Higher RANK ligand/osteoprotegerin ratio results in lower callus diameter and osteocalcin expression on the healing of femur fracture between diabetic and nondiabetic rats using intramedullary wire

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ABSTRACT

Background: Diabetes with uncontrolled glucose level offer a significant risk of acute or chronic complications. One of the chronic complications is a decrease in bone mineral density which is regulated by RANKL and OPG balance. Diabetes is estimated to slow the healing process of fracture. Both are related to fracture risk and fracture healing process. Fracture healing is characterized by measurement of callus diameter and osteocalcin expression.

Aim: This research aimed to find out the difference in RANKL/OPG ratio, callus diameter and osteocalcin expression on the fracture healing in diabetic and nondiabetic rats.

Methods: This is an experimental randomized post-test only with 32 Wistar rats. The population was divided into two groups, the control group did not get treatment and the second group was the diabetes modeling. On the 7th day, both groups were examined for their ratio of RANKL/OPG using ELISA and fracture was performed.

On the 28th day, the rats were sacrificed, and the effects were assessed using histopathology examination of callus diameter and immunohistochemistry examination of osteocalcin expression at the fracture area.

Result: Higher median ratio of RANKL/OPG in diabetic than nondiabetic rats with p = 0,000 (p <0,05). The diameter of callus on the healing of femur fracture in the nondiabetic group was higher than the diabetic group with p = 0,000 (p <0.005). Osteocalcin levels in nondiabetic group were higher than diabetic group with p = 0,000 (p <0.005). There was a negative correlation between RANKL/OPG ratio with callus diameter and osteocalcin level with a correlation coefficient of -0.701 and -0.652 respectively with p = 0,000 (p <0,05).

Conclusions: Level of RANKL/OPG ratio has a negative correlation with callus size and osteocalcin expression on healed femur fractures in diabetic rats model.

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INTRODUCTION

Diabetic condition with uncontrolled glucose level offers a significant risk of acute or chronic complications. Inflammation occurs in such condition which is signified by the increase in cytokines and osteoclast formation, and a decrease in the formation and function of osteoblast. All of these will, in turn, induce osteoclastogenetic process.

The number of diabetic patients in the world is estimated at 140 million in 1998. In 2012, Indonesia had about 7.6 million people with diabetes, the 7th largest, with relatively low prevalence (4.8% of type 1 and 2 diabetic patients were in 20-79 years of age). WHO predicted an increase in the number of diabetic population in Indonesia from 8.4 million in 2000 to 21.3 million in 2030. International Diabetes Federation (IDF) predicted an increase from 9.1 million in 2014 to 14.1 million in 2035.[1]

One of the chronic complications of DM that is not easily detected is a decrease in bone mineral density. It may be caused by inflammation process that is occurring progressively, disrupting the osteoclastic and osteoblastic balance, which is closely related to fracture risk and fracture healing process.[2]

An observational study done by Bonds in 2006 with 93,000 postmenopausal women (5,285 subjects of whom has diabetes mellitus) and seven years follow-up revealed a significant increase in the risk of fracture event in subjects with diabetes mellitus. Metanalysis studies done in the United States and Europe showed a consistent pattern on the increased risk of bone fracture in both male and female subjects. A health study on 109,983 women aged 34-59 years old and 22 years follow-up indicate that type 1 and type 2 diabetes mellitus are related to the increased risk of hip fracture.[2]

Either type 1 or type 2 diabetes is associated with a decrease in bone mineral density. One study

reported that factors such as increased expression of cytokines and chemokines, matrix destruction, damage to bone repair capacity, decreased proliferation and increased apoptosis were associated with the delayed bone healing process.[3] Clinical studies also found that there was a time impairment in both the union and fracture healing process in diabetic subjects compared to controls, particularly in the pelvic, mandibular and long bone regions.[4]

In diabetes, there is an inflammatory condition characterized by elevated levels of cytokines such as TNF-α, IL-1β, IL-6, and IL-18. This condition will, in turn, induce the process of osteoclastogenesis.[2] A study showed that mice with type 1 diabetes mellitus had two to four-fold osteoclast count as well as IL-17 and IL-23 levels, which would stimulate osteoclast formation via RANKL.[5] Diabetes increases the ratio of RANKL/OPG and TNF, both of which contribute to the bone resorption process.[6] Diabetes also decreases osteoblast formation and function. This effect is proved by a decrease in osteocalcin levels in type 2 diabetes mellitus patients compared to nondiabetic patients. It reflects a decrease in osteoblast activity, which is inversely related to IL-6 and C-reactive protein (CRP).[7]

In addition to the inflammatory cytokines pathway, the healing of bone fractures in diabetes is influenced by one of the modified proteins namely advanced glycation end-products (AGEs). AGE and hyperglycemia are associated with osteoclast formation and the RAGE expressed by osteoclasts will stimulate the process of osteoclastogenesis.[8] RAGE also suppresses the expression of osteoprotegerin (OPG) to increase osteoclastogenesis and bone resorption. Also, AGE inhibits osteoblast differentiation.[2]

Hyperglycemic conditions cause an increase in reactive oxygen species (ROS). The formation of reactive oxygen species (ROS) induces RANKL expression and promotes greater osteoclast formation.[9] The ROS production which is induced by hyperglycemia also increases RAGE expression, leading to the formation of osteoclast.[10]

One of the markers of bone formation is an increase in osteoblastic activity. Osteoblast plays a role in bone mineralization and bone remodeling.[11] Bone remodeling is closely regulated by a triad of molecules consisting of RANKL, OPG, and RANK. RANKL increases osteoclastogenesis by receptors interaction, whereas OPG inhibits this osteoclastogenesis binding to RANKL. The balance between OPG and RANKL plays an important role in bone pathophysiology.[12] This ratio increase is probably related to decreased bone density, increased risk of fracture and delayed healing process of bone fracture. This study was done to determine the relationship between RANKL/OPG ratio with the bone fracture healing process, which is characterized by callus size and osteocalcin expression in people with diabetes mellitus.

MATERIALS AND METHODS

This study is an experimental laboratory study using post-test only control group design using white rat (Wistar). The Wistar rats are divided into two groups: a control group and a treatment group. On day-1, treatment group, which acts as diabetic modeling group, was given alloxan with a dose of 140-180 mg/kgBW dissolved in 5% aqua bidest and injected intraperitoneally. The control group did not receive any treatment. Both groups were observed for seven days.

On day-7, 0.5 mL blood was sampled from the orbital sinus or rat tail using microhematocrit capillary. The blood was examined for RANKL/OPG level determination using ELISA method. A fracture on the femur diaphysis was performed on all the rats, and an intramedullary nail was placed. On day-28, the rats from each group were sacrificed, and callus diameter and osteocalcin expression were measured. The results were then compared between the control and treatment group.

RESULT

The results of data analysis included descriptive data distribution, RANKL/OPG ratio, callus diameter, and osteocalcin expression. Statistical analysis of the collected data was done using SPSS for Windows version 22.0.

Table 1 showed that the total number of study subjects was 32, with the diabetic treatment group consisting of 16 rats (50%) and the non-diabetic control group consisting of 16 rats (50%). The above data also shows that the median RANKL/OPG in the non-diabetic control group (1.35) was lower than the diabetic group (6.14). In contrast, the mean callus diameter and osteocalcin level in the non-diabetic control group (3639.67 and 56.25) was higher than in the diabetic treatment group (1660.74 and 37.3).

Based on normality test using Shapiro-Wilk test, the data distribution of callus diameter and osteocalcin levels in the diabetic treatment group (0.414 and 0.295) and the non-diabetic control group (0.898 and 0.376) followed a normal distribution with p> 0.05.

Table 1   Mean RANKL/OPG, callus diameter and osteocalcin level

Group	RANKL/OPG ratio Median	Callus Diameter Mean (sb)	Osteocalcin level Mean (sb)	Total Sample
DM	6.14 (4.19-37.61)	1660.74 (684.20)	37.3 (1.72)	16 (50%)
Control	1.35 (0.37-4.39)	3639.67 (1192.93)	56.25 (1.90)	16 (50%)
			Total	32 (100%)

Table 2   Result of comparability test on post-test data variable for each group using *independent T-test*

| Variable	Group DM (n = 16)	Group Control (n = 16)	Mean diff.	95% CI	p-value
Callus Diameter	1660.74 ± 684.20	3639.67 ± 1192.93	-1978.931	-2681.073 – (-1276.78)	0.000
Osteocalcin level	37.3 ± 1.72	56.25 ± 1.90	-19.225	-20.532 – (-17.9176)	0.000

Table 3   Result of comparability test on post-test data variables for each group using Mann-Whitney U test

Variable	Group DM (n = 16)	Group Control (n = 16)	Mann – Whitney U Z Score	Mann – Whitney U P value
RANKL/OPG ratio	6.14 (4.19-37.61)	1.35 (0.37-4.39)	-4.749	0.000

Table 4   Result of correlation test on RANKL/OPG ratio, callus diameter, and osteocalcin level in both groups

Variable	Correlation Test Correlation coefficient	Correlation Test P-value
OPG/RANKL ratio with callus diameter	-0.701**	0.000
OPG/RANKL ratio with *Osteocalcin* level	-0.652**	0.000
Callus diameter with *Osteocalcin* level	0.701*	0.000

*Pearson Test **Spearman's rho
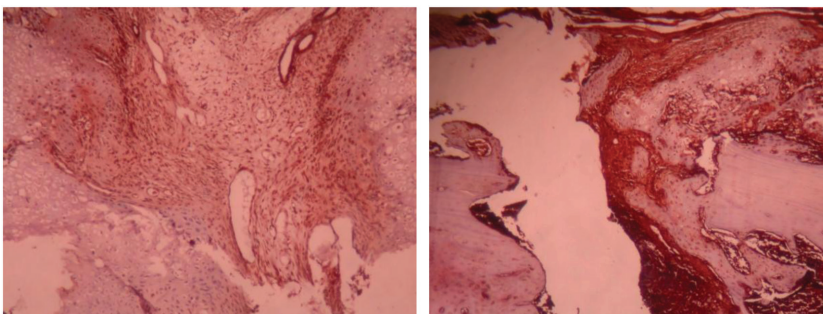
Figure 1   Histopathology results of callus diameter of the diabetic group
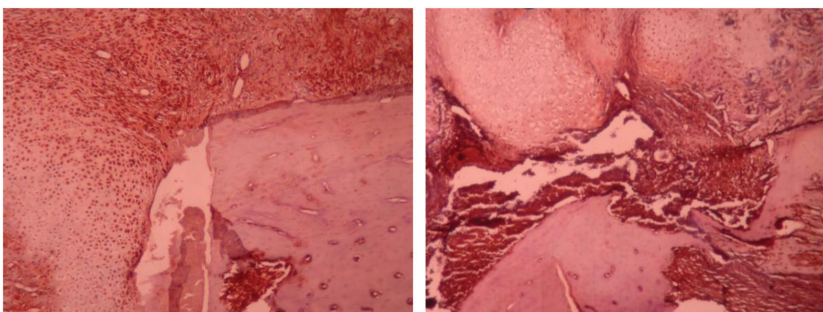
Figure 2   Histopathology results of callus diameter of the nondiabetic group

The result of independent t-test (Table 2) showed statistically significant differences in mean between both variables with p=0.000 (p<0.05).

The RANKL/OPG ratio was found to have an abnormal distribution in the diabetic treatment group and nondiabetic control group with a p-value of 0.000 and 0.006 respectively (p <0.05).

Mann-Whitney U test (Table 3) results showed that the differences in RANKL/OPG ratio in the control and treatment group were statistically significant with p = 0.000 (p <0.05).

A correlation test (Table 4) was performed to determine the relationship between the three variables: RANKL/OPG ratio, osteocalcin level, and callus diameter. Spearman's test was performed to identify the correlation between RANKL/OPG ratio with osteocalcin, and RANKL/OPG ration with callus diameter. A Pearson test was done to identify any correlation between osteocalcin level and callus diameter.

In this study, we found that the RANKL/OPG ratio had a strong negative correlation with callus diameter and osteocalcin level, and are statistically significant with p = 0.000 (p <0.05) with correlation coefficient values of -0.701 and -0.652, respectively. This result shows that the RANKL/OPG ratio has an inverse relationship with callus diameter and osteocalcin levels.

We obtained a statistically significant strong positive correlation with p=0.000 (p<0.05) and a correlation coefficient of 0.701 between the callus diameter and osteocalcin level. This result shows that the callus diameter has a linear relationship with osteocalcin levels.
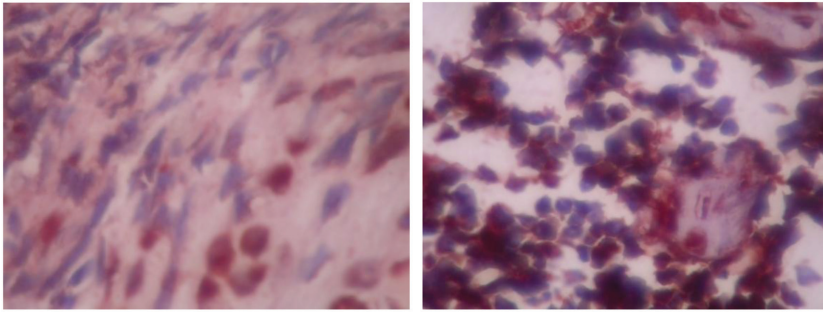
Figure 3 Immunohistochemistry results of osteocalcin expression of the diabetic group
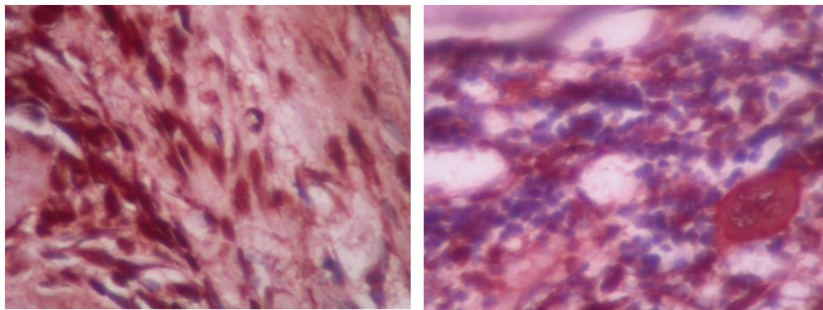

Figure 4 Immunohistochemistry results of osteocalcin expression of the nondiabetic group

DISCUSSION

Research results that have been processed and analyzed by statistical methods are in accordance with the research hypothesis. Interpretation of the data is discussed to determine the factors that influence the results of this study.

The Effect of Diabetes in RANKL/OPG Ratio

Bone remodeling is closely regulated by a triad of molecules consisting of RANKL, OPG, and RANK. NF-KB ligand receptor (RANKL) increases osteoclastogenesis by interaction with its receptors, whereas osteoprotegerin (OPG) inhibits this osteoclastogenesis binding to RANKL. The balance between OPG and RANKL plays an important role in bone pathophysiology. This study showed a significant increase in RANKL/OPG ratio in the diabetic model treatment group when compared with non-diabetic control group (p<0.001). These results are consistent with studies conducted by Stejskal et al., and Ying-Ying Wu et al., suggesting that diabetes increases the RANKL/OPG ratio and contributes to bone resorption.[5,12]

Hyperglycemic conditions in diabetes are thought to slow the healing process of the fracture by increasing the relative oxygen species (ROS) which will induce RANKL expression and promote greater osteoclast formation.[5,9] The bone healing process is also hampered by factors such as increased expression of cytokines and chemokines, an imbalance between the matrix lysis and its inhibitors, damage to bone repair capacity, decreased proliferation and increased bone apoptosis.[3]

Significant increases in TNF-α, MSCF and RANKL levels also occurred in the diabetes group. Graves et al. showed high levels of TNF-α, MSCF and RANKL have an effect on bone formation and have an adverse effect on bone strength.[13]

The Effect of Diabetes in Callus Diameter

In this study, a smaller callus diameter was identified in diabetic treatment group treated with post-fracture model and intramedullary nail fixation. This result proved to be significantly different from that of the nondiabetic control group which showed larger callus diameter (p <0.05). Independent t-test of the differences in mean callus diameter showed statistically significant results with p = 0.001.

This result is due to a decrease in osteoblast formation and function in subjects with diabetes, resulting in decreased callus diameter obtained in diabetic models. The decrease in osteocalcin levels in diabetic subjects compared with non-diabetics reflects a decrease in osteoblastic activity, which is inversely related to IL-6 and C-reactive protein (CRP).[7] This mechanism is caused by prolonged TNF expression in bones of diabetic subjects when stimulated by trauma or inflammation. It then increases the activity of the nuclear factor-kappa-B (NF-κB) and decreases expression of FRA-1 and RUNX2 in osteoblasts, as well as decreases expression of mediators that stimulate osteoblast growth and differentiation.[2] Also, diabetes-related inflammation may suppress the number of osteoblasts through apoptosis.

Several studies involving long bone fractures of diabetic mice induced by streptozotocin have demonstrated the decrease in callus diameter. The studies conducted by Gooch et al., concluded the bone healing disorder is characterized by the formation of smaller callus and minimal bone count, as well as a decrease in mechanical strength in the diabetic group when compared to the control group.[14] The same result also obtain by Krakauer et al. which shows diabetes affects the bone healing process through inhibiting bone formation and in the long run, hyperglycemia will lead to increased bone resorption that will lead to osteopenia.[15]

The Effect of Diabetes on Osteocalcin Expression

This study showed decreased levels of osteocalcin expression in diabetic treatment model group treated with post-fracture model and intramedullary nail fixation. These results proved to be

significantly different from those in the nondiabetic control group, which showed greater osteocalcin expression (p<0.05). Independent t-test on the mean difference of osteocalcin expression levels showed statistically significant results with p<0.001.

Osteocalcin is a protein synthesized by osteoblasts that bind hydroxyapatite in bone matrix and serves to regulate bone remodeling through negative feedback mechanisms. This protein is also an endocrine factor in regulating glucose hemostasis. Low osteocalcin levels are associated with an increased risk of fractures. This result is supported by Jehle et al., which shows lower serum osteocalcin and alkaline phosphatase levels in patients with type 1 diabetes.[16]

With the reduction in the number of osteoblasts in subjects with diabetes through apoptosis induced by inflammation, osteocalcin expression levels will be reduced by the number of osteoblasts available to synthesize the osteocalcin. It has been suggested that insulin insufficiency, hyperglycemia, and oxidative stress are mechanisms that affect fracture healing in subjects with diabetes. Its mechanism is through decreased osteoblast differentiation, increased osteoclast activity, and changes in apoptotic chondrocytes and osteoblasts.[11,17] Jiao et al. 's research supports this theory that decreased levels of osteocalcin in patients with type 2 diabetes reflecting a decrease in osteoblast activity, which is inversely proportional to IL-6 and C-reactive protein (CRP) levels.[2]

The diabetic model of streptozotocin-induced mice also showed a significant decrease in bone mineral density, bone mineral content, and 53% reduction in serum osteocalcin levels, as well as biomechanical strength in rat's long bone.[17] This result is supported by an increased in urinary calcium secretion and decreased calcium fraction reabsorption by the kidneys.[18]

Correlation Between RANKL/OPG Ratio, Callus Diameter and Osteocalcin Expression on Femoral Bone Fracture Healing in Diabetic Rat Model

Spearman's test between the ratio of RANKL/OPG to the number of osteocalcins showed a statistically significant negative correlation result with p <0.001 and correlation coefficient value -0.652. The same results were also obtained on Spearman's test between RANKL/OPG ratio and callus diameter which showed a statistically significant negative correlation result with p <0.001 and correlation coefficient value of -0.701. Both of these results shows the ratio of RANKL/OPG to the number of osteocalcin and callus diameter is inversely proportional. An increase in the RANKL/OPG ratio leads

to a decrease in the number of osteoblasts that will result in a decrease in the number of osteoclasts and the smaller callus diameter. It fits the results of the initial framework.

The correlation between the number of osteocalcins and callus diameter by Pearson test showed a statistically significant positive correlation with p <0.001 and correlation coefficient value 0.701, meaning the number of osteocalcin and callus diameter is directly proportional. This result reinforces the hypothesis that there is a close relationship between osteocalcin, which is a bode biomarker of bone remodeling, with callus diameter serving as an objective assessment for the process of bone formation.

In previous studies by Ying-Ying Wu et al., diabetes has been shown to increase the RANKL/OPG ratio and increase the expression of AGE, ROS, and other inflammatory mediators.[5] Those conditions lead to increased osteoclast and reduced number of osteoblasts through the process of apoptosis.[2] Graves et al. concluded that the state of hyperglycemia has also been shown to result in significant reductions in bone mineral density, bone mineral content, serum osteocalcin level, and reduced biomechanical strength in the long bones of subjects with diabetes.[13,19]

CONCLUSION

This research showed a statistically significant positive correlation between osteocalcin level and callus diameter using Pearson test (p <0.001) and a correlation coefficient of 0.701. This result signifies that the number of osteocalcin and callus diameter is directly proportional to each other. Also, a negative correlation was found in the diabetic model between RANKL/OPG ratio, callus diameter and osteocalcin expression in the healing of rat femoral bone fractures compared to the non-diabetic.

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