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Molecular Triad RANK/ RANKL/ OPG in Mandible and Femur of Wistar Rats (*Rattus norvegicus*) With Type 2 Diabetes Mellitus

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Molecular Triad RANK/ RANKL/ OPG in Mandible and Femur of Wistar Rats (*Rattus norvegicus*) With Type 2 Diabetes Mellitus

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Abstract

A successful treatment of dental implant needs a good jaw bone support, which depends on healthy bone metabolism. Bone metabolism can be affected by Diabetes Mellitus (DM). It may trigger various complications, including osteoporosis. Molecular triads consisting of Receptor Activator of NF-kappaB (RANK), Activator of nF-κB Ligand (RANKL), and osteoprotegerin (OPG), have an important role in the formation, function, and osteoclast survival. In this study, molecular triads were observed on mandible and femur bones in type 2 DM Wistar rats. The aim of this study was to observe the molecular triad RANK / RANKL / OPG expressions in type 2 DM Wistar rats. This laboratory research used 18 male Wistar rats divided into three groups: nondiabetic group (control), uncontrolled DM injected with single dose of Streptozotocin (STZ), and controlled DM treated with Metformin. On day 20, the mandible and femur were collected and specimen processing was carried out. The results of RANK / RANKL / OPG expressions were obtained from immunohistochemical staining. In both mandible and femur groups, RANK, RANKL, OPG expressions showed no difference between the control and uncontrolled DM groups. RANKL / OPG ratio in uncontrolled DM was higher than that in the control group. RANK expression was lower in uncontrolled DM group compared with controlled DM, and the RANKL expression in uncontrolled DM group was higher than that in the controlled DM group. RANKL / OPG ratio was lower in the controlled DM group**.** The study suggested that DM affects resorptive activity in mandible and femur bones which can be observed via RANK/RANKL/OPG.

Keywords: Receptor Activator of NF-kappaB (RANK); Receptor Activator of NF-κB Ligand (RANKL); Osteoprotegerin (OPG); Diabetes Mellitus; Osteoporosis.

1. INTRODUCTION

Dental implant is one of the options for Prosthodontic's rehabilitation treatment that improve patient's quality of life and satisfactory compared with conventional removable dentures [1]. A successful treatment needs healthy jaw bone to achieve a good implant attachment and stability, which is aquired by a process of bone adaptation to the implant's surfaces called osseointegration process. It depends on healthy bone metabolism [2-4].

Bone metabolism can be affected by DM. Long-term uncontrolled DM may cause bone abnormalities such as bone fracture and osteoporosis [5]. Implication of osteoporosis on prosthodotic treatment are associated with bone and tooth loss, and temporomandibular joint disorder and resorption of residual ridge crest [6]. Osteoporosis is characterized by increased osteoclastogenesis and decreased osteoblast activities and an imbalance in bone remodelling.

Osteoclastogenesis is the process of osteoclast differentiation. The process involves a molecular triad consisting of Receptor Activator of nF-κB (RANK), Receptor Activator of nF-κB Ligand (RANKL), and osteoprotegerin (OPG) [7]. Under normal conditions, bone remodeling activities are controlled by a balanced RANKL/OPG ratio. Osteoblasts can either enhance or inhibit osteoclast differentiation through RANKL/OPG ratio, simultaneously decreasing or increasing the rate of bone formation, respectively [8]. Any changes that occur in the RANKL/OPG ratio affects the bone resorption or bone formation [9].

The mandibular and femur bones were observed in this research. The mandibular bone is used for dental implants, but implantation in rat's mandible was difficult because implant had to be placed in limited space of rat's mandible [10]. Earlier studies have examined implant placement in experimental animals that carry implant insertion in the femur bone, because it had a better implantation access for simulating implantation [4, 11]. Based on this reason, it was necessary to conduct this research by observing molecular triad, RANK/RANKL/OPG, in both mandible and femur bones in type 2 DM Wistar rats (*Rattus norvegicus*).

2. METHOD(S)

This research used an experimental laboratory study design. The study has been approved by Universitas Airlangga, Faculty of Dental Medicine Health Research Ethical Clearance Commission. A total of 18 male Wistar rats (*Rattus norvegicus*) aged 3-4 months weighing ±100–130g were used. Samples were divided into 3 groups: control, STZ-induced (uncontrolled DM), and STZ-induced DM treated with metformin (controlled DM) groups.

2.1 Conditioning a Type 2 DM in Rat Models

Streptozotocin / STZ (Sigma) was administered at a dose of 50 mg/kg body weight by dissolving STZ powder in 0.05 M citrate buffer solution with a pH of 4.3–4.5. The final solution was prepared with an STZ concentration of 22.5 mg/ml citrate buffer [12].

Intraperitoneal injection of a single-dose STZ was administered to the Wistar rats after four hours of fasting. The STZ dose was 50 mg/kg of body weight. Twenty minutes before the injection, STZ was dissolved in a citrate solution. After the STZ induction, 10% sucrose solution or dextrose was administered. Hyperglycemia develops 5–12 days later. The subjects were then sacrificed after 20 days and the femur and mandibular bones were collected [13].

2.2 Procedure to Make Metformin Solution

Metformin solution was administered at therapeutic dosage, which was 100 mg/kgBW prepared by dissolving crushed metformin into 1.5 ml of distilled water [14].

2.3 Collection of Mandibular and Femur Bone Sample

After being sacrificed, the soft tissue was removed from the mandibular bone, then the left mandible was separated from the jaw of the rats. The examined mandibular area was on the interradicular septum below the first molar. The samples were immersed in a 10% buffered formalin fixation solution for at least 8 hours before the decalcification process [15, 16].

Extraction of the femur bone was performed by separating the posterior limb from the body. Beginning with the muscles, ligaments, and tendons that connect the rat's femur and pelvis in the proximal part of femur were cut. The femur of the posterior limb of the rat was preserved by completely submerging it in 15% formalin in a closed bottle.

Decalcification process used 10% EDTA solution with a volume of 50 times the volume of the material examined. EDTA solution was changed once every 3 days [15].

2.4 Immunohistochemical Examination

Samples were immunohistochemically (IHC) stained with anti-RANK serum (Thermo Scientific), anti RANKL (Thermo Scientific), and anti OPG (Thermo Scientific) to evaluate the expression of each marker. The results were performed using modified Remmele method. Data for each sample were the mean of *Immuno Reactive Score* (IRS) value observed in 5 (five) fields of view at 400× magnification using a regular light microscope (Nikon H600L, Japan) equipped with a 300 megapixel DS Fi2 digital camera and Nikon image processing software image system [16,17].

2.5 Statistical Analysis

Data normality test used Shapiro–Wilk to find out the distribution of research data. The differences for each group were analyzed by One-Way ANOVA and Post-hoc Tukey HSD.

3. RESULTS

3.1 Molecular Triad

The mean values, standard deviations, and significance values of RANK, RANKL, and OPG expressions in the mandibular and femur are shown in Figures 1 and 2. Microscopic examinations of RANK, RANKL, and OPG expressions were observed and shown in Figures 3, 4 and 5.

The normality test with Shapiro–Wilk test showed that all research groups had a p value of significance >0.05, which indicated that the data were normally distributed.

The results of the Tukey HSD test in Figures 1 and 2 marked by asterisk (*) show significant values >0.05. Significance of RANK expressions were obtained between the uncontrolled DM and controlled DM in mandible (sig = 0.006) and between the uncontrolled DM and controlled DM in femur (sig = 0.022). RANKL expressions also showed significant differences between the uncontrolled DM and controlled DM in the mandible (sig = 0.007) and between the uncontrolled DM and controlled DM in femur (sig = 0.003).

Figure 1. Molecular triad expression mean values and significance bar chart of mandible.

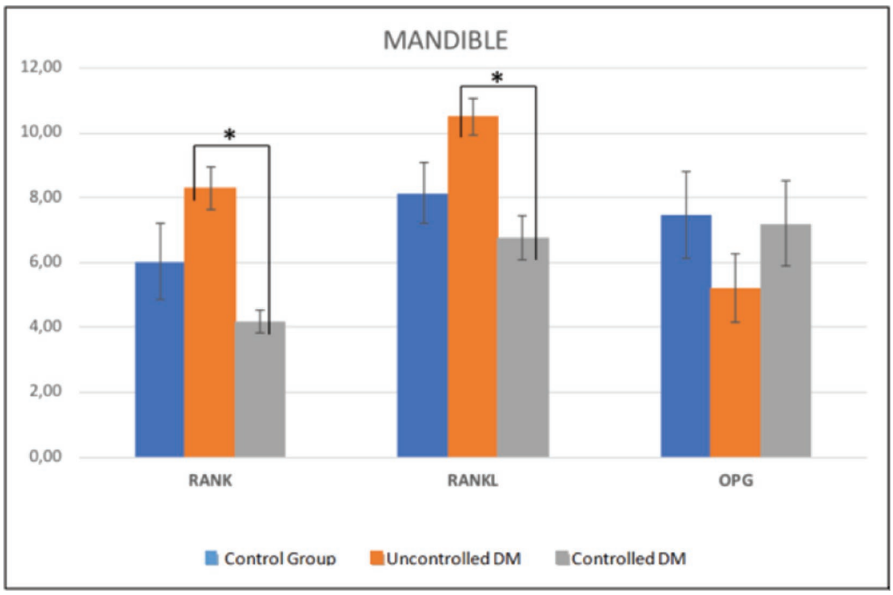
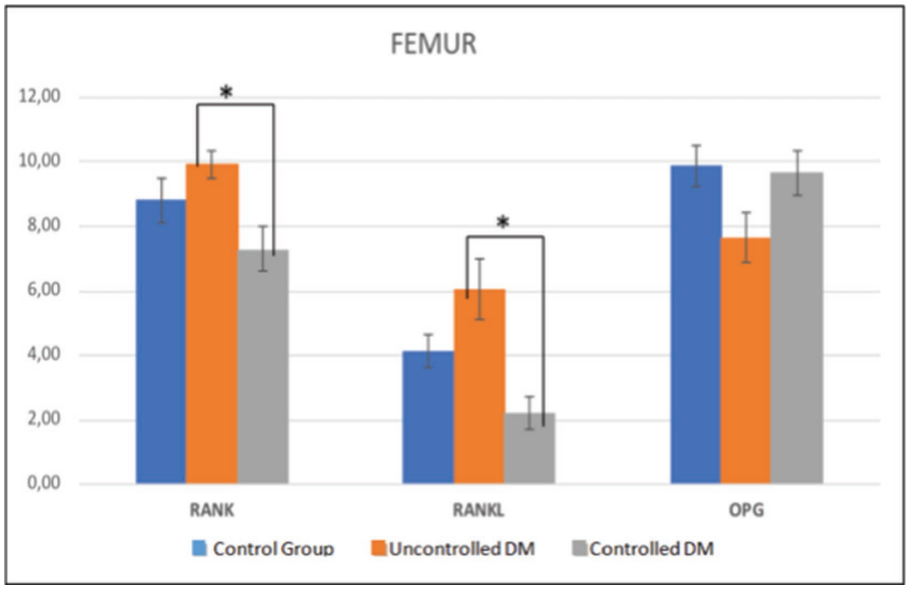


Figure 2. Molecular triad expression mean values and significance bar chart of Femur.



3.2 RANKL/OPG Ratio

In Figure 6, the mandible showed significant difference between the control and uncontrolled DM groups (sig.=0.023), uncontrolled DM and controlled DM groups (sig = 0.009), and no significant difference between the control and controlled DM (sig = 0.888), whereas in femur groups there were significant differences between the control and uncontrolled DM (sig = 0.011), uncontrolled DM and controlled DM (sig = 0.001) and nonsignificant differences between the control and controlled DM (sig = 0.308).

Figure 3. Results of IHC examination of RANK expression indicated by arrows using a light microscope at 400× magnification.

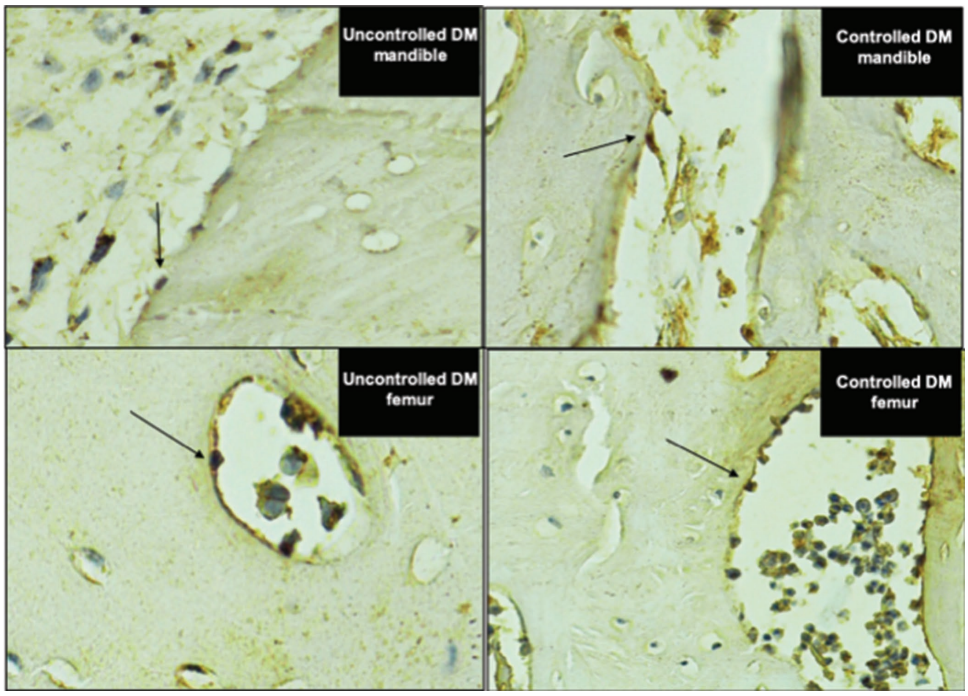


Figure 4. Results of IHC examination of RANKL expression shown by arrows using a light microscope at 400× magnification.

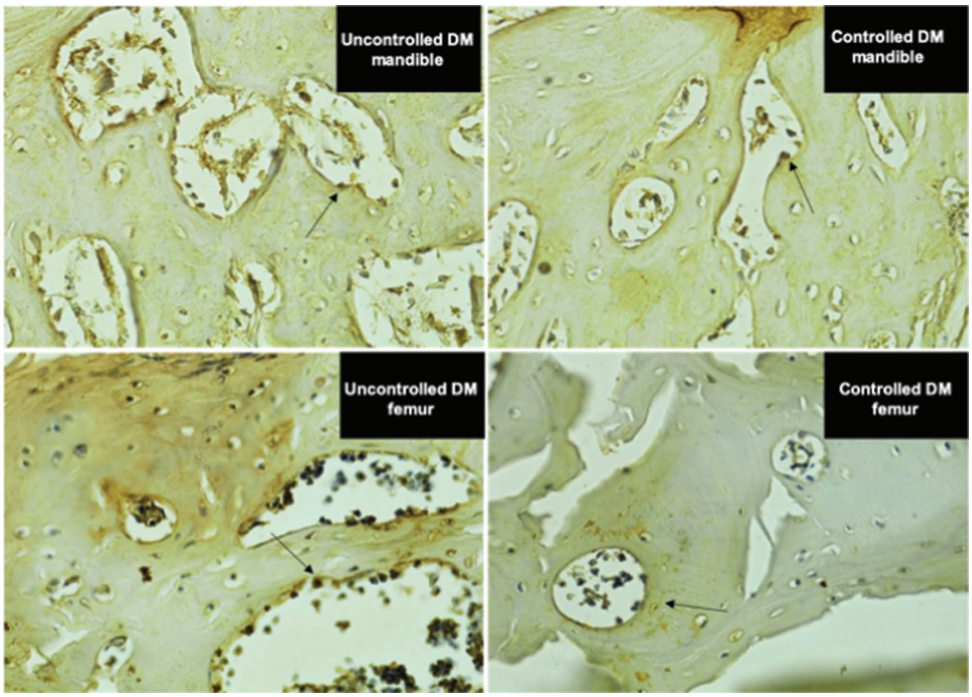


Figure 5. Result of IHC examination results of OPG expression shown by arrows using a light microscope at 400× magnification.

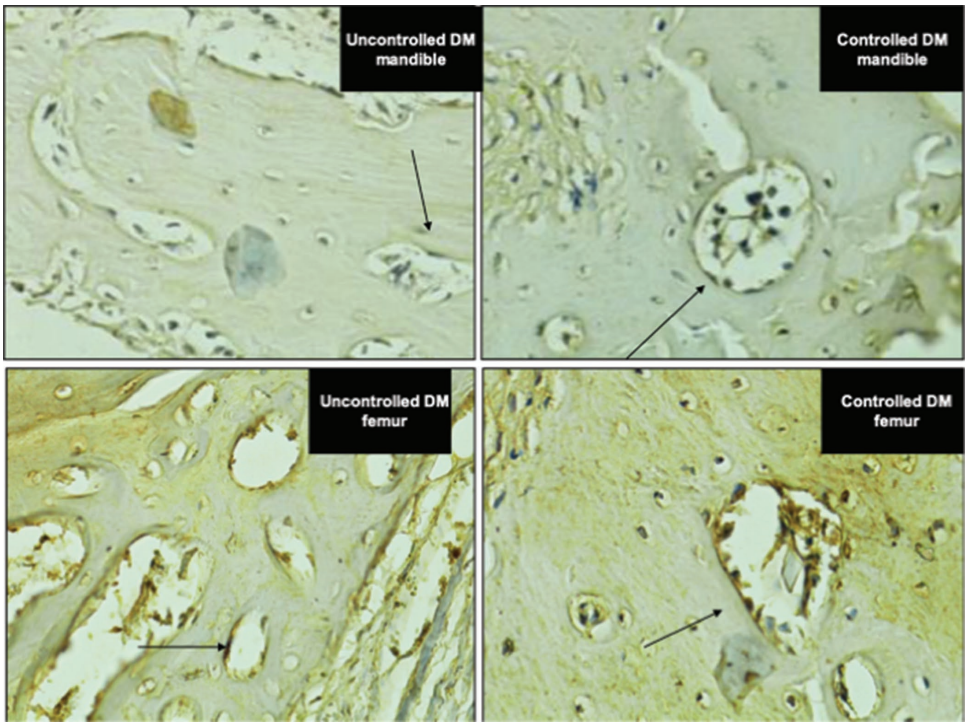
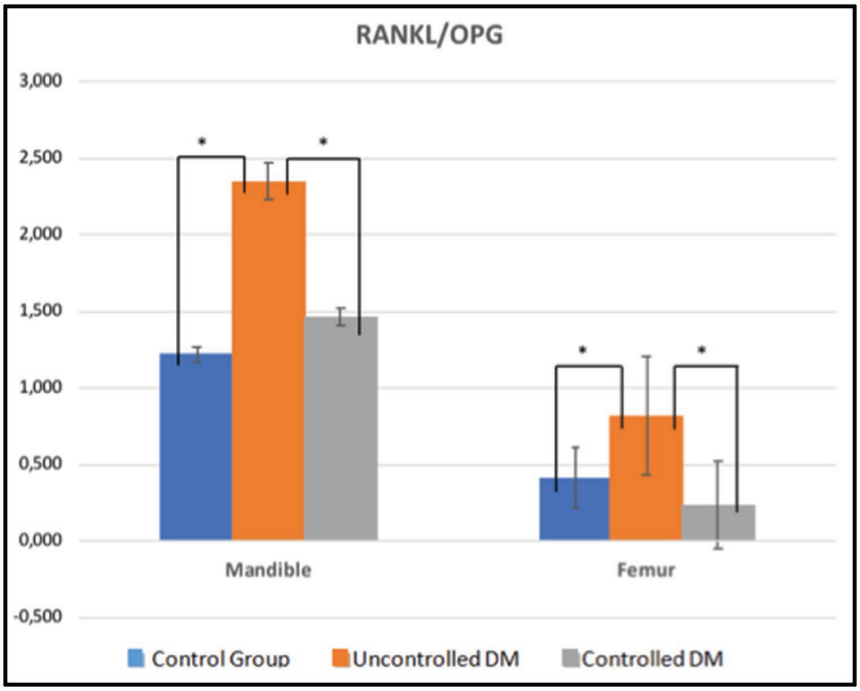


Figure 6. Mean values and significance bar chart for RANKL / OPG ratio in mandible and femur.



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4. DISCUSSION

Osteoporosis in type 2 DM patients can be caused by several conditions such as accumulation of advanced glycation end products (AGEs) due to hyperglycemic condition. AGEs bond to advanced glycation end products receptors (RAGEs) and lead to an increased production of reactive oxygen species (ROS) [18]. ROS increases oxidative stress and affect the RANK / RANKL / OPG pathway. The disturbance in the pathway causes the development of osteoporosis [19].

At the observation on day 20, we found that there was no difference on RANKL and RANK expressions between the control and uncontrolled DM groups in both mandible and femur bones, but the RANKL /OPG ratio in uncontrolled DM is higher than that of the control groups. Meanwhile this study found no difference on OPG expression in all groups in both mandible and femur bones. The result of this study is in line with the research conducted by Hie, that no difference was observed in RANKL and OPG expressions in STZ-induced diabetic rats [20].

In this study, we found that the RANKL/ OPG ratio was lower in controlled DM, indicates an inhibition of bone resorption activity [21]. Higher RANKL/ OPG ratio expression indicates an increase in resorption activity that promotes bone loss. Similar results were obtained in an earlier study that examined bone fracture healing in Alloxan-induced diabetic rats, showed that RANK, RANKL, and OPG expressions in DM groups did not differ from those of the control groups, but the RANKL/OPG ratio was higher in DM groups [22]. The results of this study are also the same as those by Suzuki; they found that RANKL/OPG ratio in diabetic patients was higher than that in the healthy populations [23].

Higher RANKL/OPG ratio in the uncontrolled DM occured due to hyperglycemic conditions. Hyperglycemia increase pro-inflammatory mediators IL-6, thus stimulating prostaglandin (PGE2) production. The PGE2 promote osteoclast formation through stimulation of RANKL expression from osteoblast cells [24].

Metformin in the controlled DM groups helps reduce ROS formation by inhibiting RAGE expression. Metformin also has a beneficial role in bone formation by decreasing RANKL expression in osteoblast through activation of AMP-activated protein kinase (AMPKs) pathway [25]. AMPKs pathway increase osteoblast differentiation, suppress osteoclast activity in bone resorption, and have a positive effect on bone formation [25-28]. This explains our findings on higher OPG/RANKL ratio and lower RANKL and RANK expressions in controlled DM groups compared with uncontrolled DM groups and indicate that metformin therapy for DM can suppress the activities and differentiation of osteoclast by inhibiting RANKL expression in osteoblast cells [29].

5. CONCLUSION

This study suggested that bone resorption activity in DM affects both mandible and femur bones, which can be observed via RANK/RANKL/OPG. DM induced the increase in RANKL and OPG, whereas a controlled DM regulated the RANKL and OPG to the normal condition.

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Authors' Contributions
This work was carried out with the collaboration of all authors. NH was responsible for the experiment design, data analysis, literature search and preparation of the research report. R, AK, MDAA, and RMS carried out the sample collection and literature search. PDH, VVA, and PYIS performed statistical analysis. R, AK, MDAA, RMS, PDH, VVA, and PYIS contributed to research report and literature search work.

Conflict of Interest
None.

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