The evaluation of the effects of natural zeolite (Clinoptilolite) in diabetic rats on bone healing in dental extracting socket

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ABSTRACT

Objectives: The fact that clinoptilolite reserves are abundant and economical in the world, its toxic effects are not observed in clinical studies in humans and animals, and it has superior physical properties suggests that clinoptilolite may be an easily accessible product for the healing of tooth extraction wounds. The aim of the study was to evaluate the effects of clinoptilolite on bone tissue healing and bone formation in the extraction socket in experimentally diabetic rats by micro-CT analysis.

Materials and methods: 36 male Wistar Albino rats were divided into 3 main groups as Healthy Control, Diabetes Control and Diabetes + Clinoptilolite. Afterwards, these groups were divided into 2 subgroups to be sacrificed on the 14th and 28th days, and each subgroup had 6 rats (n = 6). At the 14th and 28th days, the rats were sacrificed and the effects of clinoptilolite on bone tissue healing and bone formation in the alveolar socket were evaluated by micro-CT analysis.

Results: According to the statistical analysis results, the difference between the groups in terms of bone volume, bone surface to bone volume, trabecular thickness and hounsfield units values, which are the parameters of the 14th day groups, was statistically significant (p < 0.05). Among the parameters of the 28th day groups, only the hounsfield units value was statistically significant (p < 0.05).

Conclusion: The results of the study show that clinoptilolite can contribute to the healing of extraction wounds and bone formation.

1. Introduction

The healing process within the alveolar socket is generally uneventful. However, in addition to local factors that complicate wound healing in the mouth, impaired or delayed healing may develop due to smoking and use of certain drugs, immunosuppressive treatments, immune diseases, metabolic bone diseases and some systemic diseases such as diabetes mellitus.

Zeolites are microporous, hydrated aluminosilicates, the majority of which are of volcanic origin. Zeolites are biocompatible, non-toxic materials and have important properties such as molecular sieve structure, ion exchange and high absorption. Because of these properties, they are used in various medical fields, drug delivery systems, wound healing, tissue engineering, coating of implants, hemodialysis, gas absorption and removal of harmful ions from the body.

Clinoptilolite, one of the natural zeolites, is found in nature in large reserves and in pure form. In addition, it is remarkable with its features such as not containing harmful elements, non-toxic and high quality. Due to the non-toxicity of clinoptilolite, it has been reported to be safe for in vivo use by the EFSA (European Food Safety Authority) and for human use by the pharmaceutical communities in the European Union and the USA.

The aim of the study was to evaluate the effects of clinoptilolite (Froximun Toxaprevent Skin Powder®) applied locally in the extraction socket on the bone tissue healing, quality and quantity of bone formation in the extraction socket in rats with experimental diabetes using micro-CT.

2. Materials and Methods

The study protocol was independently reviewed and approved by the Institutional Review Board of Cumhuriyet University and the Committee for Ethical Treatment of Experimental Animals (date and number, 20.05.2020–405).
2.1. Determination of experimental groups

In the study, 36 male Wistar Albino rats with an average weight of 250g, 4–4.5 months old, were used. Experimental animals were divided into 3 main groups consisting of 12 rats, as the Healthy Control group (HC), the Diabetes Control group (DC) and the Diabetes + Clinoptilolite (Experimental group-D + CLNP) group. Afterwards, these groups were divided into 2 subgroups to be sacrificed on the 14th and 28th days, and each subgroup had 6 rats (n = 6).

2.2. Establishment of experimental diabetes

In order to establish experimental diabetes, streptozotocin (STZ Cayman Chemical, USA) dissolved in 0.9% sodium chloride (NaCl) was calculated as 55 mg/kg according to the weight of the animals and administered intraperitoneally to the DC and D + CLNP groups. Rats whose blood glucose concentration exceeded 200 mg/dl 3 days after STZ injection were considered as diabetic. The blood glucose level was measured higher than 200 mg/dl in all rats treated with STZ. It was observed that polydipsia and polyuria developed in rats.

2.3. Operational procedure

General anesthesia was provided by intraperitoneally administering 3 mg/kg Xylazine (Rompun 2%, Bayer, Istanbul, Turkey) to the rats. Gingival retraction was performed with a fine-tipped probe, the furcation area of the teeth was reached, and the teeth were mobilized with traction. The teeth were retained with the help of a fine-tipped hemostat and the lower right first molars of all rats were extracted (Fig. 1).

Powdered clinoptilolite (Froximun Toxaprevent Skin Powder®, Froxpharma pharmaceutical medical limited company, Istanbul, Turkey) was applied to the extraction sockets of the D + CLNP group, considering that it would remain stable in the wound (Fig. 2). In addition, the wounds were sutured with 5/0 Vicryl (Polyglactin) to keep the clinoptilolite more stable in the wound and to reduce the wound opening.

2.4. Termination of the experiment

Six animals in each group were sacrificed on the 14th post-operative day, and the remaining six animals in each group were sacrificed on the 28th post-operative day by administering high-dose sodium pentobarbital (200 mg/kg). Rat mandibles were removed together with the surrounding soft tissue by dissection and fixed with 10% formalin.

2.5. Radiological examination

Each mandible specimen was fixed with reverse styrofoam and the occlusal plane was made horizontal to the floor. The regions of interest were trabecular areas of 100 slices around the extraction sockets and were segmented by semi-manual contouring. Samples were scanned with a Bruker Skyscan 1272 micro-CT (Bruker, Kontich, Belgium) instrument using a 9 μm thick (50Kv and 500 μA) copper and aluminum filter and a 0.3 mm rotation step. In the images obtained by X-ray projection, the relevant areas were determined with NRecon (v.1.6.3 software) software and the images were reconstructed. With the CTVol (v.2.2.1 software) data viewer software, the images were reconstructed to conform to the standard positioning for all samples and to be observable in three dimensions. In this way, standardization was achieved so that test results could be repeated when necessary. Then, the relevant areas were analyzed according to gray scale for three-dimensional evaluation with CTAnalyser-CTAn (v.1.12 software).

Bone surface (BS), bone volume percent (BV/TV), trabecular number (Tb. N), bone volume (BV), bone surface to volume ratio-surface roughness (BS/BV), trabecular thickness (Tb. Th), Hounsfield Units (HU) values were measured.

2.6. Statistical analysis

Statistical analyzes of the data were performed using the Statistical Package for the Social Sciences (SPSS version:25.0) program. The normality test of the data was evaluated with the Shapiro-Wilk test. Analysis of Variance (ANOVA) was applied for normally distributed data. Tukey test was used in the significance tests for pairwise comparisons of significant groups. As to error level alpha 0.05 was considered significant.

3. Results

According to the findings, the difference between the groups in BV, BS/BV, Tb.Th and HU values, which show the bone formation and healing amount of the 14th day groups, was found to be statistically significant (p < 0.05). In general, mean values were found to be against the DC groups in all 14th and 28th day DC groups. (Table 1-Table 2). Among the 28th day parameters, only the difference between groups in HU value was found to be statistically significant (p < 0.05) (Table 2).

The findings of the study showed that locally applied clinoptilolite contributed to healing and bone formation in the early 14-day period, reduced the negative effects of diabetes on wound healing in the early period, and did not significantly contribute to the late healing at 28 days.
Table 1
Statistical evaluation table of micro-CT parameters between groups on day 14th

| Groups | Micro-CT parameter | HC | D | D + CLNP |
|--------|-------------------|----|---|---------|
| BV(mm³) | 18,17 ± 2,10 | F – 5,62 p – 0,017* | 15,07 ± 2,06 | 19,98 ± 2,90 |
| DC | 129,76 ± 8,86 | F – 2,33 p – 0,136 | 166,51 ± 16,18 | 163,38 ± 45,68 |
| BS(mm³) | 7,22 ± 1,66 | F – 5,28 p – 0,021* | 11,25 ± 2,16 | 8,28 ± 2,48 |
| DC | 63,73 ± 3,69 | F – 1,69 p – 0,222 | 57,12 ± 10,51 | 62,60 ± 8,71 |
| BS/BV(%) | 4,15 p – 0,040* | 0,41 ± 0,03 | 0,32 ± 0,04 | 0,42 ± 0,08 |
| DC | 1,85 ± 0,06 | F – 4,80 p – 0,091 | 1,74 ± 0,19 | 1,50 ± 0,47 |
| Tb.Th(mm) | -134,17 ± 85,84 | F – 6,01 p – 0,049* | -346,16 ± 27,03 | -115,14 ± 28,78 |
| DC | -88,92 ± 18,51 | F – 4,31 p – 0,045* | -182,87 ± 21,42 | -151,21 ± 19,83 |

Table 2
Statistical evaluation table of micro-CT parameters between groups on day 28th

| Groups | Micro-CT parameter | HC | D | D + CLNP |
|--------|-------------------|----|---|---------|
| BV(mm³) | 21,10 ± 4,02 | F – 1,05 p – 0,371 | 18,67 ± 2,62 | 19,26 ± 2,07 |
| DC | 158,51 ± 33,52 | F – 0,01 p – 0,990 | 162,48 ± 57,06 | 159,62 ± 54,69 |
| BS(mm³) | 7,50 ± 0,64 | F – 0,40 p – 0,674 | 8,41 ± 1,89 | 8,33 ± 2,70 |
| DC | 75,32 ± 7,42 | F – 0,91 p – 0,421 | 66,71 ± 11,49 | 71,96 ± 7,42 |
| BS/BV(%) | -134,17 ± 85,84 | F – 6,01 p – 0,049* | -346,16 ± 27,03 | -115,14 ± 28,78 |
| DC | -88,92 ± 18,51 | F – 4,31 p – 0,045* | -182,87 ± 21,42 | -151,21 ± 19,83 |
| BV/TV(%) | 2,01 ± 0,02 | F – 0,32 p – 0,602 | 2,01 ± 0,02 | 2,01 ± 0,02 |
| DC | 0,40 ± 0,11 | F – 0,01 p – 0,990 | 0,40 ± 0,11 | 0,40 ± 0,11 |
| Tb.Th(mm) | 1,61 ± 0,16 | F – 4,78 p – 0,091 | 1,61 ± 0,16 | 1,61 ± 0,16 |
| DC | 1,01 ± 0,11 | F – 0,01 p – 0,990 | 1,01 ± 0,11 | 1,01 ± 0,11 |
| HU | -18,89 ± 50,44 | F – 9,06 p – 0,011* | -19,98 ± 50,44 | -19,98 ± 50,44 |

4. Discussion

This study is noteworthy as it is the first study to evaluate the effects of clinoptilolite on healing and bone formation in a different body environment such as the mouth and in the extraction socket.

In the literature, there are similar studies examining radiologically the alveolar bone healing after tooth extraction in rats. Li J. et al. reported that socket healing time was shorter in rats than in humans and dogs, 2 weeks after extraction, new bone trabeculae filled the apical third of the alveolar socket, and after 4 weeks, the socket was largely filled with new bone trabeculae. Berkovitz B.K. reported that 30 days after tooth extraction in rats, socket outlines were invisible on the radiograph. Xu L. et al. reported that bone formation with sufficient radio-opacity was achieved in the socket within 14 days. Similar to the literature, we observed radiologically the bone formation in the socket on the 14th and 28th days. In addition, we hypothesized that there would be no statistically significant difference after the 28-day period and longer follow-up would not be significant, since clinoptilolite was applied locally and administered as a single dose and not repeated at regular intervals.

As in many studies in the literature, it can be said that the diabetes model is ideal for our study in terms of comparison of extraction socket healing in normoglycemic animals. Bell J. et al. reported that permanent hyperglycemia started 10–12 h after the application of diabetogenic agent to rats, and Furman B.L. reported that the pathology that occurred in diabetic rats was similar to diabetes in humans. Pitol Palin L. et al. showed that diabetes stimulates the inflammatory process in the first stage of the alveolar repair process 7 days after tooth extraction, which leads to disruption of the mineralization process and collagen fiber organization and a decrease in fiber count. Xu L. et al. showed in their study with 14-day follow-up that vascular endothelial cell function, osteoblastic function and matrix mineralization were impaired due to the increased inflammatory response due to high glucose levels, resulting in delayed healing of tooth extraction sockets.

Micro-CT is frequently used in research laboratories due to its ability to achieve high spatial resolution up to 10 µm and to provide highly detailed anatomical information. Micro-CT has been shown to be the most widely used technique for the evaluation of bone-related parameters such as bone mass and morphology, amount of bone formation, density and quality in small animal models. In the study, micro-CT analysis results and images showed that bone formation in the extraction socket is significantly less in diabetic conditions than in normoglycemic conditions, and clinoptilolite reduces bone tissue destruction in diabetic conditions.

In the literature, it has been reported that Clinoptilolite has an autobioregulatory effect by absorbing harmful substances in the body environment and releasing the beneficial substances in its structure, mainly due to its absorption and ion exchange properties in local and systemic applications, and then it is excreted from the body by fecal route or from the wound surface with wound exudate in local applications. It can be said that it is not excreted via kidney or liver like other pharmacological drugs. EFSA, Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) reported that clinoptilolite is not absorbed through the gastrointestinal tract and is not metabolized. Samekova K. et al. showed that clinoptilolite given orally to individuals reduces enteral lead absorption. In addition, they reported that clinoptilolite grains do not pass into the bloodstream, are not absorbed due to their size and chemical inertness, and that the main mechanism of action of clinoptilolite is to bind heavy metals in water and food in the gastrointestinal tract, and the heavy metal-loaded clinoptilolite is then excreted through the feces. Kuruca D.S. et al. predicted that the mean particle size of clinoptilolite is 40 µm, therefore, clinoptilolite cannot pass through the intestinal lumen into the blood.

In our study, we assume that clinoptilolite will not cause any long-term toxic effects and organ damage due to local and single dose application. Kraljević Pavelić S. et al. investigated the short, medium and long-term effects of clinoptilolite on humans at 28 days, 12 weeks and 4 years. At the end of the relevant periods, blood values showed that toxic accumulations of metal ions such as sodium (Na), calcium (Ca), lead (Pb) and arsenic (As) did not occur in the organs. Kraljević Pavelić S. et al. reported that individuals with osteoporosis who were treated with 9 g of clinoptilolite per day for 5 years had a good general health condition, a low incidence of fractures, and no adverse blood value...
changes or significant side effects were observed during this period. Rodríguez-Fuentes G. et al.\textsuperscript{19} reported that the natural clinoptilolite-containing drug called Enterex, which is still used in the treatment of diarrhea in humans in Cuba, does not cause biological damage to the gastrointestinal system and other organs of animals. Zhakov Y.I.\textsuperscript{20} showed that even after long-term administration of zeolites, physiologically, no significant changes were observed in the homeostasis of the relevant trace elements and vitamins.

In the literature, it is seen that clinoptilolite has local applications similar to our study. Aksyö S.O. et al.\textsuperscript{21} applied Froximun Toxaprevent Skin powder\textsuperscript{®} in powder form according to the wound size after mastectomy and axillary dissection in rats, and closed the wound area with sutures after local application. Uraloğlu M. et al.\textsuperscript{22} applied Froximun Toxaprevent Skin powder\textsuperscript{®} to the wounds opened on the back skin of the rats locally, in powder form and according to the wound size, and did not suture the wounds. Becher G. et al.\textsuperscript{23} reported that Froximun Toxaprevent Skin powder\textsuperscript{®} applied to wounds in people with acute or chronic wounds provides hemostasis in the wound area and closes the wound physically as a protective shield. They applied clinoptilolite locally, in powder form and according to wound size, but did not suture the wounds.

According to the study findings, the most basic mechanism in the contribution of clinoptilolite to healing and bone formation in the extraction socket; It can be said that clinoptilolite has a detoxification effect due to its ion exchange and absorption properties in the attraction socket, attracting inflammatory proteins, toxic substances, heavy metals and exudate in the socket. Becher G. et al.\textsuperscript{23} reported that clinoptilolite (Froximun Toxaprevent Skin Powder\textsuperscript{®}) binds to toxic substances, heavy metals and inflammatory proteins (eg histamine) in the wound area in a completely selective physical way, thanks to its reversible ion exchange and absorption properties. They also reported that by absorbing harmful substances and exudates in the wound area, they provide detoxification of these substances, thereby relieving inflammation and accelerating wound healing. Aksyö S.O. et al.\textsuperscript{21} attributed the mechanism of reducing seroma formation and accelerating wound healing of clinoptilolite (Froximun\textsuperscript{®}) to the fact that clinoptilolite exerts a detoxification effect thanks to its absorbing structure with micropores, attracting the seroma with inflammatory exudate into its structure and increasing the formation of granulation tissue.

Other mechanisms in the contribution of clinoptilolite to the healing and bone formation in the tooth extraction socket; it can be attributed to the fact that it provides the mineral support necessary for wound healing and bone formation due to the calcium and silica in its structure. Deinsberger J. et al.\textsuperscript{24} reported in their study on humans that clinoptilolite has the ability to absorb wound exudate and bind bacteria irreversibly, as well as absorb bacterial toxins. Kraljević Pavelić S. et al.\textsuperscript{2} reported in their study on osteoprototic rats that cations such as Ca, Mn, Zn, Fe, and Mg were physiologically released from the clinoptilolite framework during the ion exchange process of clinoptilolite, and these cations were then used in antioxidative mechanisms. Wu Q et al.\textsuperscript{25} reported in their in vivo study that decreased serum malondialdehyde (MDA) values can be associated with the absorption, ion exchange and cation binding properties of clinoptilolite, and that clinoptilolite neutralizes free radicals by absorbing them. Oschilwski M. et al.\textsuperscript{26} reported that silica in the structure of clinoptilolite had a protective effect against the destruction of the islet of langerhans in their study on diabetic rats. Price C.T. et al.\textsuperscript{27} stated that silica in the zeolite structure is an essential material for bone formation, and that silica participates in the cross-linking of proteoglycans and collagen in the bone matrix. Akkınemmedov R. et al.\textsuperscript{28} stated in their in vitro study that clinoptilolite caused a local increase in the Ca\textsuperscript{2+} ions they released into the biological environment, which could induce osteogenic differentiation.

The early contribution of clinoptilolite to healing and bone formation; although the beneficial cations released from the clinoptilolite structure are absorbed to some extent in the wound environment, this can be attributed to the fact that the absorbed amount is too low to create a long-term systemic effect, and that it is administered as a dose and not repeated at regular intervals. In addition, it can be said that the contribution of clinoptilolite to healing in the early period may be related to the fact that it is applied locally immediately after tooth extraction and has an antioxidant effect in the early period, and similarly, the other biological effects of clinoptilolite occur in this early period when inflammatory events are most intense. According to literature; immediately after injury, neutrophils and macrophages come to the wound site and produce large amounts of ROS (Reactive Oxygen Species) to protect the organism against invading bacteria and other microorganisms, as well as proteolytic enzymes and proinflammatory cytokines.\textsuperscript{28} It has been reported that macrophages and fibroblasts gradually increase in number in the early stage of the healing process and reach their highest level and gradually decrease as the healing process progresses, and the antioxidant effect of clinoptilolite occurs at this early stage.\textsuperscript{31} García Godoy F. et al.\textsuperscript{29} reported that clinoptilolite added locally to the graft in the bone defect created in the mandible, similar to our results, provided positive effects on bone regeneration in the early period. In addition, they showed that there was no significant difference between the groups in terms of healing and bone formation in the late 30 and 60 days. Çoban Z. et al.\textsuperscript{30} reported that externally applied St. John’s Wort oil had a positive effect in the early period (first 7 days) use, but this effect was not observed in the late period (second 7 days) use. Park J.J. et al.\textsuperscript{31} concluded that local diode laser irradiation of extraction sockets in diabetic rats promoted early healing, especially for the initial stages of alveolar bone healing in both diabetic and normal rats. The results of this study were found to be consistent with these studies on the effects of clinoptilolite on healing in bone tissue and soft tissue.

5. Conclusion

The results of this study show that clinoptilolite applied locally to the alveolar socket after tooth extraction can contribute to the healing of extraction wounds and bone formation. Therefore, clinoptilolite can be recommended as a local agent that can be used after tooth extraction. Considering the biological effects of clinoptilolite due to its superior physical properties, its use as a locally applicable agent that may also be effective in the treatment of alveolar osteitis may be the subject of future studies.

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Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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