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SPILANTHES ACMELLA AND PHYSICAL EXERCISE INCREASED TESTOSTERONE LEVELS AND OSTEOBLAST CELLS IN GLUCOCORTICOID-INDUCED OSTEOPOROSIS MALE MICE

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Background: Glucocorticoid-induced osteoporosis is leading cause of secondary osteoporosis by decreasing formation activity and increasing resorption activity. Spilanthes acmella, is one of Indonesia medicinal plants that contain of polyphenol and flavonoids. Previously in vitro study showed that buthanol and water fraction from this plant have increased alkaline phosphatase that known as marker of bone formation. The objective of this study to analyze the effect of *Spilanthes acmella* and physical exercise in increasing testosterone and osteoblast cells of femoral's trabecular glucocorticoid-induced osteoporosis male mice. **Method:** This study using a posttest control group design, 36 male healthy mice (5 months old) were randomizely devided into 6 groups, there are : 1.Healthy control group (without induction dexamethaxone), 2.Osteoporosis groups (induction with dexamethaxone without treatment), 3.Positive control receive suspension alendronat, 4.70% Ethanol extract of Spilanthes acmella group, 5.Combination group of 70% extract ethanol of Spilanthes acmella and exercise, and 6.Exercise group (walking using mice treadmill 10m/minute, 5-12 minutes 3 times a week). All of the intervention were given for 4 weeks. The serum levels of testosterone were determined using immunoserology (ELISA) and osteoblast cells were determined histomorphometry by light microscopy. All statistical test were carried out using SPSS 23 and statistical significance was set at $p<0.05$ for all analysis. The testosterone levels between group were compared using Mann-Whitney test and osteoblast cells between group were compared with multiple comparison. **Results:** It showed that the alendronate group, combination group and the exercise group increasing testosterone level ($p<0.05$) from that osteoporotic group. There were also increasing osteoblast cells ($p<0.05$) in the alendronate group and combination group. There was no correlation between testosterone level and osteoblast cells ($p>0.05$). **Conclusion:** It proved that 70% ethanol extract of Spilanthes acmella have an additive effect to weight bearing exercise in glucocorticoid-induced osteoporosis male mice.

Keywords: Glucocorticoid; Induced; Osteoporosis; Spilanthes acmella; Testosterone

INTRODUCTION

Osteoporosis is defined as a skeletal disorder of compromised bone strength predisposing to an increased risk of fracture.[1] Recent epidemiological studies have shown that osteoporosis become as a worldwide major public health problem not only for women population but also in men population. It is estimated that the total number of hip fractures in women and men in 2025 will be similar.[2] In men the distribution of prevalence of osteoporosis is bimodal, the early peak (before age 50) is mostly due secondary osteoporosis, while the later peak (after age 60) mostly represents primary osteoporosis.[3] According to the World Health Organization (WHO) by applying the standard from The International Society for Clinical Densitometry it is estimated that 1 to 2 million men in the United

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Background: Glucocorticoid-induced osteoporosis is leading cause of secondary osteoporosis by decreasing formation activity and increasing resorption activity. Spilanthes acmella is one of Indonesia medicinal plants that contain of polyphenol and flavonoids. Previously, in vitro study showed that buthanol and water fraction from this plant have increased alkaline phosphatase that known as marker of bone formation. The objective of this study is analyze the effect of Spilanthes acmella and physical exercise in increasing testosterone and osteoblast cells of female's underwent a glucocorticoid-induced osteoporosis male mice. **Method:** This study using a positive control group design, 36 male healthy mice (5 months old) were randomly divided into 6 groups, there are 1 Healthy control group (without induction dexamethasone), 2 Osteoporosis group (induction with dexamethasone without treatment), 3 Positive control receive suspension alendronate, 4 70% Ethanol extract of Spilanthes acmella group, 5 Combination group of 70% extract ethanol of Spilanthes acmella and exercise, and 6 Exercise group (walking using rate treadmill 10m minute, 5-12 minutes 3 times a week). All of the intervention were given for 4 weeks. The serum levels of testosterone were determined using immunocerology (ELIS V) and osteoblast cells were determined histomorphometry by light microscopy. All statistical test were carried out using SPSS 23 and statistical significance was set at $p<0.05$ for all analyses. The testosterone levels between group were compared using Mann-Whitney test and osteoblast cells between group were compared with multiple comparison. **Results:** It showed that the administration group, combination group and the exercise group increasing testosterone level ($p<0.05$) from that control group. There were also increasing osteoblast cells ($p<0.05$) in the alendronate group and combination group. There was no correlation between testosterone level and osteoblast cells ($p>0.05$). **Conclusion:** It proved that 70% ethanol extract of Spilanthes acmella has an additive effect to weight bearing exercise in glucocorticoid-induced osteoporosis male mice.

Keywords: Glucocorticoid; Induced; Osteoporosis; Spilanthes acmella; Testosterone

INTRODUCTION

Osteoporosis is defined as a skeletal disorder of compromised bone strength predisposing to an increased risk of fracture.[1] Recent epidemiological studies have shown that osteoporosis occurs as a

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States have osteoporosis (T-score <-2.5) and 8 to 13 million have osteopenia (T-score between -1.0 and -2.5) or the prevalence are 6% for osteoporosis and 47% for osteopenia.[2] In aging population, morbidity and mortality from hip fractures are higher in men than in women with fatality rates among over 75 years is 20.7% in men versus 7.5% in women.[1] The causes osteoporosis in men are related to genetics, environmental, hormonal and disease-specific factors, and approximately 50% of men with osteoporosis are secondary osteoporosis.[2] The three major causes of secondary osteoporosis in men are alcohol abuse, glucocorticoid excess (Cushing's syndrome or long-term glucocorticoid therapy) and hypogonadism. The prevention and treatment according Recommendation of American College of Rheumatology Ad Hoc Committe including supplementation with calcium and vitamin D, antiresorptive agents (bisphosphonates),calcitonin, replacement of gonadal sex hormone (testosterone replacement therapy), and modify lifestyle risk factors.[4] Clinical evidence suggests a role for phytoestrogen in the treatment of post-menopausal osteoporosis.[5,6] Based on screening of 32 Indonesian tradisional medicinal plants Spilanthes acmella aerial parts stimulated ALP activity. Previously in vitro study showed that buthanol and water fraction from this plant have increased alkaline phosphatase (ALP) that known as marker of osteoblast differentiation.[7] Phytochemistry study showed the major constituent in this plant was spilanthol (N-isobutylamide) and there also triterpenoid.[8] Suthikrai *et al.*,(2010) reported that Spilanthes acmella contains 0.59-1.39 ng/g of phytotestosterone.[9] There have been many reports the effect of phytoestrogen and exercise in invivo studies, but still few invivo studies which reports the effect of phytotestosterone and exercise. The objective of this study to analyze the effects of *Spilanthes acmella* and physical exercise in increasing testosterone and osteoblast cells of femoral's trabecular in glucocorticoid–induced osteoporosis male mice.

MATERIALS AND METHOD

This study using a posttest control group design. Thirty six male healthy mice (5 months old) with the mean body weight 19.208 ±10.265 gram, were randomizely devided into 6 groups, there are : healthy control group, osteoporosis group, alendronate group: osteoporosis received alendronate suspension (0.026 mg/20 g BW/day), Spilanthes acmella group : osteoporosis received 70% ethanol extract of Spilanthes acmella (4.14 mg/20 g BW/day), exercise group: osteoporosis with intervention walking with velocity 7-10m/min for 5-12 minutes, 3 times/week, and combination group: osteoporosis received 70% ethanol extract of Spilanthes acmella and exercise. To determined the effect of dexamethasone (0.002 mg/20g BW/day for 4 weeks) the trabecular area of proximal femur from six normal male rats and six male rats who received 4 weeks dexamethasone were determined histomorphometry. Four weeks after intervention the serum testosterone levels were determined with immunoserology (ELISA). After femurs were removed, immediately fixed in 10% neutral-buffered formalin, and placed in decalcifying solution for 24 h at 37°C, continuous with dehydrated and embedded in paraffin. The proximal femur section dyed with a haematoxylin-eosin (HE) stain. Osteoblast cells were determined histomorphometry by light microscopy, magnifying 2000 times. The amount of osteoblast cells can be counted as a total osteoblast per five field area. Because the test of normality in osteoporosis and spilanthes acmella groups significant ($p<0.05$), the testosterone levels between the groups were compared using Mann-Whitney test. Multiple comparison test was applied to determine the specific difference between the groups of osteoblast cells. All statistical test were carried out using SPSS 23 and statistical significance was set at $p<0.05$ for all analysis.

RESULTS

Histomorphometry were determined based on the effect of dexamethasone (0.002 mg/20g BW/day for 4 weeks) the trabecular area of proximal femur from six normal male rats and six male rats who received 4 weeks. The result was presented in Figure1.

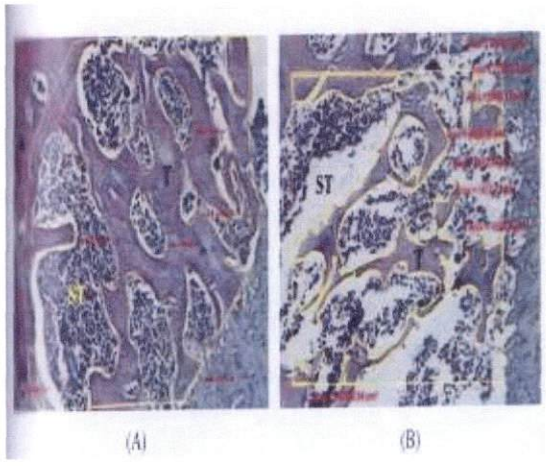

Figure 1
Histomorphometry trabecular area of the proximal femur of the normal control male rat (A) and after received dexamethasone for 4 weeks (B). The section dyed with a haematoxylin-eosin, magnifying 100 times. There was decrease in the thickness of trabeculae (T) in the dexamethasone group (B) compared to the normal control groups (A). BM: bone marrow, T: trabecular area

There is a significant increase of testosterone levels after intervention compared to osteoporosis and Spilanthes acmella groups (p<0.05). The result can be seen in Figure 2.

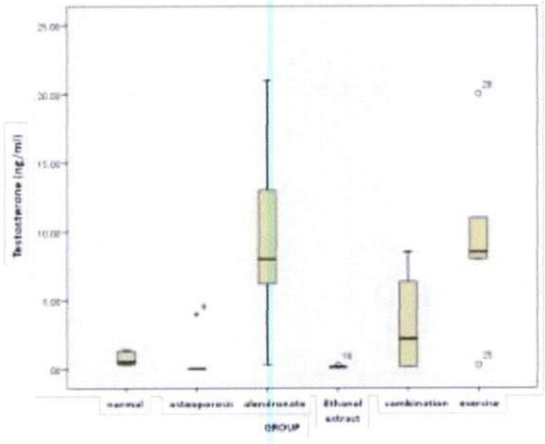

Figure 2
Testosterone levels after intervention. Note there is a significant increase (p<0.05) the testosterone levels in the alendronate group, combination group and exercise group, compared to osteoporosis and Spilanthes acmella groups.

In this study we obtained that osteoblast cells after interventions is a significant increase osteoblast cells in the alendronate and combination groups (p<0.05), compared to the osteoporosis, spilanthes acmella and exercise groups (Figure 3)

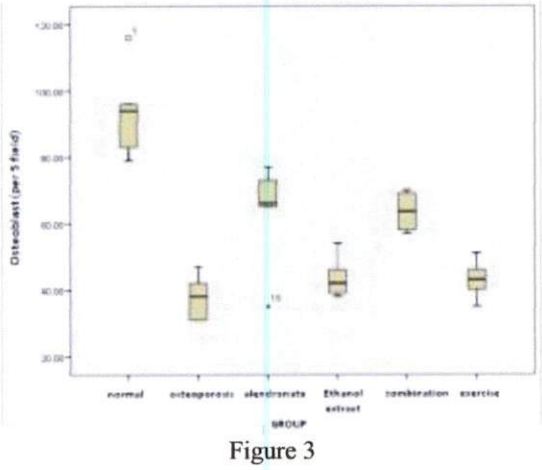

Figure 3

Osteoblast cells after interventions. Note there is a significant increase osteoblast cells in the alendronate and combination groups (p<0.05), compared to the osteoporosis, spilanthes acmella and exercise groups.

Histomorphometry trabecular area of the proximal femur can be seen in Figure 4.

DISCUSSION

There were significancy difference of testosterone levels on alendronate group (p=0.016), combination group (p=0.048) and exercise group (p=0.016) from osteoporotic group but not significance difference in ethanol extract group (p=0.112). The effect of combination group same with the effect of alendronate group (p=0.789) and exercise group (p=0.895).

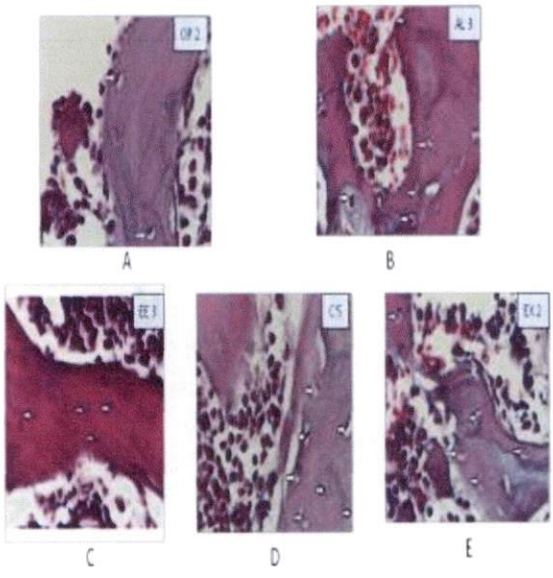

Figure 4
Histomorphometry trabecular area of the proximal femur. The section dyed with a haematoxylin-eosin, osteoblast cells was counted by light microscopy, magnifying 2000 times. Note there is an increase osteoblast cell (arrow) in the alendronate (B) and combination group (D) compared to the osteoporosis (A), Spilanthes acmella (C) and exercise (E) groups.

This study showed that in the osteoporotic group (after intervention with dexamethasone for 4 weeks), the testosterone level is lower than healthy group and ethanol extract group but not significant. In human, after 3 months glucocorticoid administration will decline sex steroid production by supression of gonadal function and pituitary gonadotropin secretion.[3] Low serum total testosterone (below 3 ng/ml) was the cause of osteoporosis. There is less information regarding hypogonadism secondary to glucocorticoid treatment.

There was an increase of testosterone level in alendronate group. Not too much information regarding the mechanism. However the possibility that its action not directly from alendronate. Alendronate was found increase bone mass in lumbar spine and femoral neck in androgen-

replaced men with long-term hypogonadism after 12 months of alendronate treatment. The urinary marker of bone resorption (urinary deoxypyridinoline) decreased significantly after 6 months of therapy with alendronate.[10] This condition will result balance of bone remodeling and increasing of serum marker of bone formation (osteocalcin). Osteocalcin promotes testosterone production in the Leydig cell by activating steroidogenesis enzymes.[11,12]

In this study 4.14 mg/20g BW/day ethanol extract of Spilanthes acmella treatment was not increase testosterone level. The possibility of the result in this study could cause by the effect of intestinal metabolism of phytotestosterone[13] or the other possibility in osteoporotic condition several cytokines such IL-1β, IL-11 and TNFα stimulated aromatase activity of osteoblast-like cells in vitro, convert testosterone to estrogen.[14] This result contrary with Sharma *et al.*,(2011), they found that in healthy male rats who received 50, 100 and 150 mg/kg Spilanthes acmella extract, serum testosterone level increased significantly in comparison to the control group.[15] Peripheral aromatization of testosterone into estrogen may a key role in maintaining estrogen level in osteoporotic condition. In this study was not measure the estrogen level.

In exercise group the testosterone levels was increase may cause by induction the hormonal and immune respons.[16] Lane reported that moderate and high intensity exercise caused an increase in both salivary and serum testosterone level.[17]

In combination group the testosterone level also increased signficantly. The result same with Laswati in vivo study (2007), in postmenopause mice, the estrogen levels increased highest significantly in combination group than only phytoestrogen treatment or exercise intervention.[18]

From one-way ANOVA analysis the level significancy p=0.000, there was minimally one pair group have significant different of osteoblast cells. From multiple comparison analysis there were significant different between osteoporotic group and alendronate group (p=0.001) and combination group (p=0.001) but not significant different with spilanthes acmella group (p= 0.378) and exercise group (p=0.444). The spilanthes acmella and exercise group have the same effect (p=0.906), but the combination group have the same effect with alendronate group (p=0.967).

In this study showed that osteoblast cells in the osteoporotic group without intervention ethanol extract and exercise is lower than the other groups. Glucocorticoid blunt intestinal calcium absorption directly and secondary hyperparathyroidism develops, increasing osteoclast life span and activity and skeletal turnover, also directly blunt osteoblast activity, decrease in the lifespan osteoblasts and induce osteocyte apoptosis.[3,19] (2,3 Licata, Weinstein). Isoenzyme 11β-hydroxysteroid dehydrogenase (11β-HSD1) expression, a prereceptor modulator of glucocorticoid action increases with glucocorticoid administration.[19] Ma *et al.*,(2011) reported that in vivo glucocorticoid may increase the expression and signaling activity of β2-adrenergic receptors (β2AR) in osteoblasts as antianabolic effect of sympathetic neuron. Stimulation of the β2-adrenergic receptors (β2AR) in osteoblasts by norepinephrine or isoproterenol inhibits osteoblast proliferation, stimulates osteoclastogenesis and up regulation of nuclear factor-κB ligand (RANKL) expression. Study with pharmacological and genetic β2AR blockade in mice significantly reduced the bone catabolic effect of high-dose prednisolone in vivo. In vitro study shows a direct effect and genomic effect of the glucocorticoid receptor (GR) on the Adrβ2 promoter.[20]

There was increased significantly osteoblast cells in alendronate group. Shimon *et al.*, (2005) reported that alendronate treatment 10 mg daily for 6 and 12 months in osteoporotic men with long-standing hypogonadism and receiving standar testosterone replacement treatment increased lumbar-spine bone mineral density (BMD) significantly (p<0.005).[10] A 2-year double-blind, placebo-controlled trial of 10 mg of alendronate daily was carried out in 241 men with osteoporosis who were aged 31 to 87. After 2 years, men in the alendronate group showed a 7.1% increase in bone density at the lumbar spine, but those in the placebo group showed a 1.8% increase.[21] Alendronate is an anti-resoptive agent, inhibit farnesyl diphosphate (FPP) synthase, thus blocking the prenylation of small signalling proteins essential for osteoclast function and survival.

In this study showed that spilanthes acmella group increased osteoblast cells but not significant. Gonadal and adrenal testosterone (C19 steroid) can be converted into estrogen (C18 steroids) by P450 aromatase (CYP 19) which present in bone. Studies in knock-out mice show estrogen receptor (ER) activation, but not androgen receptor (AR) activation is involved in the regulation of skeletal growth in mice . However studies in rats with aromatase inhibition is associated with osteopenia, suggesting that androgen also regulate bone metabolism either directly by stimulation of AR or indirectly by aromatization of androgen.[22] Testosterone has a dual mode of action on different bone surfaces with involvement of both the ER and AR. One animal study have investigated the bone phenotype of transgenic male animal with KO of AR (ArKO), ERα (ERKO) , ERβ (BERKO) and ERα and ERβ (DERKO). AR and ERα can independently mediate the cancelous bone-sparing effects of sex steroid in male mice.[22] Okazaki

(2002) reported that estrogen treatment through bone morphogenetic protein-2 (BMP-2) was increased osteoblast cell.[23] In vivo study with glucocorticoid-induced osteoporosis female rats, genistein aglycon showed a greater increased in bone mineral density, and significantly increased bone-alkaline phosphastase as a marker of osteoblast differentiation.[24] The possibility of the result in this study could cause by the effect of intestinal metabolism of phytotestosterone[13] and may depend on aromatization of phytotestosterone to estrogen.[22]

In exercise group we found osteoblast cells increase but not significantly. During physical activity, mechanical forces are exerted on the bones through ground reaction forces and by the contractile activity of muscles. Osteocytes are highly mechanosensitive, alter the production of a multitude of signaling molecules when triggered by a mechanical stimulus. Mechanically activated osteocytes produce signaling molecules like bone morphogenetic proteins (BMPs), Wnts, prostaglandin E2 (PGE2), and NO, which can modulate the recruitment, differentiation, and activity of osteoblasts.[25] Cheng at al., (2002) reported that the anabolic effect of strain on osteoblast cell numbers is mediated by IGF's action through the IGF-1 receptor (IGF-1R) within the cell membrane and this responsiveness to a ligand is regulated by integrins.[26] This result in this study might be an effect of the low sensitivity of the mice skeleton to moderate intensity for 4 weeks. Other factors such as stress have probably influenced the results.

Combination of exercise and spilanthes acmella treatment showed increased osteoblast cells significancy. This may cause by cross-talk mechanism of the IGF-1 and estrogen. The number and activity of ER were regulated by estrogen. The effect of mechanical force from exercise on osteoblast cell numbers is mediated by IGF's action through the IGF-1 receptor (IGF-1R). IGF-1R requires association with ligand-bound ERα that will results in IGF-1R autophosphorylation and activation downstream mitogen activated regulated kinase (MAPK) and extracellular regulated kinase (ERK) signaling cascade for osteoblast survival and proliferation.[26] In this study the effect of combination group have the same effect to increasing osteoblast cells with alendronate group (p=0.967)

There is no correlation between testosterone level and osteoblast cells (r=0.177; p=0.358). A study on androgen supplementation in eugonadal men with osteoporosis, the increase in BMD and the reduction in bone turnover positively correlated with estradiol, but not in testosterone levels indicating of conversion androgen to estrogen.[2] In this study peripheral aromatization of testosterone into estrogen may have a role in osteoporosis condition.

CONCLUSIONS

Seventy percentage of ethanol extract of Spilanthes acmella have an additive effect to weight bearing exercise through increasing testosterone and osteoblast cell of trabecular proximal femur in glucocorticoid-induced osteoporosis male mice. The results in this study suggest a need for further researches to investigate the role of phytotestosterone and AR in bone cells.

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