Investigation of the effects of quercetin and xenograft on the healing of bone defects: An experimental study

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ABSTRACT

Objective: In this study, it was aimed to histologic and immunohistochemical examined that the effects of quercetin on new bone formation and bone regeneration in critical size rat tibial bone defects.

Material & methods: In the study, 56 rats were divided into 4 groups with 14 rats in each group. Control (C) (n = 14): A defect was created in the cortico cancellous bone in the metaphyseal part of the tibia bones of the rats and no additional procedure was applied until the end of the experimental setup. Xenograft (X) group (n = 14): Bone defects were created in the tibia bones of the rats and the defects were filled with xenograft. No additional process was applied until the end of the experimental setup. Quercetin (Q) group (n = 14): A defect was created in the tibia bones of the rats and 0.1 mg/kg quercetin was administered by oral gavage until the end of the experimental setup. Quercetin and Xenograft (Q + X) group (n = 14): A defect was created in the cortico cancellous bone in the metaphyseal part of the tibia bones of the rats and the defect was filled with xenograft.

1. Introduction

The defects in oral and maxillofacial area due to congenital defects, trauma, bone loss due to advanced periodontitis, tumour, could be treated with reconstructive surgery. While small bone defects heal spontaneously, it may be necessary to use various biomaterials to treat large defects. Studies have been conducted to ensure success in the incorporation of these biomaterials into the bone structure.1

In literature to accelerate bone regeneration, agents such as antioxidants, bisphosphonates, hormone and other bone regenerative materials applications have been shown to play a significant part.2-4 In vitro and in vivo studies have shown that a plant-derived polyphenol quercetin has a wide range of biological activities, including antineoplastic, anti-inflammatory and antiviral activity, as well as reducing lipid peroxidation, platelet aggregation and capillary permeability.4-9

Quercetin is widely in of vegetable origin (fruit, seed, root, flower, tea and wine). The main property of quercetin, which is the positive effects on wound healing, is its antioxidant capacity. This antioxidant capacity is due to a series of structural properties that allow transition metals such as Fe, Cu or Zn to sequence their ions and catalyse electron transport.10 Recent studies show that it has positive effects on osteoblast and osteoclast activities.3,4,7

While there are many studies in the literature on the anti-inflammatory, antioxidant and antiangiogenic effects of quercetin, few studies have investigated its effects on the bone.7,9-11 Pang et al.

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1. Introduction

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aimed to study the effect of quercetin on the differentiation and proliferation of bone marrow mesenchymal stem cells in mice. The results showed that quercetin significantly increased bone marrow mesenchymal stem cell proliferation, alkaline phosphatase activity, extracellular matrix production and mineralization. In addition, they found that quercetin increased the osteogenic differentiation of mesenchymal stem cells.7-11

The aim of this study is to examine the effects of quercetin on bone regeneration performed with or without xenograft in experimental critical size defects created in rat tibia by histological and immunohistochemical methods.

2. Materials and methods

2.1. Animals and experimental design

This study was carried out in Dicle University Sebahattin Payzın Experimental Animal Production and Research Laboratory, Diyarbakır, Turkey with permission obtained from Dicle University Experimental Research Institute Experimental Animals Ethics Committee (2018/15 protocol number, 28.06.2018). The recommendations of the Helsinki Declaration regarding the protection of laboratory research animals and Arrive guide were followed. The number of animals required for the experiments was determined by power analysis; 8% deviation, type 1 error (α) 0.05 and type 2 error (β) (Power = 0.80), and for experiments in which the animals were divided into groups, at least 7 animals were required in each group.

In the study, 56 Sprague-Dawley adult male rats weighing an average of 300 g ± 50 g were used. The rats were kept at a temperature of 21 ± 10 °C, relative humidity of 40-60%, throughout the experiment. Experimental animals were fed standard rat chow containing 21% protein and water. Subjects were randomly divided into four groups.

Control group (C): A bone defect was created in the tibia. Subjects in this group were injected intraperitoneally with 0.1 mg/kg saline solution.

Xenograft group (X): A bone defect was created in the tibia, and a xenograft was applied to the prepared defect.

Quercetin group (Q): A bone defect was created in the tibia. Rats in this group were given 0.1 mg/kg of quercetin by gavage after the operation until the day of sacrifice.

Quercetin and xenograft group (Q + X): A bone defect was created in the tibia, and only xenograft was applied to the prepared defect. After the operation, 0.1 mg/kg of quercetin was given by gavage until the day of sacrifice.

Seven animals from each group were sacrificed at the end of the fourth week and the other seven animals from each group at the end of the eighth week. The removed tissues were stored in a fixative solution and examined histopathologically.

2.2. Surgical method

General anesthesia was provided to the subjects by intraperitoneal injection of 70 mg/kg ketamine (Ketalar, 500 mg enjectable 1 facon, Turkey) and 13 mg/kg xylazine (Xylazinbeho, Rometar 20 mg/ml, Turkey). The tibia of the rats was shaved, wiped with antibacterial povidone-iodine solution and made ready for the surgical procedure. Subcutaneous tibial crest incision was made. The outer surface of the tibia was reached by removing the soft tissues with blunt dissection and lifting the periosteum. In order to create the bone defects, first the initial drill with a diameter of 1.8 mm, then an intermediate drill with a diameter of 2.5 mm and a final drill with a diameter of 4 mm were used under sterile saline perfusion. Post-operative 10 mg/kg ceftazolin (Cefoxin 1 gr intramuscular flacon) was administered intramuscularly (i.m.) for infection control. The rats were taken to the recovery room in metal cages. In the postoperative period, 10 mg/kg of ceftazolin and 1 mg/kg tramadol hydrochlorure (Contramal Ampul 100 mg) was administered (i.m.) once a day for the first five days after surgery to the subjects, who were fed orally.

2.3. Histochemical and immunohistochemical staining protocol

At the end of the fourth and eighth weeks, the rats were euthanized by cervical blockage with high-dose anesthesia. For histopathological analysis, the tibial bone was resected and fixed with a zinc–formalin solution (catalogue no. Z2902, Sigma-Aldrich, St. Louis, MO, USA).

2.4. Hematoxylin eosin staining

Tibiae kept in zinc–formalin were exposed to tap water for 2–3 h. Tissues were kept for 24 h in 70%, 80%, 90% and absolute alcohol series. The tissues were then taken into xylol solution for 2 × 30 min to remove alcohol, and paraffin incubation was performed. Tissues were embedded in paraffin blocks, after which sections of 4–6 μm thickness were cut with a microtome (catalogue no. Leica RM2265, Wetzlar, Germany). The sections were placed in an oven set at 60 °C and kept there for 3–4 h. Then, they were passed through xylol for 2 × 30 min and thereafter in turn through 96%, 90% and 70% alcohol series for 10 min. After passing through distilled water for 2 × 5 minutes, the sections were kept in hematoxylin staining for 8 min. They were then washed under running water for 5 min. After removing excess water from the sections, they were kept in eosin dye for 5 min. The sections were passed through increasing alcohol series and kept in xylene for 2 × 30 min. For histoch

2.5. Immunohistochemical staining

Tibiae kept in zinc–formalin were exposed to tap water for 2–3 h. Tissues were kept for 24 h in 70%, 80%, 90% and absolute alcohol series. They were then placed into a xylol solution for 2 × 30 min to remove alcohol, after which they were incubated in paraffin. The tissues were embedded in paraffin blocks, then sections of 4–6 μm thickness were cut with a microtome (catalogue no. Leica RM2265, Wetzlar, Germany). The sections were placed in an oven set at 60 °C and kept there for 3–4 h. Then, they were passed through xylol for 2 × 30 min and in turn through 96%, 90% and 70% alcohol series for 10 min. Sections were kept in a phosphate-buffer saline (PBS) for 3 × 5 minutes and then sequenced in an immunohistochemistry bar. Hot water was added to ensure the humidity of the bar, and operations were carried out in a humid environment. The sections were dropped into hydrogen peroxide solution and then incubated for 20 min. Then, the sections were washed with PBS for 3 × 5 minutes and kept in Ultra V Block (catalogue no. TA-015-UB, Thermo Fischer, Fremont, CA, USA) solution for 7 min. Primary antibodies prepared with a dilution were osteopontin (OPN) (catalogue no: sc-21742, Santa Cruz, Biotechnology, Dallas, USA) and secreted protein acidic and cysteine-rich (SPARC) (catalogue no: sc-25574, Santa Cruz, Biotechnology, Dallas, USA) dropped on to the washed sections. Then, streptavidin-peroxidase (catalogue no. TS-015-HR, Thermo Fischer, Fremont, CA, USA) was dropped on to the washed sections and the reaction was monitored under a microscope and stopped with PBS. After counterstaining with Harris hematoxylin, the sections were closed with Entellan®, evaluated and visualized under the Zeiss Imager A2 light microscope.
2.6. Statistical method

The data obtained in this study were at a 95% confidence level and analysed with the SPSS 22 package (IBM). When investigating the status of the variables coming from the normal distribution, the results of the normality test were examined using the Kolmogorov–Smirnov or Shapiro–Wilk test according to the unit numbers. In interpreting the results, 0.05 was set as the significance level; if p < 0.05, the variables did not come from the normal distribution. In examining the differences between groups, if the variables did not come from the normal distribution, the Kruskal–Wallis H test was used. In case of significant difference, the groups with significant differences were determined with the help of post-hoc tests. The Wilcoxon test between two dependent variables was used if the variables did not come from the normal distribution. The significance level was set at p = 0.05.

3. Results

3.1. Histopathological findings

As can be seen in Supplemental Tables 1,2,3; in control group a few bone regeneration; bone trabeculae and low bone marrow formation compared with the treatment groups; Q, X, and Q and X groups (P < 0.05) (Fig. 1A). In Group Q, bone trabecula formation in the defect of the tibial bone detected higher (P < 0.05) compared to Group C, but bone marrow formation was found to be at similar levels (Fig. 1B). In the Q + X group, an increased level of bone formation was detected compared to controls, and bone marrow formation was observed higher when compared with the control group (P < 0.05) (Fig. 1C), (Fig. 1D). In the eighth week in controls, bone regeneration was increased compared with the fourth week (Fig. 1E). In Group Q, trabeculat bone formation was higher compared to Group C (P < 0.05) (Fig. 1F). In Group Q + X and Q new bone trabecula level detected highly when compared with group control (P < 0.05) (Fig. 1G) (Fig. 1H). There was no difference between the other groups in terms of bone healing parameters (P > 0.05).

3.2. Immunohistochemical findings

As seen in Supplemental Table 4; at the fourth week a low level of osteonectin immunoreaction was observed in the newly formed bone trabeculae in controls (P < 0.05) (Fig. 2A). Osteonectin immunoreactivity detected lower in Group Q when compared the controls but not statistically (P > 0.05) (Fig. 2B). In Group Q + X and Q osteonectin detected higher compared to Group C, but not statistically (P > 0.05) (Fig. 2C). The osteonectin level in the in Group X was not found to be statistically significant different when compared the other groups (P < 0.05) (Fig. 2D and E). Although Group Q had a low level of osteopontin level compared the with the controls (P < 0.05)(Fig. 2F). While a significant increase in immunoreactivity was observed in Group Q + X compared to Group C (P < 0.05), the increase was not statistically significant when compared to Group Q (P > 0.05) (Fig. 2G). The osteopontin level in Group X was significantly increased compared to Group C (P < 0.05), but there was no statistically significant difference compared with the other groups (P > 0.05) (Fig. 2H).

As seen in Supplemental Table 5; in the eighth week in controls, a low level of osteonectin immunoreaction was observed in the newly formed bone trabeculae (P < 0.05) (Fig. 3A). In Group Q, the immune reactivity of osteonectin was moderate, and a statistically significant increase was found compared to controls (P < 0.05) (Fig. 3B). Group Q + X exhibited a non-significant increase in immunoreactivity compared to controls (P > 0.05) and a significant increase in immunoreactivity compared with Group Q (P < 0.05) (Fig. 3C). While the osteonectin immunoreactivity was similar in Groups Control and Q + X, the increase in immune reactivity in Group Q was statistically significant (P < 0.05) (Fig. 3D). When the tibia sections belonging to controls were examined at the eighth week, a low level of osteopontin immune reaction was observed in the newly formed bone area (P < 0.05) (Fig. 3E). In the Q group, osteopontin immune reactivity exhibiting a statistically significant increase compared to the Group Control (P < 0.05) (Fig. 3F). While a non-significant increase in immunoreactivity was observed in the Q + X group compared to the Group Control (P > 0.05), the increase in the immunoreactivity of Q was statistically significant when compared with

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**Fig. 1.** Defect area of the A) Control (Bar scale: 50 µm), B) Quercetin (Bar scale: 50 µm), C) Quercetin and xenograft (Bar scale: 200 µm), D) Xenograft (Bar scale: 100 µm) groups tibial bone in the fourth week view. (O): bone trabeculae. The defect area of the E) Control F) Quercetin G) Quercetin and xenograft H) Xenograft groups tibial bone in the eighth week view. (O): bone trabeculae. Bar scale 100 µm.
the Q + X group (P < 0.05) (Fig. 3G). The osteopontin immunoreactivity treated with only xenograft was similar to the Group C and the Q + X group, while the osteopontin level in the Q group exhibited an increase that was statistically significant when compared to the Q + X group (P < 0.05) (Fig. 3H).

4. Discussion

Endochondral ossifying tibial bone was used as the bone model, notably because of its morphological structures and the ease of histopathological preparations. The advantage of the tibial defect model is that it offers a more suitable physiological condition for healing and inducing bone formation. To test different conditions in bone healing, a model defect that can normally heal should be created. This defect should be reproducible and the recovery period should be as short as possible. The literature contains no study conducted with Q + X in rats for the healing of tibial bone defects showing endochondral ossification; thus, the present study is original.

Phytoestrogens are plant-derived dietary chemicals found in many vegetables and fruits, consisting of isoflavonoids, lignans, stilbene and the flavonoid Q. Some studies have shown their role in the prevention and treatment of cardiovascular diseases, osteoporosis, diabetes, obesity
and menopausal symptoms.\textsuperscript{10,11} Chemically speaking, osteogenic effects occur through interaction with ERα and ERβ, both subtypes of estrogen receptors (ERs) located on the surface of osteoblast cells. They show structural similarity with 17-β-estradiol (E2), which enables them to modulate the mechanism.\textsuperscript{12} It has been reported in various studies that Q has an effect on osteogenesis.\textsuperscript{13,14}

Pang et al.\textsuperscript{15} studied the effect of Q on proliferation and osteoblastic differentiation in in vitro cultures of bone marrow mesenchymal stem cells (BMSCs) produced from mice. Since they are stromal cells that can differentiate into various cell types, including osteoblasts, they are thought to play an important role in the induction of osteogenesis. A significant increase in BMSC proliferation and ALP activity was found to occur when Q concentrations increased (0.1 μM–5 μM).\textsuperscript{16} The researchers suggested that this situation results in a dose-dependent increase in osteoblasts. They also suggested that Q stimulates osteogenic differentiation and interacts with the estrogen signalling pathway, which leads to the upregulation of transcription factor 2 (RUNX2) and Osterix (OXS) related to osteogenic genes.\textsuperscript{17} It was investigated by Pruillet et al.\textsuperscript{18} that human osteoblastic cell incubated at 1, 10, and 50 μM with Q for 24 and 48 h, which suggested that Q can rapidly increase ALP activity. In a study conducted by Taskan et al.\textsuperscript{19} to examine the effects of quercetin on alveolar bone loss and histopathological changes in rats with periodontitis, it was found that low (75 mg/kg) and high (150 mg/kg) doses of quercetin increased osteoblast cells and osteoclastic cells. They reported that inflammation was statistically significantly less in the quercetin-administered groups compared to the controls. The group with the highest levels of inducible nitric oxide synthase (iNOS), matrix metalloproteinase-8 (MMP-8) and cysteine-aspartic proteases 3 (caspase-3) and the lowest TIMP-1 (tissue inhibitor of metalloproteinase-1) expression was the control group; again, the differences were statistically significant. Within the limits of the study with an experimental periodontitis model, they suggested that administration of quercetin could reduce alveolar bone loss by increasing osteoblastic activity and decreasing osteoclastic activity, apoptosis and inflammation.\textsuperscript{20} In our study, a bone defect was created and quercetin was applied. Analysis found that the new bone formation in 56 days was higher in Groups Q and Q + X compared to controls, and the difference was statistically significant. In addition, significantly high values in bone marrow formation were obtained in controls on the 28th day. This shows that quercetin has a positive effect on bone healing, similar to the abovementioned studies.

Bone regeneration involves the interaction between blood vessels and bone cells.\textsuperscript{21} It has been reported that critical nutrients and oxygen accelerate bone healing by promoting angiogenesis and playing an important role in bone tissue repair and regeneration.\textsuperscript{22,23} Osteopontin is involved in different processes such as bone regeneration and tumour growth. It has been suggested that it is an important factor supporting the angiogenesis of endothelial cells.\textsuperscript{24,25} In addition, osteopontin reportedly has the ability to regulate vascular endothelial growth factor (VEGF) secretion and angiogenesis.\textsuperscript{26} In the present study, osteopontin values were compared and a significant increase was observed in both the fourth and eighth week in Group Q compared to controls. In this case, we think that quercetin application causes an increase in osteopontin, which consequently has a positive effect on bone healing, thanks to the increase in angiogenesis.

Ram et al.\textsuperscript{27} reported that osteonectin is a calcium-binding glycoprotein makes up 25% of non-collagen protein. Osteonectin plays a role in the angiogenesis mechanism and is believed to cause an increase in the mineralization of the collagen matrix. Studies have reported that osteonectin is high during development and wound healing.\textsuperscript{28} In the present study, there was no statistically significant difference in immunohistochemical values between the groups in the fourth week, whereas a significant increase was found in Group Q in the eighth week compared to the other groups. This shows that the application of Q at the eighth week has a positive effect on the bone healing mechanism.

In this study, bone healing was investigated by histological and immunohistochemical methods in which living tissues were examined. Bone histomorphometry is defined as the numerical evaluation of the microarchitecture of the bone. Histomorphometric analyzes allow measuring bone healing, new bone formation and bone density in guided bone regeneration studies. While evaluating the findings in this study, there are some limitations are encountered. In this study, only quercetin was evaluated and the effects of quercetin at different doses were not evaluated. In addition, long bones such as the tibia and femur have different osteogenic properties compared to the jaw bones (mandible-maxilla) and may therefore respond differently to quercetin. In addition, we think that studies in which quercetin is given at different doses and investigation of its effects on bone tissue in different regions such as the jaw will be more effective.

5. Conclusion

Based on the limited results of this study, it can be stated that quercetin can increase bone healing, and in addition, the use of quercetin with xenografts placed in defects can increase the rate of healing and ossification. It can be considered that quercetin can be used with a graft material in the future or as a material that positively affects bone healing. However, that further studies are needed for more accurate and comprehensive results.

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Note

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Declaration of competing interest

The authors declare there is no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jobcr.2022.10.008.

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