. It has been found that the collagen matrix produced by ascorbic acid-treated cells provides a permissive environment for tissue-specific gene expression and this is the mechanism by which vitamin C induces pre-osteoblast proliferation [15].

Vitamin C deficiency can induce osteoclastic differentiation and inhibit osteoblastic differentiation of bone marrow cells [14].

It has been shown that vitamin C deficiency during orthodontic treatment reduces tooth movement because of its effect on healing [16-18] and results in complete cessation of osteogenesis [16].

Vitamin C is not synthesized in human body [19], but it is found in some foods. Furthermore, it is commercially available as a nutritional supplement. The present study was designed to evaluate the effect of dietary vitamin C on osteogenesis of expanding midpalatal suture in rats.

MATERIALS AND METHODS

According to the formula for sample size calculation and based on a previous study [16], 48 rats were sufficient for perfoming this study. In this animal study, fifty-four male Wistar rats, six to eight weeks old and weighing 2.5-3.1 kg, were collected and kept in cages at a temperature of 25°C and humidity of 55% under alternate twelve-hour periods of light and dark conditions for seven days to acclimatize with the new conditions.

The animals were divided by blind randomization method into three equal groups according to the time of sacrificing which was selected for the groups at three, nine and 17 days after the beginning of expansion (G1: three days, G2: nine days and G3: 17 days). Each of these groups was randomly divided into control (without vitamin C: -vc) and experimental (with vitamin C: +vc) subgroups (Table 1). Pelleted rat diet (with normal nutritional levels) and water were provided ad libitum and was similar for all groups. In order to achieve proper blood levels of vitamin C (L(+)-ascorbic acid, Acros Organics, Geel, Belgium) in the experimental groups, it was added to the water of the animals seven days prior to starting the expansion at a 10 kg/m3 concentration, [20,21] and was continued until sacrificing the animals.

All the experiments were performed according to the guidelines of the US National Institutes of Health (publication 85-23, revised 1985) and approved by the Ethics Committee in the Research Center of Hamadan University of Medical Sciences.

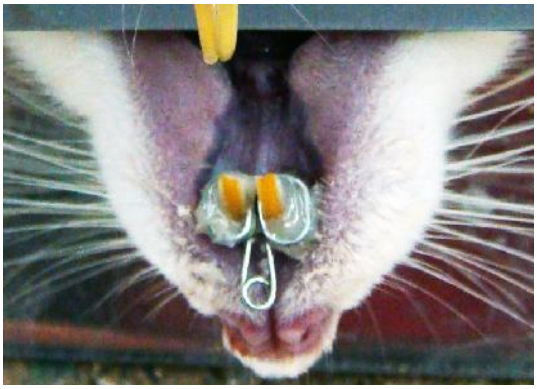

Fig 1. The spring bonded to the maxillary incisors of a rat.

Fig 2. Plane of sectioning.

All the rats were anesthetized with an intramuscular injection of ketamine (Ketamine hydrochloride, Rotex Medica, Trittau, Germany), 50–60 mg/kg of body weight. Sutural expansion was carried out for all the rats with expansion spring made of 0.35 mm stainless steel wire (Dentaurum, Ispringen, Germany). This spring consisted of an 8-mm arm and a 1.5-turn helix with 2.5 mm of diameter. The springs were activated to apply 100 g of force measured with a force gauge. According to previous studies, 100 g of force is optimum for opening the suture in rats [22]. The spring was bonded with a self-etch bonding agent (Transbond Plus, 3M Unitek, Monrovia, CA) and flowable light-cure composite resin (Heliomolar Flow, Ivoclar Vivadent, Liechtenstein) to the maxillary incisors under general anesthesia, with a 3-mm distance from the incisal edges (Figure 1). In groups G1 and G2, the spring remained in place until the animals were sacrificed. In group G3, the springs were removed nine days after bonding in order to evaluate relapse of the expanded suture.

The animals were sacrificed with an overdose of ether and then premaxilla was dissected, placed in a fixative solution (Finefix, Milestone, Fatebenefratelli, Italy) and then in 10% nitric acid for decalcification.

The samples were embedded in blocks; then six transverse sections, 3–5 micron in thickness, were made with a microtome (RM 2135, Leica, Nussloch, Germany) at the premaxillary area, parallel to the incisor axis, at 1 mm distance from the labial surface of the upper incisors (Figure 2). The sections were stained with hematoxylin& eosin. Furthermore, immunohistochemical staining was performed with avidin-biotin method and sandwich technique using osteoblast marker [osteopontin antibody (NCL-O-PONTIN, Novocastra, Newcastle Upon Tyne, United Kingdom)]. Osteopontin is an extracellular glycoprotein, which is secreted by osteoblasts in the early stages of bone formation and it has been reported that mechanical stimuli increase the expression of osteopontin in osteoblasts [23,24]. The best section was selected for each specimen.

Table 1. Groups, subgroups and sacrificing time.

Group	Experimental	Control	Sacrificing time
G1	G1+V_c	G1-V_c	3 days
G2	G2+V_c	G2-V_c	9 days
G3	G3+V_c	G3-V_c	17 days

+V_c:vitamin C group , -V_c: control group

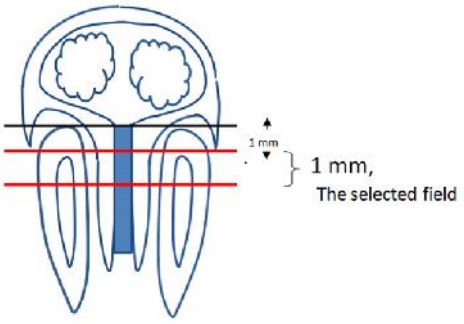

Fig 3. The standard field selected for counting osteoblasts and osteoclasts.

Then, osteoblasts and osteoclasts were counted in a standard field of the midpalatal suture (Figure 3) by an observer blinded to the clinical information, under a light microscope (Olympus BX41, Tokyo, Japan) at ×400 magnification.

In order to prevent missing any cell, all of the cells with the morphology of osteoclasts and all of the immunostained cells with the morphology of osteoblasts were counted in this selected field [6].

Fifteen sections were randomly selected and the cells were recounted after one week to evaluate systematic error. The distance between the mesioincisal points of the upper incisors were measured with Boley gauge (Digimatic caliper, Mitutoyo, Aurora, USA) at the time of spring removal.

Statistical analysis:

Data were analyzed with the statistical package SPSS 13. Wilcoxon test was used for estimating the systematic error.

Two-way ANOVA was used for analyzing the differences in interincisal distance between the groups G1,G2 and G3 and Tukey's test was used as post-hoc test.

Independent t-test was used for evaluating differences between each vitamin C and its matched control group.

Friedman analysis was used for evaluating the differences in osteoblast and osteoclast numbers between the groups G1,G2 and G3. Mann-Whitney-U test was used as post-hoc test and also for evaluating differences between each experimental and its matched control group.

The level of significance for all tests was set at $P<0.05$. Normality of variables was checked with the Kruskal-Wallis test.

RESULTS

Two rats in group G3 died during the second anesthesia at the time of spring removal. There was no evidence of diarrhea or other gastrointestinal symptoms in any of the animals. The body weight of the rats decreased on the third and ninth days but subsequently increased on the 17th day.

Furthermore, there were no significant differences in body weight change between the control and experimental groups ($P>0.05$). The systematic error was not significant ($P=0.317$). Descriptive data of the variables are presented in Table 2.

Two-way ANOVA and Tukey's test revealed that the distance between the incisors, was significantly greater in group G2 than G1, and it was significantly greater in G1 than G3.

Table 2. The mean and standard deviation of interincisal distance (mm), osteoblast number and osteoclast number.

Group	Interincisal distance	Osteoblast	Osteoclast
G1+V_c (n=9)	1.73±0.19	5.44±2.00	0.44±0.52
G1-V_c (n=9)	2.02±0.38	1.55±1.42	0.44±0.52
G2+V_c (n=9)	2.17±0.26	34.40±10.34	9.7±2.83
G2-V_c (n=9)	2.33±0.4	56.50±4.00	11.8±2.61
G3+V_c (n=8)	0.74±0.49	9.25±2.96	4.37±1.06
G3-V_c (n=8)	0.63±0.51	6.62±4.83	4.62±1.76

Fig 4. H &E stained section. Histological features of the suture in group G1: Amorphous tissue of the suture, smooth bony margins and ruptured fibers (asterisk) are evident. Sharpey's fibers near the suture margins (arrows) are aligned in the direction of tension; ×100.

Independent t-test showed that the interincisal distance was not significantly different between the experimental and control subgroups of G1, G2 and G3 (Table 3).

Histological features of the sections:

Microscopic examination of hematoxylin & eosin stained sections revealed some histological changes of the suture in the samples. In group G1, the margins of the suture were smooth with no interdigitation.

Sutural tissue between the bony margins was amorphous and with low cellularity. Hemorrhage was evident in most of the sections and in some of them the fibers of the suture were ruptured as well as the fibers in the PDL of the incisors.

The Sharpey's fibers near the suture margins were aligned in the direction of the tension (Figure 4).

In group G2, bony projections in the form of interdigitations were evident along the suture. These interdigitations were aligned transversely across the suture, corresponding to the direction of tension.

The sutural width (from base of the projections, not tip of them) seemed greater than in group G1 when compared objectively under the microscope (Figure 5a).

The sutural tissue had more fibers and high cellularity with increased cellular activity. There was a cellular rim at the margin of the bony interdigitations, including cuboidal to round cells representative of osteoblasts. In the middle area, sutural tissue consisted of spindle-shaped cells with plumb nuclei, which resembled fibroblasts and mesenchymal cells. In fact, the histological features of the suture at this time were a combination of fibrogenesis and osteogenesis (Figure 5b).

Furthermore, there were some dispersed areas of osteoid matrix at the margins of the suture. Osteoclasts were seen as large cells with one or more nuclei at the margins of the suture and in Howship's lacunae. They were more frequently seen in the lower areas of the suture near the crest of bone between the incisors.

In group G3, a decrease in the width of the suture in most of the areas, compared to the former groups and its tortuous form, were obvious.

Table 3. Comparison of the distance between mesial points of incisal edges (mm). P-values of two-way ANOVA, Tukey's test and independent t-test are presented.

	Main effect of time of sacrificing	**Main effect of vit. C**	**Interaction effect of vit. C and time of sacrificing**
Two-way ANOVA	<0.001 *	0.283	0.329
Tukey's test	G1-G2 0.012*	G1-G3 <0.001*	G2-G3 <0.001*
Independent t-test	G1-v_c - G1+v_c 0.59	G2-v_c - G2+v_c 0.329	G3-v_c - G3+v_c 0 .686

*Statistically significant
Level of significance: P<0.05

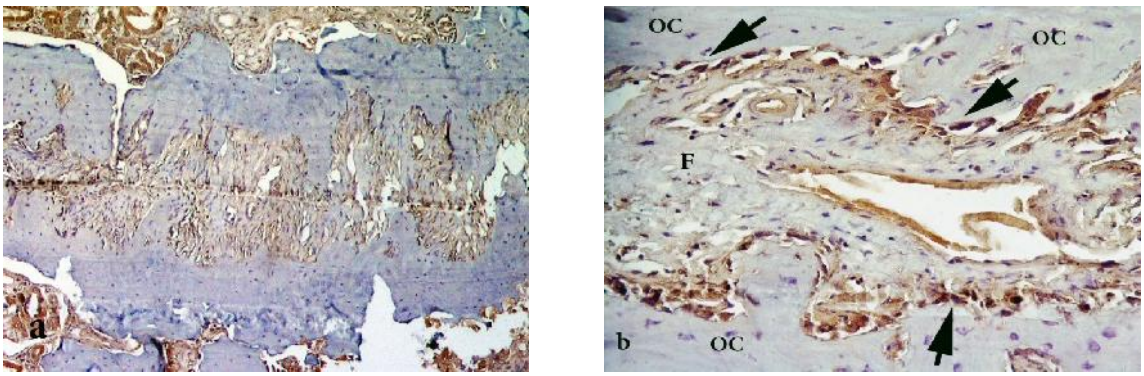

Fig 5. Immunohistochemical sections. Histological features of the suture in group G2. **a**: interdigitation of bony margins; ×100. **b**: High cellularity of the sutural tissue , OC: osteocytes, F: fibroblast and mesenchymal cells, Arrows: osteoblasts in a cellular rim at the suture margin; ×400.

The sutural tissue between the serrated bony margins had low cellularity. There were reduced numbers of cells in the osteoblastic rim, most of which had the morphology of inactive cells.

The histological features indicated some reconstruction of the suture (Figure 6).

Number of osteoclasts:

Friedman test revealed that the number of osteoclasts was significantly different among groups G1, G2 and G3 (P<0.001). Mann-Whitney U test showed that the number of osteoclasts in group G3 was significantly higher than that in G1 and it was significantly higher in group G2 compared to G3(P<0.001).

The number of osteoclasts was not significantly different between the experimental and control samples in each group (Table 4).

Immunohistochemical sections:

For counting the osteoblasts, positively immunostained cells at the margins of the suture were considered as osteoblasts (Fig 5).

Number of osteoblasts:

Friedman test revealed that the number of osteoblasts was significantly different among the groups G1, G2 and G3 (P<0.001).Mann-Whitney U test showed that the number of osteoblasts in group G3 was significantly higher than G1(p=0.001) and it was significantly higher in group G2 compared to G3 (p<0.001). The number of osteoblasts was significantly higher in group G1+vc compared to G1-vc. Furthermore, it was significantly higher in group G2-vc compared to G2+vc, but it was not significantly different between groups G3+vc and G3-vc (Table 4).

Table 4. Comparison of osteoblast number and osteoclast number in the groups according to the results of the Mann Whitney U test.

Group	Osteoclast Number		Osteoblast Number	
	Mean Rank	P value	Mean Rank	P value
G1-v_c	8.69	0.815	5.17	0.000*
G1+v_c	9.28		13.31	
G2-v_c	12.70	0.105	15.40	0.000*
G2+v_c	8.30		5.60	
G3-v_c	9.00	0.721	7.50	0.277
G3+v_c	8.00		10.33	

*Statistically significant
Level of significance: P<0.05

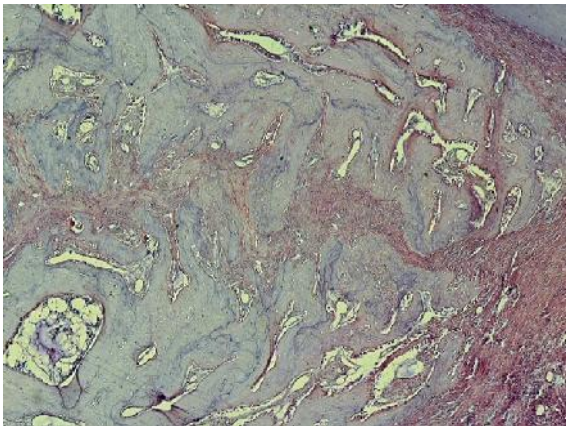

Fig 6. H-&-E stained section. Histological features of the suture in group G3: Low cellularity of sutural tissue; tortuous form and reduced width of the suture can be seen; ×100.

DISCUSSION

Sutural expansion with mechanical force results in a chain of events that lead to bone formation in order to adapt to the increase in width of the suture and maintain its original architecture [22]. Rats and rabbits are ideal animals for investigating sutural changes under stress [3].

Some studies have found that the peak number of cells in the DNA synthesis stage is at one day after the beginning of expansion in rats [22,25,26]. New bone formation initiates three to four days after beginning of the expansion [27] and the new osteoid needs one week for mineralization [28].

After two to three weeks, with reduction and cessation of the expanding force, remodeling of bone occurs [27]. Therefore, the selected intervals for sacrificing the animals in the present study were three, nine and 17 days after bonding the springs.

In the present study, the effect of vitamin C on bone regeneration of rat midpalatal suture was investigated through histomorphometric cell counting method.

The number of osteoblasts is considered a kinetic index of bone formation [29].

According to the histological evaluation, in group G1 the changes were predominantly due to trauma to the suture from the force applied by the spring. The expanded suture experiences hemorrhage, necrosis and wound healing before the onset of bone formation [28]. This may explain why the number of osteoblasts and osteoclasts after three days was significantly less than that after nine and 17 days.

The number of osteoblasts in group G1+vc was significantly greater than in G1-vc. This might be attributed to the positive effect of vitamin C on osteoblast differentiation. Surprisingly, in G2-vc sample, the number of osteoblasts was significantly greater than in G2+vc sample. This may be due to some adverse effects of prolonged intake of vitamin C shown in some studies.

It has been reported that vitamin C can inhibit cell death or cause apoptosis depending on the culture medium used [30]. It has been found that high concentrations of ascorbic acid in culture medium are cytotoxic [31]. Ascorbic acid can act as an antioxidant or as a prooxidant [32,33].

On the other hand, it has been reported that vitamin C supplements (above the normal level) in guinea pigs result in copper deficiency [34] and this might lead to decreased osteogenesisand bone deformities [35,36].

Van den Berg et al. [20] found that long-term vitamin C feeding lowers copper absorption in rats but short-term intake does not have this effect. Furthermore, vitamin C enhances iron absorption [21,37] and iron overload may cause osteoporosis [26] through its inhibitory effect on osteoblastmaturation and function [38]. Therefore, in group G2, prolonged intake of vitamin C may have influenced these essential elements. But, in the initial stages of applying tension forces (group G1), the presence of vitamin C might have resulted in more osteoblast differentiation. Therefore it seems that for the positive effect of vitamin C on osteoblasts in rats, we may need to lower its dose or shorten the period of intake. It must be kept in mind that, in contrast to animals, vitamin C does not affect copper or iron metabolism in human beings [39].

According to the results, the amount of movement of the incisors in group G1 was significantly less than that in group G2. The movement of the teeth continued between the third and ninth days of experiment because the spring was still active in this period. In the current study, vitamin C had no effect on the amount of incisor movement as expansion indicator. This may be attributed to the time needed for osteoid formation and mineralization [27,28]. In G3 samples after removal of the springs, the incisors moved toward each other and the interincisal distance decreased regardless of vitamin C intake. In histological examination, the suture had tortuous form. The number of osteoblasts and osteoclasts decreased significantly due to the pressure force of maxillary halves. Regarding the clinical relapse of expansion, it seems that remodeling of the expanded suture is taking place toward resorption of new bone. Storey [40] found that after removal of the helical expansion springs, the bones moved together rapidly and after two or three weeks the suture was reconstructed. Histological examinations have revealed that the new bone formed within the suture could slow down the process of relapse while maintaining the bones apart[40]. However, in quickly expanded premaxilla, it is not uncommon to see rapid resorption of this newly formed bone [40].

The number of osteoblasts and osteoclasts was significantly greater in group G2– when bone formation was higher – compared to groups G1 and G3 because bone formation and resorption are coupled phenomena [41]. Although vitamin C deficiency increases bone resorption and osteoclast differentiation [18], in the present study additional vitamin C did not affect the number of osteoclasts. Therefore, it seems that vitamin C consumption had no significant effect on osteoclasts.

CONCLUSION

Vitamin C had an initial stimulatory effect on osteoblast differentiation during expansion of the suture in rats, but it was later followed by an adverse effect on osteoblasts, which might be attributed to the effect on metabolism of essential elements. It was concluded that vitamin C did not have a positive effect on bone formation and retention of midpalatal suture after expansion in rats. Considering the difference of vitamin C metabolism between animals and humans, similar studies are suggested on human beings.

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