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# Effectiveness of Short-Term Use Denosumab and Risedronate Using β-Crosslaps and Histopathology as a Parameter in Osteoporotic Rat Model

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**Abstract**

Osteoporosis is a condition of decreased bone mass density. Pharmacological management uses drugs that decrease bone resorption or increase bone formation. The most commonly used drugs are bisphosphonates. Risedronate is one of these bisphosphonates. It inhibits osteoclasts, resulting in osteoclast apoptosis. Denosumab is a human monoclonal antibody that inhibits receptor activator kappa-B ligand, inhibiting osteoclast activation. Both drugs are widely used. Research on the efficacy of these two mechanisms has yet to obtain conclusive results. β-cross-laps is a parameter that can be used to evaluate the effectiveness of therapy through monitoring the bone resorption process. This experimental study used female rats >9 weeks old and was conducted at the Pharmacology and Therapeutic Laboratory, Universitas Padjadjaran Bandung, Indonesia, from June to September 2021. This study utilized a simple random sampling to allocate 24 experimental animals into three groups: control, risedronate, and denosumab. β-Crosslaps expression values before ovariectomy, post ovariectomy before receiving medication, and post ovariectomy and receiving medication was recorded and statistically analyzed using the SPSS version 24.0. The analysis of 24 samples revealed a statistically significant decrease in the median value of β-Crosslaps after ovariectomy in the denosumab group (p=0.036) when compared to the control group, whereas the decrease in the risedronate group was not significant (p=0.687). Administration of denosumab in rat models is more effective in reducing bone resorption compared to risedronate.

**Keywords:** β-cross-laps, denosumab, risedronate, osteoporosis

## Introduction

Osteoporosis is a public health problem that will increase as the population ages. Osteoporosis is a condition of decreasing bone mass density (BMD) to the reference. According to WHO, osteoporosis is when bone mass density has a T-score < –2.5; normal if the T-score > -1 and osteopenia if the T-score is between -1 to -2.5. [1]

Osteoporosis is caused by a combination of excess bone structure resorption, inadequate bone formation, and an imbalance in activity between bone cells that play a role in the remodeling process.[2] Osteoporosis occurs as a result of an increase in the number and activity of osteoclasts, the cells responsible for bone resorption; decreased number and activity of osteoblasts, bone-forming cells; or the presence of areas of bone that show both characteristics of abnormal bone cells.[2]

Bone mineral density (BMD) is the most important predictor for predicting fracture risk in osteoporosis. There are various methods available to evaluate bone loss. β-Crosslaps is a C-terminal telopeptide of type I collagen, a major component of the protein matrix of bone. β-Crosslaps are released into the bloodstream during resorption and are almost entirely excreted via the kidneys. Its level can be a specific parameter for the degradation of mature type I

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collagen from the bone.[3]

Pharmacological management of osteoporosis focuses on targeted remodeling through drugs that decrease bone resorption.[3] Bisphosphonates belong to the group of antiresorptive agents that have an inhibitory effect on osteoclasts, which reduces bone resorption.[4] Risedronate can inhibit bone resorption by reducing the action of the enzyme farnesyl pyrophosphate synthase (FPPS) at the osteoclastic level. Risedronate is also widely distributed in bone and has a half-life of up to weeks. Risedronate can reduce bone resorption by up to 50%.[5]

Denosumab is a human monoclonal antibody that inhibits the receptor activator kappa-B Ligand (RANKL) from binding to osteoprotegerin (OPG) so that the process of inhibiting osteoclast activation occurs. Denosumab has specific properties on certain TNF groups, including TNFa, TNF, and TNF associated with apoptosis. Denosumab is used for the management of metabolic bone diseases, including postmenopausal osteoporosis and osteoporosis due to glucocorticoid consumption.[6] Both risedronate and denosumab have the goal of reducing bone resorption. Research on the efficacy has yet to obtain conclusive results, so further research is still needed. In addition, no studies have been found that examine the effectiveness of denosumab with risedronate, which was assessed histopathologically. Thus, based on the description above, the authors conducted a study comparing the effectiveness of the short-term use of denosumab with risedronate using β-Crosslaps and histopathology parameters in a rat model of osteoporosis.[7]

## Methods

This study is an animal experimental research with a parallel design and a double-masked method, aimed at investigating causal correlations using experimental animals. The research received approval from the Health Research Ethics Committee of Universitas Padjadjaran (No. 683/UN6.KEP/EC/2021). It was conducted at the Pharmacology and Therapeutic Laboratory, Faculty of Medicine, Universitas Padjadjaran, Bandung, from June to September 2021. The study utilized female rats (*Mus musculus L.*) aged over nine weeks. Sample size calculations using the Federer formula determined that 8 samples per group were required.

Inclusion criteria for this study were female rats aged over nine weeks, weighing between 250 and 290 grams, in good health, and having undergone ovariectomy and dexamethasone injection to induce osteoporosis. Exclusion criteria included noticeable behavioral changes in the rats, such as refusal to eat or lack of movement. Dropout criteria comprised rats that died during the adaptation period or throughout the experiment.

The materials used in this study included experimental animals, risedronate tablets, denosumab injection, carboxymethyl cellulose powder, aquabides, dexamethasone injection, ketamine for surgery, a mouse β-Crosslaps ELISA kit, and food and water for the rats. The equipment required included animal cages, a minor surgical instrument set, a nasogastric tube, a 1 cc syringe, a plain vacuum tube, and general rat rearing equipment.

Experimental rats were obtained from the Pharmacology Laboratory of Universitas Padjadjaran. The rats underwent a one-week adaptation and conditioning period prior to the study. All rats were subjected to ovariectomy and received dexamethasone injections (0.1 mg/kg body weight, subcutaneously) to induce osteoporosis.

Randomization was achieved by dividing 18 rats into two initial groups of nine rats each. An additional group of nine rats, which did not receive any treatment, was included. This resulted in three groups: the control group (G1), the Risedronate group (G2), and the Denosumab group (G3). Rats in the control group (G1) did not receive any treatment for osteoporosis after the induction phase. Rats in the Risedronate group (G2) were administered risedronate orally at a dosage of 0.24 mg/kg body weight once daily for four weeks. Concurrently, rats in the Denosumab group (G3) received Denosumab injections subcutaneously at a dose of 6 mg/kg weekly for four weeks.

Blood samples will be drawn three times before ovariectomy, one month after ovariectomy and administration of dexamethasone, and one month after administration of denosumab or risedronate. The first and second samples were drawn from the tail blood/serum using a one cc syringe, while the third sample was taken from the heart after termination. Each blood draw is inserted into a vacuum tube (plain), recording the time from the start of inserting the blood into the tube. The sampling tube is a disposable, non-pyrogenic, and non-endotoxin tube. Next, the sample is sent from the Pharmacology Laboratory to the Clinical Pathology Laboratory using a bag/box at room.

The termination of the animals was carried out anesthetically using Pentobarbital, administered intravenously at a dosage of 100-150 mg/kg b.w. temperature and stored at -80°C. After all the samples were collected, they were stored at -80°C. Once ready for analysis, the samples were removed from the freezer and allowed to reach room temperature before being examined with the β-Crosslaps ELISA kit. The β-Crosslaps method uses a competitive ELISA to quantify C-terminal telopeptides of type I collagen (CTx-I), markers of bone resorption. Serum or plasma is introduced to antibody-coated wells, followed by a secondary, enzyme-linked antibody that competes with CTx-I for binding. The resulting color intensity from a chromogenic substrate is inversely related to CTx-I levels, providing a measure of bone turnover, crucial for diagnosing related conditions. Histopathological examination was carried out after euthanasia in rats using an anesthetic agent. The fragments of the femur bone were taken and then washed using 70% alcohol, purified with butyl alcohol (n-butanol), and added to paraffin. After that, axial and vertical sections with a thickness of 5 mm were taken and stained using the modified Masson Goldner's Trichome method and examined under an optical microscope to see bone components such as the haversian system and bone structure.31 Histopathological assessment was assessed by assessing the volume of the trabeculae bone as follows: 0= none, 1= mild, 2=moderate, 3=severe.

Statistical analysis was conducted to evaluate the significance of each variable using the Mann-Whitney test, performed with SPSS version 23.

## Results

The study was carried out from June to September 2021. Statistical test results in the research group on variable post-ovariectomy results, as shown in Table 1, obtained a p-value of 0.052 which is considered marginally significant. Post hoc analysis was continued to determine the more effective mechanism of action between risedronate and denosumab. In addition, it can be explained that there is no statistically significant β-Crosslaps difference between the three groups post-ovariectomy. The absence of statistically significant differences in β-Crosslaps levels among the three groups post-ovariectomy suggests effective therapeutic intervention. β-Crosslaps, a marker of bone resorption, typically increases following ovariectomy due to the resultant estrogen deficiency, which accelerates bone turnover. However, the introduction of treatments such as risedronate and denosumab in the study groups appears to have mitigated this effect.

The β-Crosslaps levels measured after ovariectomy in the control group, compared to those in the risedronate group, yielded a p-value of 0.052, which is marginally significant. The median β-Crosslaps values were 100.05 and 77.80, respectively. This suggests a decrease in β-Crosslaps in the risedronate group, indicating reduced bone resorption, although the result does not reach statistical significance under the strict $p<0.05$ criterion.

In contrast, the comparison between the control group (G1) and the denosumab group (G3) produced a p-value of 0.038, indicating a statistically significant difference. The median β-Crosslaps values for these groups were 100.05 and 70.75, respectively. This demonstrates a significant reduction in β-Crosslaps in the denosumab group, reflecting a reduction in bone resorption that is statistically significant.

Meanwhile, the group given risedronate and denosumab showed a p-value greater than 0.05 ($p>0.05$), indicating no statistically significant difference between these two treatment groups. .Based on a repeated ANOVA analysis with a p-value of 0.027 in the control group (G1), there is a statistically significant difference between Outcome II and III. The median β-Crosslaps

**Table 1 Comparison Results of β-Crosslaps (ng/mL) After Ovariectomy Outcome III In Three Groups**

| Variable | n | Median | Range (min.-max.) | p value |
|---|---|---|---|---|
| G1 | 8 | 100.05 | 62.90-148.80 | |
| G2 | 8 | 77.80 | 62.60-112.10 | **0.052**** |
| G3 | 8 | 70.75 | 51.50-120.70 | |

Description: Numerical data was tested with the Kruskal Wallis test—the significance value is based on $p<0.05$. The * sign indicates the p value<0.05 means significant or statistically significant. The ** sign indicates the value for marginally significant. G1= control group; G2=Group receiving risedronate; G3=Group receiving denosumab

**Table 3 Comparison of Changes in β-Crosslaps Results Between Outcome II and III**

| Group | n | Outcome (ng/mL) | | p value |
|---|---|---|---|---|
| | | II | III | |
| G1 | 8 | | | |
| Median | | 76.95 | 100.05 | **0.027*** |
| Range (min-max) | | 61.10-93.50 | 62.90-148.80 | |
| G2 | 8 | | | |
| Median | | 74.05 | 77.80 | **0.687** |
| Range (min-max) | | 38.10-85.70 | 62.60-112.10 | |
| G3 | 8 | | | |
| Median | | 77.80 | 70.75 | **0.036*** |
| Range (min-max) | | 61.40-88.60 | 51.50-120.70 | |

Description: The Repeated Anova test tested numerical data of p-value. The value of significance is based on the p-value of <0.05. The * sign indicates the p value<0.05 means significant or statistically significant. G1= control group; G2=The group that received risedronate; G3 = Group receiving denosumab. Outcome I = before ovariectomy, Outcome II = after ovariectomy, before medication is given, Outcome III = after ovariectomy, after medication

value increased from 76.95 pre-medication to 100.05 post-medication, indicating successful osteoporosis induction through the immobilization process during the two-month maintenance period in the treatment cages.

Comparing post-ovariectomy β-Crosslaps values before medication between the control group (G1) and the group given risedronate (G2) shows median values of 76.96 and 74.05, respectively. With a p-value greater than 0.05 (p>0.05), this indicates no statistically significant difference between these groups' pre-medication, suggesting that while risedronate inhibits bone resorption, its effect was not significant enough to differ statistically from the control.

The results in the group given denosumab (G3) revealed a p-value of 0.036, indicating a statistically significant decrease in bone resorption, consistent with findings from a 2012 study by McClung et al., which documented a persistent increase in bone mineral density (BMD) in the lumbar spine and hip over 8 years with denosumab treatment, resulting in final increases of 16.5% and 6.8%, respectively.[8]

Table 3 presents the median and range values for β-Crosslaps at two different time points: Outcome II (prior to medication) and Outcome III (after medication). In the Control group (G1), the median β-Crosslaps levels increased from 76.95 ng/mL at Outcome II to 100.05 ng/mL at Outcome III. The range also widened from 61.10-93.50 ng/mL to 62.90-148.80 ng/mL. This change was statistically significant, with a p-value of 0.027*, indicating a rise in β-Crosslaps levels following ovariectomy without any pharmacological intervention.

In the Risedronate group (G2) the β-Crosslaps levels showed a minor increase from a median of 74.05 ng/mL at Outcome II to 77.80 ng/mL at Outcome III, with the range extending from 38.10-85.70 ng/mL to 62.60-112.10 ng/mL. This increase was not statistically significant, as reflected by a p-value of 0.687.

In contrast, the Denosumab group (G3) exhibited a decrease in median β-Crosslaps levels from 77.80 ng/mL at Outcome II to 70.75 ng/mL at Outcome III. The range varied from 61.40-88.60 ng/mL to 51.50-120.70 ng/mL. This reduction was statistically significant, with a p-value of 0.036*.

## Discussion

This study evaluated the effects of short-term administration of denosumab and risedronate on β-Crosslaps levels and histopathological changes in an experimental osteoporosis model. The data indicate that neither denosumab nor risedronate demonstrated a significant advantage over the other in reducing β-Crosslaps levels over three months. However, denosumab exhibited a slightly more pronounced effect in decreasing bone resorption markers compared to risedronate, as evidenced by changes observed from pre-medication to post-medication phases. These findings suggest the need for further research comparing short-term and long-term pharmacological interventions for osteoporosis treatment. Extended observation periods are necessary to fully assess the therapeutic potential and histological impact of these drugs.

In another study conducted by Saag et al. where a comparison of the administration of risedronate with denosumab resulted in a significantly more significant increase in BMD in the femoral neck, lumbar spine, and total hip with denosumab than with risedronate as measured at 12 months of follow-up and 24 months.[9] The same study also reported the results of denosumab administration were compared with risedronate on bone resorption markers, assessed by the CTX and P1NP markers showing that denosumab was superior in increasing bone mineral density (BMD) at all assessed time points.

Previous studies have typically evaluated the effects of denosumab and risedronate over extended periods (greater than one year), with limited research focusing on short-term administration. For instance, a study by Murat Arslan et al. compared the effects of bisphosphonates administered for six months and found that β-Crosslaps levels had a significance value of 0.034, indicating a significant reduction compared to treatments involving hormones.[1]

Histopathological results showed no significant difference in the number of osteoclasts, osteoblastic rimming, trabecular thickness, lamellar meshwork density, and bone matrix percentage between the control group and groups receiving risedronate or denosumab. These findings suggest that short-term administration does not significantly alter these histological features, which is consistent with the β-Crosslaps results shown in Tables 2 and 3, indicating similar efficacy in bone resorption reduction between risedronate and denosumab. However, these results of this study differ from the study conducted by David et al. in 2018, which showed that the biopsy results of patients given denosumab for ten years showed normal histology.[9]

A study in the United States in 2010 showed that Denosumab significantly reduced bone resorption and reduced fracture rates. The results of the qualitative histological evaluation of the biopsy showed no significant results. In the Freedom study, median eroded surface was reduced by more than 80% and osteoclasts did not there were more than 50% of the biopsy results in the denosumab group.[10] The mean bone formation rate was reduced by 97%.42 The McClung et al. study, also demonstrated that denosumab significantly improved the trabecular bone score in postmenopausal women with osteoporosis. The percentage change in FFB was statistically significant compared to baseline ($p < 0.001$) and placebo (p 0.014). [11]

The results of previous studies differ from this study because there are differences in the time of the intervention. In this study, denosumab and risedronate were given for 3 months, whereas in the previous study, the average denosumab was given for 3 years every six months, and risedronate was given for 1 year every 6 months. The results of this study, as summarized in Table 2, indicate that denosumab and risedronate did not show a significant difference in terms of β-Crosslaps levels, with both treatments demonstrating similar effectiveness in reducing bone resorption ($p>0.05$). However, as shown in Table 3, denosumab was more effective in reducing β-Crosslaps levels from the pre-medication to post-medication phases compared to risedronate. This suggests that, although both treatments are similarly effective overall, denosumab may have a slightly superior effect on bone resorption in the short term. Further research with extended study periods is needed to fully assess the long-term effects of denosumab and risedronate on β-Crosslaps levels and histopathological features, as the short-term administration (less than one year) may not capture the complete effects of these treatments.

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