Thymoquinone accelerates new bone formation in the rapid maxillary expansion procedure

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ABSTRACT

Objective: The aim of this study was to evaluate the effect of systemic thymoquinone (TQ) in a rat rapid maxillary expansion (RME) study.

Design: Thirty-two Wistar albino rats were divided into 4 equal groups: only-expansion (OE), expansion plus TQ (TQ1 group, TQ given to the rats during their nursery phase and during the expansion and retention period), expansion plus TQ (TQ2 group, TQ given to the rats only during the retention period), and control group (no procedure done). Expansion appliances were placed on the maxillary incisors of all animals for 5 days. The appliance was deactivated during the 12 day retention period. The rats were sacrificed at the end of the retention period. Histomorphometric evaluation was carried out in order to compare the number of osteoclasts, osteoblasts, and capillaries, as well as the intensities of inflammatory cells, and new bone formation amongst the groups.

Results: New bone formation, number of capillaries and the ratio of intensities of inflammatory cells in maxillary sutures was higher in the TQ groups than in the other groups. Statistical analysis also demonstrated that osteoblast and osteoclast numbers were also highest in the TQ1 group.

Conclusion: Histomorphometric analysis demonstrated that systemic use of thymoquinone may be effective in accelerating new bone formation in the RME procedure and that TQ may be beneficial in preventing relapse following the RME procedure.

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1. Introduction

Rapid maxillary expansion is a common procedure in orthodontic practice which is used when orthopaedic widening is necessary due to maxillary transverse deficiency which can occur in a variety of malocclusions. Rapid maxillary expansion can exert significant force on the mid-palatal suture, which results in the stretching of collagenous fibres, accompanied by new bone formation with associated mitotic figures.1

Although this technique offers solutions for the treatment of several abnormalities such as transverse maxillary deficiency, pseudo-Class III malocclusion, rhinologic and respiratory ailments, and cleft lip and palate, there are some drawbacks to the method including external root resorption,
microfractures at the mid-palatal suture, and microtrauma of the temporomandibular joint and post-expansion relapse.\(^2\,^3\)

Of all of these potential drawbacks, relapse is one of the most challenging issues involving the RME procedure. Although, the actual causes of relapse are not fully understood, there are likely many factors involved. Regulation of bone metabolism, stresses accumulated between the articulations of the craniofacial complex, and tension produced in the palatal mucosal and supracrestal fibres were amongst some of the causes suggested by researchers.\(^2\,^3\) Additionally overcorrection, scar tissue contraction, age, local and systemic status of patient, devices type, length of retention period can also effect relapse.\(^3\,^5\) Authors\(^2\,^3\) also stated that the velocity and the quality of bone formation in the intermaxillary suture may influence early post-expansion relapse.\(^6\)

Mechanical forces are lead to sutural expansion, which is accomplished by stretching the collagenous fibres accompanied by new bone regeneration in company with mitotic figures. In post-expansion period, the suture undergoes remodelling through bone formation and resorption, and fibre rearrangement. This continues until the architectural environment achieves equilibrium.\(^1\)

The expanded maxillary arch width relapses unless followed by a long retention period. Therefore, it is crucial to describe the mechanism of active bone regeneration in the mid-palatal suture in response to expansion, which will lead to understand the causes of relapse and develop more effective expansion methods.\(^7\)

Therefore, accelerating and enhancing the growth of new bone in the intermaxillary suture after expansion may help to prevent relapse of arch width and to shorten the retention period. Because of this, various approaches were reviewed that might hold potential for the acceleration of new bone formation in the expanding suture including low-power laser therapy,\(^8\) transforming growth factor,\(^9\) vitamins\(^1\) and antioxidant.\(^1\) Recently, antioxidant therapies have been widely investigated by researchers due to their effect on bone metabolism. These methods are especially promising since they inhibit osteoclastic activity and promote osteoblastic activity.\(^7\,^10\)

Thymoquinone (2-methyl-5-isopropyl-1,4-benzoquinone, TQ), is the bioactive constituent of the volatile oil of black seed (Nigella sativa) which has been used for centuries to treat a variety of diseases such as rheumatoid pain, hypertension, asthma, cough, and bronchitis.\(^11\) TQ has a variety of pharmacological properties which include antithistamine, antibacterial, antihypertensive, hypoglycemic, anti-inflammatory, and immunopotentiating actions. TQ has also been shown to have a potent anti-oxidant effect which is considered to be one of its most important properties. TQ induces an antioxidant response through its potent superoxide scavenging ability.\(^12\)

Superoxides play a role as intermediate molecules in the activation of osteoclasts which is especially important in the area of bone resorption (ROS).\(^13\) TQ decreases the ROS production and the level of pro-inflammatory cytokines such as IL-1α and -6 and TNF-α. These cytokines can lead to differentiation of osteoclast precursors and osteoclast activity which then causes bone resorption.\(^14\) In light of these potent anti-oxidant properties, TQ may prove to have an important role in the acceleration of bone formation and in shortening of the retention period involved in RME. We expect the study of the effects of TQ on RME to provide additional new insights into strategies for preventing relapse after RME treatment.

2. Material and methods

An animal cohort comprised of 12 week-old adult male Wistar albino rats, weighing 200 g (± 10 g) was provided by the Animal Laboratory at Cumhuriyet University Faculty of Medicine. The animals were restricted to plastic cages under artificial lighting from fluorescent lamps, with a 12-h light photoperiod and a 12-h dark photoperiod. The room temperature was maintained at 25°C and food and water were provided ad libitum. All procedures were approved by the Animal Ethics Committee at Cumhuriyet University.

Thirty-two rats were randomly divided into four groups: only-expansion (OE) group (\(n = 8\)), expansion plus TQ (TQ1) group (TQ was given to the rats 40 days before the procedure and additionally during the expansion and retention periods), expansion plus TQ (TQ2) group (TQ was given to the rats during the 12 days retention period only), and no-expansion (NE) group (the control group which received no procedure). In the TQ groups, TQ was administered systemically through orogastric way, at a rate of 10 mg/kg/day.

The rats were anesthetized using a ketamine xylazine (Rompun, Bayer, Leverkusen, Germany, 3 mg/kg) and ketamine (Ketalar, Pfizer, USA, 90 mg/kg) combination. Expansion appliances were then affixed to the maxillary incisors of all animals. A helical spring fabricated from a 0.012-inch piece of stainless-steel wire was used to perform the expansion of the premaxillary suture. The springs were placed on a grid and activated using pliers. The spring force was measured with a gauge and found to be 30 g. To obtain retention, a stainless-steel disc was used to prepare a groove at the level of the gingival papilla on the distal sides of the incisor teeth. Then a 0.009-inch stainless-steel ligature wire was used to fix the spring.

The rats were observed for signs of weight loss, infection or appliance failure throughout the duration of the study. In the event of the appearance of infection, rapid decrease in their body weight, or appliance failure the animals were excluded from study and renewed.

After the 5-day expansion period was complete (Fig. 1), the TQ1, TQ2 and EO groups underwent 12 days of mechanical retention. At the end of the retention period the animals were sacrificed under general anaesthesia using 200 mg/kg sodium pentobarbital (Pentothal; Abbott, North Chicago, IL, USA) and their pre-maxillae were dissected and fixed in a 10% neutral buffered formalin solution at room temperature for 24–48 h. After fixation, the springs were removed and the samples were de-mineralized in an aqueous 10% formic acid solution. These specimens were then dehydrated and embedded in paraffin. The paraffin blocks were sectioned (5 μm thick) and prepared for Haematoxylin and Eosin staining.

Histomorphometric evaluation was carried out by a single examiner in a blind study and the results were taken as an average of the counts. Three histological sections from each animal were analysed. The study and control groups were
compared in order to establish the number of osteoclasts, osteoblasts, and capillaries as well as the number and intensities of inflammatory cells, and new bone formation. The intensities were rated as mild (+), moderate (++) or strong (+++).

2.1. Statistical analysis

Statistical analysis was performed using a commercially available software program (SPSS 14.0, SPSS Inc., Chicago, IL, USA). Differences amongst the four groups with regard to the number of osteoclasts and the number of osteoblasts were evaluated using the Kruskal–Wallis test and pair-wise comparisons were made by the Mann–Whitney U test. A value of p less than .05 was considered statistically significant. The results were presented as the mean ± standard error of mean.

3. Results

Serious weight loss was not encountered in members of either the treated or the control groups. Neither was there any infection noted in any group during the study. Deep mucosal infection, dehiscences, or other adverse effects were not observed in any animals. However, three animals were excluded from study due to appliance failure. They were replaced with three other rats. The inter-maxillary suture was successfully expanded following application of the activated helical spring.

3.1. Histological findings

3.1.1. Number of osteoblast

The histologic findings revealed that the number of osteoblasts was significantly higher amongst the TQ1 group than the other groups (p < 0.05). No significantly differences were found between the TQ2 and OE groups. Whilst the lowest number of osteoblastic cells was found in NE group (Tables 1 and 2 and Fig. 2).

3.1.2. Number of osteoclast

Osteoclast cells were found in all four groups. Table 2 gives the number of osteoclasts present in the groups. Osteoclast numbers for TQ1 group were significantly higher than those found in the other groups (p < 0.05). No significant differences were found in the remaining three groups (Tables 1 and 2).

3.1.3. New bone formation

When the groups were compared for new bone formation, considerable differences were found amongst members of the TQ1 group compared to all other groups. The results showed that there was an increase in new bone formation in the TQ1 group that was significantly greater than in the other groups. The TQ2 group also showing increased growth which exceeded that of the remaining groups (Table 3 and Fig. 2).

3.1.4. Number of capillaries

The TQ1 group also showed a similar increase in capillaries relative to the other groups. It was also found that the number of capillaries was higher in the TQ2 group than in the EO and NE groups (Table 3 and Fig. 2).

3.1.5. Intensities of inflammatory cells

Intensities of inflammatory cells in the TQ1 group were significantly higher than those found in the TQ2, OE and NE groups, respectively. Increased intensities of inflammatory

Table 1 – Effects of thymoquinone on the number of osteoclasts and osteoblasts at the end of the 12th day of experimental period.

<table>
<thead>
<tr>
<th></th>
<th>OE (x ± SD)</th>
<th>TQ1 (x ± SD)</th>
<th>TQ2 (x ± SD)</th>
<th>Control (x ± SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Num. of OC</td>
<td>1.28 ± 0.48</td>
<td>2.71 ± 0.48</td>
<td>1.57 ± 0.53</td>
<td>1.00 ± 0.57</td>
<td>0.001*</td>
</tr>
<tr>
<td>Num. of OB</td>
<td>10.1 ± 4.67</td>
<td>19.14 ± 5.04</td>
<td>11.0 ± 2.88</td>
<td>4.42 ± 0.53</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

* Kruskal–Wallis variant analysis, p < 0.01.

Table 2 – Pair-wise comparisons of the groups.

<table>
<thead>
<tr>
<th></th>
<th>Group OE-TQ1</th>
<th>Group OE-TQ2</th>
<th>Group OE-control</th>
<th>Group TQ1-TQ2</th>
<th>Group TQ1-control</th>
<th>Group TQ2-control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Num. of OC</td>
<td>p:0.002**</td>
<td>p:0.298</td>
<td>p:0.334</td>
<td>p:0.005**</td>
<td>p:0.002**</td>
<td>p:0.081</td>
</tr>
<tr>
<td>Num. of OB</td>
<td>p:0.011*</td>
<td>p:0.898</td>
<td>p:0.003**</td>
<td>p:0.007**</td>
<td>p:0.007**</td>
<td>p:0.001**</td>
</tr>
</tbody>
</table>

* Mann–Whitney-U test, p < 0.05.
** Mann–Whitney-U test, p < 0.01.

cells were also found in the TQ2 group relative to the NE and EO groups (Table 3).

4. Discussion

Relapse is one of the important problems clinicians have to cope with in the RME procedure. Although the precise reasons for relapse are not yet known, researchers have been examining the effects of the velocity and the quality of bone formation in the intermaxillary suture as they relate to the problem. Thus, numerous approaches have been investigated for hastening new bone formation. Sawada and Shimizu evaluated the local effects of transforming growth factor-beta 1 (TGF-beta 1) in a rat RME study and suggested that TGF-beta 1 may play an important role in bone formation at the active site of the suture in response to RME. They noted that the application of TGF-beta 1 during the early stages of RME may accelerate bone formation. Ozturk et al. examined the radiographical (computed tomography) changes associated with experimental sagittal suture expansion in rats responding to zoledronic acid, and demonstrated that the systemic administration of zoledronic acid stimulated bone formation and decreased the relapse ratio after expansion in the rat sagittal suture. Saito and Shimizu aimed to test the effectiveness of low-power laser irradiation on bone regeneration during expansion of the midpalatal suture in rats. The results of the histomorphometric evaluation, confirmed that laser therapy accelerated bone regeneration in this case, thus it may have a potential therapeutic benefit with regard to preventing relapse and decreasing the retention period in RME. The aim of this study was to investigate the potential use of TQ for accelerating the formation of bone in the mid-palatal suture in RME thus minimalizing the risk of relapse following the procedure.

Finally, numerous researchers and studies have demonstrated that there is a correlation between oxidative stress and bone metabolism. Oxidative stress caused by excessive generation of intracellular reactive oxygen species (ROS) can exert adverse biological effects on bone through inhibiting bone cell differentiation in the preosteoblastic cell line and in the marrow of the stromal cell line. ROS can also directly promote osteoclast formation and activity. Increasing

| Table 3 – Effects of thymoquinone on number of capillaries, intensities of inflammatory cell infiltration and new bone formation. |
|-----------------|--------|--------|--------|--------|
|                 | OE     | TQ1    | TQ2    | Control |
| Num. of cap.    | +      | +++    | ++     | +/+    |
| Inflam. cell infil. | +      | +++    | ++     | +      |
| New bone form.  | +      | +++    | ++     | -      |

Fig. 2 – H&E staining photomicrographs from groups. Increased osteoblastic activity, new bone and capillary formation observed in TQ1. A: OE, B: TQ1, C: TQ2 and D: Control. Ob: osteoblast, Oc: osteoclast, Nb: new bone and Cp: capillary. 400× (original magnification).
the intracellular level of ROS can play a crucial role for the up-regulating of NF-kappaB (nuclear factor-kappaB) ligand (RANKL) which is essential for the formation, as well as the survival and resorbing activity of osteoclasts.\textsuperscript{15} Upregulation of RANKL can also lead to osteoclast differentiation induced by osteoclastogenic factors including IL-1, TNF-a, and LPS.\textsuperscript{17} Furthermore, ROS and TNF-a also suppress osteoblastic differentiation.\textsuperscript{18} Thus, considering the detrimental effect of oxidants, various host-modulatory agents such as antioxidants have been widely investigated for their ability to cope with the oxidant-related breakdown of hard tissues and for their possible role in the promotion of bone healing.

Antioxidant agents have been commonly used as food additives and may play an important role in the treatment of many degenerative and chronic diseases such as atherosclerosis, brain dysfunction, immune system decline, metabolic bone diseases, and cancer.\textsuperscript{19,20} It has also been demonstrated that antioxidants may accelerate new bone formation. For this reason, antioxidant agents continue to generate interest particularly in regard to their effect on new bone formation. Uysal et al.\textsuperscript{8} assessed the effects of dietary boron, which is an antioxidant agent, on bone formation in response to expansion of the interproximal suture during different retention periods in rabbits. Their work demonstrated that dietary boron is effective for enhancing bone growth in the early phase of bone regeneration in the maxillary expansion procedure. As an antioxidant, N. sativa and its main constituent, thymoquinone have been widely investigated due to their rich and diverse chemical composition. N. sativa has been used for many centuries in folk medicine for the promotion of good health and for the treatment of many acute, as well as, chronic conditions. Several beneficial pharmacological effects have been attributed to TQ including anti-oxidant, anti-inflammatory, anti-microbial, anti-tumour, immunomodulatory, bronchodilation, hypotensive, anti-diabetic, hepatoprotective, gastroprotective, anti-histamine, and neuroprotective effects. Amongst these, the antioxidant effect is the most prominent.\textsuperscript{21-23}

The antioxidant response of TQ originates from its ability to scavenge free radicals; its scavenging power is as effective as that of superoxide dismutase in its ability to neutralize superoxide anions.\textsuperscript{12} It has been reported by Tekeoglu et al.\textsuperscript{22} that TQ is effective for decreasing serum levels of TNF-alpha and IL-1 beta. El-Mahmoudy et al.\textsuperscript{24} have demonstrated that TQ suppresses the production of nitric oxide (NO) from lipopolysaccharide-induced peritoneal macrophages in normal rats. TQ can also effectively normalize the levels of lipid peroxide, lactate dehydrogenase, glutathione, and superoxide dismutase.\textsuperscript{25}

Kirui et al.\textsuperscript{26} examined the physiological responses associated with sustained delivery of TQ in the femoral defect animal model (bone healing). According to their results, TQ can enhance bone healing with little or no side effects involving major vital and reproductive organs noted. In this study we evaluated the effect of TQ on accelerate bone formation and in shortening the retention period in a rat RME model. Our results indicate that increased new bone formation and higher osteoblast numbers were encountered in the TQ groups relative to the control. This corroborates the results of a previous study done by Kirui et al.\textsuperscript{26} We found that the osteoclast number was also higher in the TQ groups relative to the others. This elevation of the osteoclast number is most likely related to the acceleration of bone turnover. These histological results demonstrated that systemic administration of 10 mg/kg of TQ can promote bone formation and may be effective in the prevention of relapse following the RME procedure.

According the literature feeding antioxidant-rich diet per day prior to and after surgery would benefit adult patients. Gavino et al.\textsuperscript{27} was aimed to investigate the influence of dietary antioxidants on lipid peroxidation and liver regeneration in partially-hepatectomized rats. Rats were fed for 6 weeks with the antioxidant-rich diet before the surgery. In conclusion, it was demonstrated that elevated levels of dietary antioxidants can be beneficial in terms of reduced lipid peroxidation and increased rates of liver regeneration following liver surgery. Uysal et al.\textsuperscript{8} started the administration of antioxidant (boron) to the rats 40-days before the operation and suggested that boron had a positive effect on the early phase of bone regeneration. We began giving TQ before 40 days prior to the operation as recommended by Uysal et al.\textsuperscript{8} in TQ1 group. The results indicated that the TQ1 group showed a significant increase in the acceleration of bone formation relative to the other groups including the group for which TQ was given only during the retention period.

The rat model of maxillary expansion is a well-established model for RME. This model was also used for the evaluation of bone regeneration in other animals including cats, monkeys and rabbits. Although the maxillary sutures of cats and monkeys more closely resemble those of humans, rats and the rabbits are considered to be suitable for animal models designed to achieve a clear picture of bony and sutural changes under stress.\textsuperscript{6}

Some authors have measured the distance between the mesial corners of the maxillary incisors at the beginning and on the fifth-day of the expansion with a calliper.\textsuperscript{3} Because of it was clearly defined that 5 day-expansion period enough for sutural expansion,\textsuperscript{1,3,8} distance between maxillary incisors was not measured in our study. But the required space between the incisor teeth of all rats was confirmed by researchers. Additionally it was also confirmed by a histologist during histomorphometric evaluation that required amount of expansion was done at palatal sutures of all animals.

Because of we aimed the evaluate effect of TQ in the acceleration of bone formation and in shortening of the retention period in RME procedure, histological analyses limited to the pre-maxillary suture area in this study. Besides this, generalized effects of TQ on bone metabolism and differences in external root resorption between the groups would be very interesting. So, further studies can be designed.

The preferred dosage of TQ in these studies varies amongst researchers. The most frequently used doses of TQ for experiments involving the assessment antioxidant, anticancer, anti-inflammatory, and cytoprotective effects, vary from 5 to 12.5 mg/kg. No significant adverse effects have been noted at these levels.\textsuperscript{28} No adverse reactions have been encountered with the use of TQ except in children receiving high doses.

(80 mg/kg). The dose used in the current study was 10 mg/kg TQ administered oro-gastrically.

According to Al-Ali et al.’s report, in the case of orally administered TQ, LD50 levels were 10 times higher than they were when the drug was administered intraperitoneally, in both mice and rats. It was also suggested that oral administration of TQ in experimental animals is considered to be relatively safe.

5. Conclusion

The results of this study have demonstrated the effectiveness of using systemic TQ for increasing new bone formation in the RME procedure in rats. Therefore, systemic administration of TQ may be effective in the prevention of relapse following the RME procedure.

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None.

Competing interest

None declared.

Ethical approval

Animal Ethics Committee at Cumhuriyet University, B.30.2.CUM.01.00.00-50/18.

References
