

## LONG-TERM GLUCOCORTICOID EFFECTS OF BONE LINING CELLS APOPTOSIS

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### ABSTRAK

*Tujuan dari penelitian ini adalah untuk menghitung jumlah apoptosis bone lining cells setelah pemberian glukokortikoid jangka panjang. Penelitian ini menggunakan Rattus norvegicus perempuan berusia 3 bulan yang dibagi menjadi 3 kelompok, masing-masing kelompok terdiri dari 7 ekor tikus. Kelompok-kelompok tersebut adalah: (1) kelompok kontrol, (2) kelompok perlakuan 1 diberi glukokortikoid 0,01 mg/hari, (3) kelompok perlakuan 2 diberi glukokortikoid 0,2 mg/hari. Pengobatan dilakukan selama 4 minggu dan pada akhir pengobatan, tikus dikorbankan dan dilanjutkan dengan persiapan, dan jumlah bone lining cells yang mengalami apoptosis dihitung melalui pemeriksaan bagian metafisis jaringan tulang femur menggunakan teknik imunohistokimia. Semua data dianalisis dengan analisis statistik Anova. Hasil penelitian menunjukkan bahwa jumlah sel-sel tulang yang melapisi apoptosis meningkat pada kelompok dengan pemberian glukokortikoid 0,01 mg/hari dan 0,2 mg/hari dibandingkan dengan kelompok kontrol dengan  $p = 0,000$  ( $p < 0,05$ ). Jumlah bone lining cells yang apoptosis pada kelompok glukokortikoid dosis 0,2 mg/hari lebih tinggi daripada kelompok dosis glukokortikoid 0,01 mg/hari dengan  $p = 0,000$  ( $p < 0,05$ ). Kesimpulannya pemberian jangka panjang glukokortikoid meningkatkan apoptosis dari bone lining cells. (FMI 2012;48:12-16)*

**Kata kunci:** Bone lining cells, apoptosis, glucocorticoid

### ABSTRACT

*The aim of this study was to count the amount of apoptotic bone lining cells after long term glucocorticoid distribution. This study used Rattus Norvegicus females aged 3 months that were divided into 3 groups, each group consisted of 7 rats. The groups are: (1) control group; (2) treatment group 1 were given glucocorticoid 0,01 mg/day; (3) treatment group 2 were given glucocorticoid 0,2mg/day. The treatment carried out for 4 weeks and at the end of treatment, mice were sacrificed and continued with preparation, and the number of bone lining cells that undergoing apoptosis was calculated through examination of the femur bone tissue metaphysis section using immunohistochemical technique. All data were analyzed with statistical analysis Anova. The result showed that the number of apoptotic bone lining cells is increased in group with glucocorticoid administration 0.01 mg/day and 0.2 mg/day compared to control group with  $p = 0.000$  ( $p < 0.05$ ). The number of apoptotic bone lining cells on the group of glucocorticoid dose 0.2 mg/day higher than the group of glucocorticoid dose 0.01 mg/day with  $p = 0.000$  ( $p < 0.05$ ). In conclusions long term glucocorticoid distribution increase apoptosis of bone lining cells. (FMI 2012;48:12-16)*

**Keywords:** Bone lining cells, apoptosis, glucocorticoid

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### INTRODUCTION

Glucocorticoids are produced and released by the adrenal glands as response to stress and regulates several physiological processes network. Among others, these hormones cause immunosuppression and anti-inflammatory action and affect apoptosis multiple cell types include T-lymphocytes and monocytes. Therefore, glucocorticoids are widely used in medicine as therapeutic immune system and inflammatory processes. Glucocorticoid therapy may save the patient's life but in the long-term use will cause side effects that can be life threatening, one of which is the loss of bone fracture risk (Adler & Hochberg 2003, Dvorak et al 2006).

Bone is a specialized connective tissue that make up the tissues and organ systems, serves as a means of motion, protection, and mineral homeostasis. Consists of four cells are osteoblasts, osteocytes, bone lining cells, and osteoclasts. Bone lining cells derived from mesenchymal stem cells or cells osteoprogenitor, which is a relatively inactive form of osteoblast cells as "resting cells of osteoblasts". Other researchers reported that bone lining cells may be the precursors of osteoblasts that regulate the growth of bone crystals or as a barrier between the extracellular fluid and bones (Downey & Siegel 2006, Datta et al 2008, Lorenzo et al 2008). Each organ get spare cells from stem cells, such as muscle satellite cells, basal cells of the skin and

lining cells on the bone. In the event of damage or apoptosis then any manipulation will not be able to fix up the cells so that the number will decrease. Similarly with bone, if the bone lining cells is reduced due to increased apoptosis of the backup process for the formation of bone cells will decrease the risk for an increased risk of fractures. Epidemiological data indicate that glucocorticoid therapy increases the risk of hip fractures and distal radius doubled and quadrupled spine. This is due to the provision of long- term glucocorticoid therapy causes rapid bone loss and weight resulting in increased risk of bone fractures. From the analysis of the General Practice Research Database (UK) on Veterans Affairs Medical Centers, from 40 % patients with respiratory disease who received glucocorticoid therapy with an increased daily dose found an increased relative risk of fracture of the femur and spine (Abu et al 2000, Adler & Hochberg 2003, Takuma et al 2003, Jia et al 2006, Kim et al 2006, Swanson et al 2006, Lu et al 2007). Although it has been many laboratory studies about the effects of glucocorticoids on bone but the molecular mechanisms underlying the onset of decline in bone formation process described with increased apoptosis of bone-lining cells, has not been disclosed. Based on these facts, the research will be conducted to prove whether long - term distribution of glucocorticoids externally will improve the process of apoptosis of bone lining cells.

**MATERIAL & METHODS**

This study is an experimental study to examine the causal relationship between the treatment outcome after treatment in a time period. In this study as the experimental unit is mice (*Rattus norvegicus* Wistar strain), female, age 3 months. The number of samples used is 21, divided into 3 groups: control group, treatment group 1 low-dose glucocorticoid distribution (0.01 mg/200 g body weight/day), treatment group 2 high-dose glucocorticoid distribution (0.2 mg/200 g weight/day). The study was conducted for 4 weeks, then the mice were sacrificed for its femur and then doing preparation for immunohistochemistry. After that counting the number of bone-lining cells that undergo apoptosis, characterized by chromatin condensation and fragmentation of nuclei by immunohistochemistry (Weinstein et al 2000), calculate each microscopic point of view.

**RESULTS**

The data obtained from the studies of bone lining cells undergoing apoptosis due to low dose glucocorticoid

distribution of 0.01 mg/200 g BB and high dose distribution 0.2 mg/200 g BB in the long term (4 weeks) were calculated based on test results on the metaphysical basis immunohistology femur bone tissue. The higher the dose was given, the greater the number of cells undergoing apoptosis. These variables were tested by using a statistical test one way analysis of variance (one way anava), which aims to look at the effect of treatment between groups on the dependent variable. The results of the analysis of variance calculations show that there is a very significant difference,  $p = 0.000$  ( $p < 0.05$ ). To find out where the different between groups, followed by a different test (multiple comparisons) through Post Hoc Tests

Table 1. Results of different test variables between groups of apoptotic cells

mGROUP		p
Control	Dose 0.01 mg	0.000
	Dose 0.2 mg	0.000
Dose 0.01 mg	Dose 0.2 mg	0.000

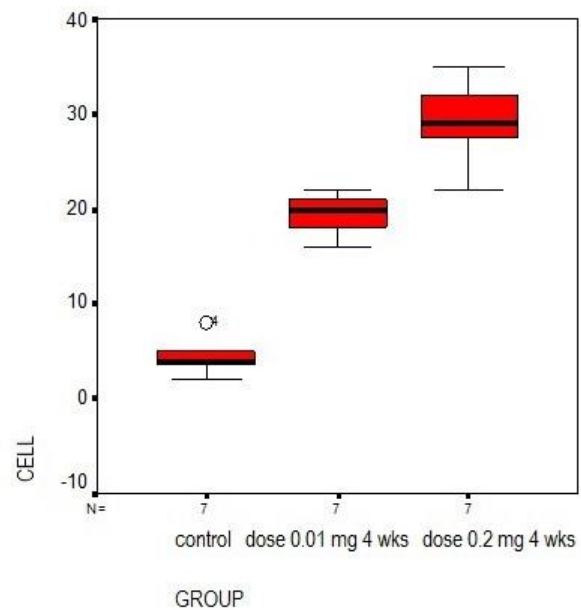


Figure 1. The results of the analysis of the mean apoptotic cell groups

The results of the analysis of variance calculations (Table 1), there are differences in bone lining cells undergoing apoptosis between the control group and the treatment group. Bone lining cells in mice that received 0.2 mg dose glucocorticoid treatment experienced more apoptosis than 0.01 mg-dose glucocorticoids. Mice that received moderate-dose glucocorticoid treatment of 0.01

mg of bone-lining cells undergoing apoptosis greater than untreated mice (Figure 1).

Immunohistochemistry in the control group showed that bone lining cells appear normal on the surface of trabecular bone (Figure 2). Immunohistochemical examination results in the group with administration of 0.01 mg dose glucocorticoid Tunnel assay using cells undergoing apoptosis showed dark brown color changes to black in the cell nucleus that indicates the occurrence of DNA fragmentation (Figure 3).

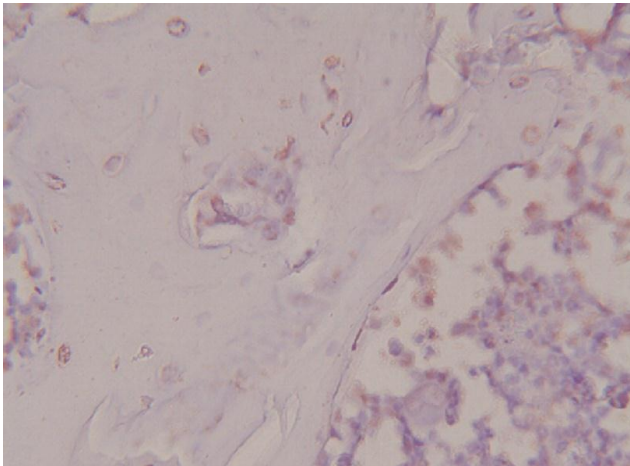


Figure 2. Bone-lining cells in the control group. X 400

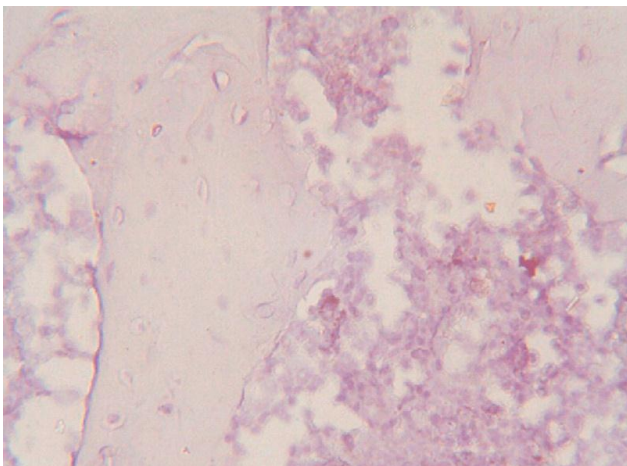


Figure 3. Apoptosis of bone lining cells in the 0.01 mg group glucocorticoid administration. X400

Overview immunohistochemistry using methods Tunnel assay for group glucocorticoid distribution 0.2 mg, cells undergoing apoptosis show a dark brown color changes to black in the cell nucleus that indicates the occurrence of DNA fragmentation. At the 0.2 mg group number of apoptotic bone-lining cells more than 0.01 mg group (Figure 4).

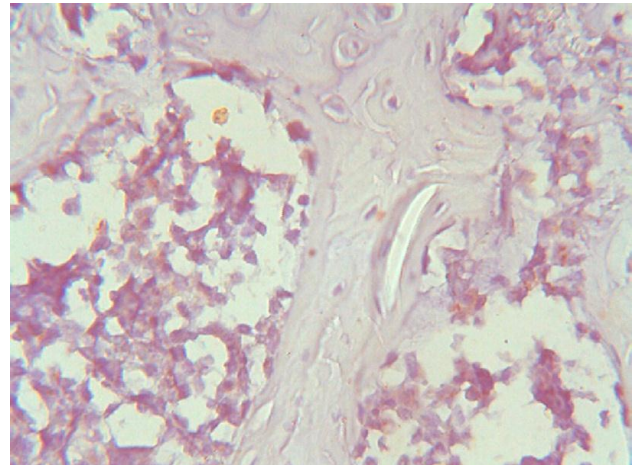


Figure 4. Apoptosis of bone lining cells in the 0.2 mg group glucocorticoid administration. X400

## DISCUSSION

The objective from this study was to examine the causal relationship between treatment and treatment outcome after a time period. The treatment given to the experimental animals, in this study using drugs from glucocorticoid group that is Dexamethasone with low dose variation of 0.01 mg per 200 g body weight of rats and high dose of 0.2 mg per 200 g body weight of rat based dose calculation of conversion table of various animals and humans.

Dexamethasone is used in this study is drugs from glucocorticoid group that easily available, cheap prices and a group of drugs commonly used in treatment in the community, especially for chronic diseases. Weinstein et al (2000), states that bone destruction occurs in 25 % of patients who received glucocorticoid treatment. Although it has been done before treatment observation of femoral head collapse and severe osteoporosis but an attempt to reduce the dose or discontinue treatment usually unsuccessful because of the need for glucocorticoid treatment, such as in patients with transplants, lung disease, arthritis, autoimmune, haemopoietic and gastrointestinal. Consequently, the patient will receive treatment in months, years and even throughout his life.

Increased risk of bone damage with increasing dose and duration of treatment. In this study performed in a mice model of glucocorticoid distribution in long term that is 4 weeks. According Suatmadji (2001), the mice who received glucocorticoids in 4 weeks equivalent to 3-4 years in humans, showed a decrease in bone mineral density associated with a decrease in the number of osteoblasts, progenitor inside bone marrow, and

dramatic reduction in trabecular bone. These changes were associated with a significant reduction in osteoid area and reduced mineral apposition and bone formation. Glucocorticoids affect bone destruction through fat embolism, tampon de vascular on femoral caput blood vessels through the retention of fat or bone marrow fluid, another possibility is programmed cell death or apoptosis that are part of the mechanism of bone destruction. Weinstein et al (2000) reports mention that mice who received glucocorticoids for 4 weeks showed a threefold increase osteoblast apoptosis in vertebral cancellous bone and 28 % apoptosis of osteocytes in cortical bone metaphysis.

Examination of apoptosis of bone lining cells using immunohistochemistry way through the Tunnel assay (terminal deoxyuridine nucleotide end labeling assay), a development examination of methods apoptosis through DNA fragmentation, which can overcome the detection difficulty of apoptosis by usual hematoxylin eosin staining. DNA fragments detected by enzymatic labeling of the end 3' nucleotides OH. Ends of the DNA fragments are found in the nucleus cells undergoing apoptosis and apoptotic bodies. Tunnel assay can detect early stages of apoptosis because DNA fragmentation occurs before the morphologic changes seen in histological preparations. Apoptosis usually occurs in groups of cells close to normal cells, appear on the picture with the cell nucleus condensation and chromatin fragmentation (Weinstein et al 2000). Counting the number of apoptotic cells in this study bone lining seen in the femur bone tissue. After the manufacture of femur tissue preparation, count the number of bone lining cells undergoing apoptosis.

Animal body part being examined is the metaphysical part of the femur. Each type of bone consists of cortical and trabecular parts that have a certain proportion depending on the type of bone. In general, bone contains more trabecular bone that have a wider surfaces, so metabolic activity greater then when compared to cortical bone. Therefore, trabecular bone is more often experience changes, so have a tendency to shortage of bone mass. This is one reason why vertebrae, collum femur and metaphysis of long bones are is a place that often experience fractures in people with osteoporosis. Based on research from, proved that these bones have significance to the variation in the structure and density of the bone tissue. Therefore, this study conducted checks on the femur metaphysis region, the area of ??the femur close to the femoral collum where there is more trabecular bone than cortical bone, that has a higher metabolic capability, so it making easy to see changes in bone cell metabolism.

In general, males and black skin have a higher bone density than women. While another study states that the levels of glucocorticoids (corticosterone) in women and animals higher than men. So in this study were selected mice that are animal which has many similarities with the human metabolism, thus chosen as the model of this study. 3-month-old mice with consideration that the age is adult age of animals, with the consideration that at adult age growth hormone effect is not as big as the effect of growth hormone during pre- adult age, so it does not much affect bone growth.

According to Weinstein et al (2000), the long-term glucocorticoid will cause osteoblast apoptosis in patients with osteoporosis. Past research has shown that glucocorticoids work directly on the bone-forming cells (osteoblastic lineage) through the glucocorticoid receptor (GR?) on bone forming surface, where the remodeling process occurs in cultured osteocytes and osteoblasts (Abu et al 2000). Glucocorticoids modulate the activity of intracellular kinase Proline-rich tyrosine kinase 2 (Pyk2) members of the focal adhesion kinase (FAK) family of non- receptor tyrosine kinase. Although Pyk2 and FAK are highly homologous, but these proteins showed different effects on cell fate. FAK activation causing cell multiplication and cell defense, Pyk2 affect the reorganization of the cytoskeleton, cell damage and apoptosis (Park et al 2004, Plotkin et al 2007). In the event of damage or apoptosis then any manipulation will not be able to fix the cells so that the number will decrease. Similarly to the bone, if the bone lining cells are reserve cells (resting cells of osteoblasts) is reduced due to an increase in the apoptotic process, the process of bone formation decreases with a decrease in bone density are at risk of osteoporosis.

Osteoblasts are the cells that secrete organic matrix, place for precipitate calcium phosphate crystals (hydroxyapatite) which among other collagen as an ingredient that determines bone density (Sherwood 2004). Meanwhile, according to Downey and Siegel (2006), the osteoblasts, osteocytes and bone lining cells derived from stem cells or mesensimal known as Osteoprogenitor. Osteoblasts development will follow three forms: (1) active osteoblasts, (2) surrounded by matrix become osteocytes, or (3) become relatively inactive and form bone lining cells. Based on this statement the long-term distribution of glucocorticoids can also occur at the cell bone lining from same given with osteoblasts. This study proves that long-term glucocorticoids distribution can lead to apoptosis in bone lining cells .

Bone lining cells appear as flat form cells, thin, elongated, covering most of the surface of mature bone. Cytoplasmic protrusions or its gap junctions connected

each other or with osteocytes. Because having an inactive metabolism, bone lining cells contains fewer organelles and cytoplasm than osteoblasts. At one point bone lining cells will be "resting osteoblasts" or "surface osteocytes" (Downey & Siegel 2006). On this basis the results of this study can be said to be important because bone lining cells are reserve cells for osteoblasts. Long-term glucocorticoid treatment causes apoptosis of osteoblasts and osteocytes (Weinstein et al 2000) and proved in this study, also occur in bone lining cells will further exacerbate the resulting decrease in bone density. Other researchers say that the bone lining cells as osteoblast precursors governing growth of crystal bone (hydroxyapatite) (Downey & Siegel 2006), then in the event of an increase apoptosis of bone lining cells, due to long- term glucocorticoids distribution, bone density will decrease.

## CONCLUSION

Giving glucocorticoids 0.01 mg /day and 0.2 mg /day for four weeks in *Rattus norvegicus* will increase the number of apoptotic bone lining cells. Giving glucocorticoid dose 0.2 mg/day showed the number of apoptotic bone lining cells is higher than the dose of 0.01 mg/day.

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