GREEN CLOVER POTENTIATES DELAYING THE INCREMENT OF IMBALANCE BONE REMODELING PROCESS IN POSTMENOPAUSAL WOMEN

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ABSTRAK

Phytoestrogen memiliki kemampuan selektif dalam organ jaringan, tidak menghasilkan efek samping pada payudara, rahim dan pembuluh darah. Semanggi (Marsilea crenata Presl) adalah tanaman khas Jawa Timur. Menggunakan radioimmunoassay, konsentrasi senyawa estradiol di daun semanggi terdeteksi cukup tinggi. Tujuan penelitian untuk mengetahui apakah semanggi berpotensi menunda kenaikan proses ketidakseimbangan remodeling tulang pada wanita postmenopause. Dua puluh delapan wanita pascamenopause berusia 49-66 tahun diperiksa menggunakan pretest-posttest control group design. Mereka secara acak dialokasikan ke 4 kelompok, kontrol, kelompok yang menerima ekstrak semanggi, kelompok treadmill, dan kelompok yang menerima semanggi hijau serta berolahraga treadmill. Tingkat estrogen diukur menggunakan teknik RIA fase padat, IGF-1 menggunakan IRMA, N-Mid osteocalcin (NMid) dan C-telopeptide (CTx) menggunakan ECLIA. Data dianalisis menggunakan analisis deskriptif dan inferensial dengan uji korelasi komparatif dan statistik. p < 0 .05 dianggap signifikan secara statistik. Hasil penelitian menunjukkan bahwa ekstrak semanggi meningkatkan secara signifikan konsentrasi estrogen pada wanita pascamenopause. Gabungan intervensi lebih efektif untuk meningkatkan konsentrasi estrogen. Kelompok kombinasi juga menunjukkan perbedaan signifikan dalam konsentrasi NMid sebelum dan sesudah intervensi dibandingkan kontrol (p = 0,003), kelompok semanggi saja (p = 0,009), dan kelompok latihan saja (p = 0,057). Tidak ada perubahan signifikan dari CTx pada semua kelompok intervensi dan tidak ada perubahan signifikan kadar IGF-1, kecuali pada kelompok treadmill. Sebagai kesimpulan, ekstrak semanggi memperkuat efek latihan fisik dan menunda peningkatan ketidakseimbangan remodeling tulang pada wanita postmenopause.

ABSTRACT

Phytoestrogen has selective ability in tissue organs, producing no side effect in breast, uterus and vascular. Green clover (Marsilea crenata Presl) or Semanggi is unique plant in East Java. Using radioimmunoassay , estradiol-like compound concentrations in green clover leaves was detected quite high. The aim was to know whether Green clover have potentiates in delaying the increment of imbalance bone remodeling process in postmenopausal women. Twenty-eight postmenopausal women aged from 49-66 years were examined using pretest-posttest control group design. They were randomized and allocated to 4 groups, control, the group receiving green clover extract, treadmill exercising group, and group receiving green clover as well as treadmill exercising. The estrogen level were measured using solid phase RIA technique, IGF-1 using IRMA, N-Mid osteocalcin (NMid) and C-telopeptide (CTx) using ECLIA. Data were analyzed using descriptive and inferential analyses with comparative and correlation statistical test. A two-tailed p < 0 .05 was considered statistically significant. Results showed that geen clover extract administration increased estrogen concentration significantly in postmenopausal women. Combined intervention was more effective to increase estrogen concentration. The combination group also showed significant difference in difference NMid concentration before and after intervention from that of control (p = .003), of semanggi group only (p = .009) and of exercise group only (p = .057). There was no significant changes of CTx in all intervention groups and no significant of changes of IGF-1 level except in exercised group. In conclusion, green clover leaves extract potentiates effects of physical exercise, delaying the increment of imbalance bone remodeling in postmenopausal women.

Keywords: *green clover, postmenopausal women, biochemical marker*

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INTRODUCTION

Estrogen deficiency in the postmenopausal period is the one of the most important factors in the increment imbalance of bone remodeling (Manolagas 2000, Tobias 2003). The lack of either estrogen increased quantity of bone multi-cellular units and decrease ability of osteoblasts to fill resorption lacunae caused by reduction of bone formation. The increment of bone resorption is caused by the changes in proinflammatory cytokine balance activity that will contribute to unbalanced remodeling, leads to decreased bone mass and increased risk for osteoporosis (Pacifi 1996, Lerner 2006, Tobias 2003, Weitzmann and Pacifi 2006). In

USA, an estimated 10 million Americans over 50 years old have osteoporosis and there are around 1.5 million fragility fractures each years. Another 34 million Americans are at risk of the disease (Cooper et al. 2008). Remodeling rate, remodeling balance and bone turnover can be assessed by histomorphometric analysis of histological sections of bone. Products of bone breakdown and formation that released in the blood can be measured in blood and/or urine and used to assess bone turnover (Compston 2008). Axial-load bearing exercise has been much reported by many researches to give benefit to increase bone mass (Nieves et al. 2003, AACE 2003). Chilibeck (2004) reported that in order to increase the bone mass, the intervention combination of hormone replacement therapy (HRT) and axial-load bearing exercise are more effective than non-combination one. Since the use of HRT have linked with breast cancer and uterus, so that many experts nowadays has focused on phytoestrogen. Phytoestrogen has been reported to increase bone density besides their other benefits (Mei et al. 2001, Baziad 2003, Fitzatrick 2003). In Indonesia, especially in East Java, Green clover or semanggi plant (Marsilea crenata Presl), as one of East Java's most popular unique food, has been proved to have phytoestrogen in it (Laswati 2007). This study was aimed to analyzed the potentiate effects of Green colver in delaying the increment of imbalance bone remodeling process in postmenopausal women.

MATERIAL AND METHODS

Research design in this experiment study was randomized pretest-posttest control group design (Campbell and Stanley 1963). The total samples were twenty-eight people determined by replication formula (Hanafiah 2004). The inclusion criteria was postmenopausal women (including bilateral oovarectomy) aged ≥ 45 years, BMI 18.5-29.9, were willing to discontinue any of their other physical exercises, and also willing to follow all the planned research programs by sign-up the informed consent. The exclusive criteria are if: 1.The subject suffers hyper-thyroid, hyperparathyroid, kidney disorders 2. The subject are using anticonvulsant, immunosuppres-sant/cytostatic, antacid, diuretic, antiresorptive and anabolic drug; 3. Being treated with estrogen, progest-erone, androgen;4. Has drank coffee, alcohol, soft-drink, smoked everyday and in long-term use; 5. Suffer joint degenerative diseases, neuromusculo-skeletal disorder, lung diseases, cardiovascular diseases, which impossible to follow physical exercises; 6. Has been bone fractures on small accident. The subjects of the research were randomized and allocated into four groups :1. The controlled group which will be given 10 mg B1 vitamin in capsule once a day; 2. The green

clover leaves extract group which had been given 0.8 gram extract of green clover leaves and 10 mg B1 vitamin in capsule, once a day; 3. The exercise on treadmill group with the intensity of 60-75% of the maximum heart rate in 30 minutes, three times a week; 4. The combination group which contain combination of the group 2 protocol and group 3 protocol. These experiments were conducted in four weeks. Before the experiment and after four weeks of experiment, there the data recollecting from subjects serum which covers estrogen amount by using radioimmunoassay (RIA) solid phase, insuline-like growth factor-1 (IGF-1) using sandwich immunoradiometric assay (IRMA) test, N-Mid oesteocalcin (NMid) and C-telopeptide (CTx) using electrochemiluminescence immunoassay (ECLIA). The research had received ethical clearance from Ethical Commitee dr Soetomo General Hospital. Data gained from the research was analyzed by using descriptive analyses, and inferential analyses by using comparative statistic test and correlation statistic test as well. Before analyses, all continuous variables were tested for normal distribution.

RESULTS

The average age of this research subjects showed initial postmenopausal period with low average initial estrogen levels, low of calcium, phosphor and isoflavon consumption (table 1).Calcium consumption of the research subjects showed lower than calcium consumption recommended by RDA from Food and Nutrition Board (1989) stated the aged of 50 is 1200 mg per person per day. Recommended for phosphor consumption that is 700 mg per person per day (Anderson 2000) and isoflavon 40-160 mg per person per day (Baziad 2003). The average of walking speed on treadmill was 4.572 ± 0.428 km/hour and the average achievement distant were 2.105 ± 0.190 km in 30 minutes. The total length of steps per minute showed increase $27.786 \pm 11.866\%$ from the initial total step per minute.

The homogenesity experiment using ANOVA showed no significant difference ($p > 0.05$) in each groups in the matter of all variables. Before conducting the inferential analyses the data normally statistic is being done. The comparative test showed that green clover extract leaves administration (the group 2), physical exercise (the group 3), and the combination of both (the group 4) may result in significant difference in estrogen concentration to the controlled-group ($p = 0.008$, $p = 0.011$ and $p = 0.001$ in succession). The difference of estrogen concentration before and after experiment of the group 2,3 and 4 also differ significantly towards controlled-group ($p = 0.004$, $p = 0.045$ and $p = 0.004$ in succesion).

Only the physical exercise group showed significant difference of IGF-1 concentration towards the control group. The difference of NMid ingroup 2 and 4 has significant difference toward the controlled group (p = 0.009 and p = 0.003 in succession), but not significant in the group 3 (p = 0. 057). Figure one show the profile of the difference of estrogen, IGF-1, NMid and CTx concentration before and after the experiment.

Table 1. The variety and distribution data of the research subject of menopausal women before the experiment.

| Variable	N	Mean	SD	Minimum	Maximum
Age (year)	28	55.214	3.891	49.00	66.00
Menopause start (year)	28	49.393	3.213	42.00	54.00
Menopause term (year)	28	5.893	4.516	1.00	16.00
Height (cm)	28	155.077	5.436	145.00	166.00
Weight (kg)	28	59.893	8.126	45.00	77.00
BMI	28	24.949	2.799	19.39	29.93
Length of step (cm)	28	47.300	7.824	28.00	64.20
Cadence (total step/min)	28	108.554	6.720	100.00	125.50
IPAQ score(METmin/week)	28	2998.364	2284.822	495.00	8376.00
MCS-SF36 score	28	57.893	3.890	52.00	67.00
Calori consumption (kcal)	28	1620.277	319.928	974.80	2399.50
Protein consumption (gr)	28	41.389	10.014	27.60	70.80
Calcium consumption(mg)	28	367.307	152.421	186.60	940.90
Vit C consumption(mg)	28	80.757	53.667	13.80	266.60
Phosphor consumption (mg)	28	491.571	100,736	293.70	724.50
Isoflavon consumption (mg)	28	21.543	8.782	6.30	36.60
Estrogen concentration (pg/ml)	28	4.286	3.101	0.00	12.00
IGF-1 concenration (ng/ml)	28	212.750	139.718	46.00	460.00
CTx concentration (ng/ml)	28	0.563	0.107	0.410	0.733
NMid concentration (ng/ml)	28	29.910	6.542	20.900	47.790
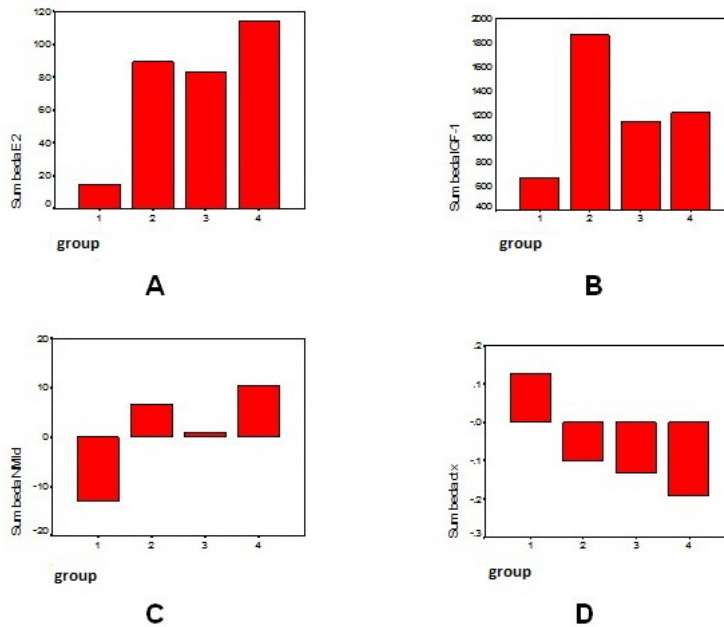
Figure 1. Profile of the difference of estrogen (A), IGF-1(B), NMid(C) and CTx (D) concentration before and after intervention from the research subjects of menopausal women.

The correlative test showed that there was a strong enough correlation between estrogen concentration after the experiment with the difference IGF-1 concentration (r = 0.549, p = 0.002) and the difference NMid concentration (r = 0.532, p = 0.004). There is an equal effect of estrogen concentration after the experiment and the difference IGF-1 towards the difference of NMid with significant value of p = 0.014. But the effect can only explain the variation of the difference of NMid of 29.1%, while the rest are explained with difference causes. Partially, only the estrogen variable after the experiment has an effect towards the difference of NMid (p = 0.007) (Table 2). There is a correlation in the difference of estrogen and IGF-1 concentration (r = 0.463, p = 0.013) and the difference of NMid (r = 0.549, p = 0.001) (table 3). The close correlation of estrogen difference and NMid difference is not freed significantly (p = 0.015) from the correlative difference of IGF-1 (Table 4).

Table 2. The effect of estrogen concentration variable after the experiment and the difference of IGF-1 towards the difference of NMid research subject of menopausal women.

Independent variables	R^2	F	Sig	t	Sig
Estrogen concentration	0.291	5.126	0.014*	2.925	0.007*
IGF-1 difference concentration				-0.518	0.609

Depedent variable: different NMid; F: value of calculated F; *significant $p \leq 0.05$; R2 :determination coefficient; t: value of calculated t

Table 3. The correlation of estrogen difference with IGF-1 difference, CTx difference and NMid difference concentration before and after experiment in postmenopausal women.

Variables	E_2 difference concentration	IGF-1 difference concentration	CTx difference concentration	NMid difference concentration
E_2 difference concentration	1	r =0.463 p = 0.013*	r = 0.002 p = 0.990	r = 0.594 p = 0.001**
IGF-1 difference concentration	r = 0.463 p = 0.013*	1	r = 0.065 p = 0.742	r = 0.319 p = 0.098
CTx difference concentration	r = 0.002 p = 0.990	r = 0.065 p = 0.742	1	r = -0.151 p = 0.443
NMid difference concentration	r = 0.594 p = 0.001**	r = 0.319 p = 0.098	r = -0.151 p = 0.443	1

*significant $p \leq 0.05$ (2-tailed), **significance $p \leq 0.01$ (2-tailed)

Table 4. The association IGF-1 difference in the close variable correlation in estrogen difference and NMid difference of the research subject of menopasal women.

Variables	Estrogen difference concetration	NMid difference concentration	IGF-1 difference concentration
E_2 difference concentration	1	r =0.412 p = 0.003**	r = 0.332 p = 0.015*
NMid difference concentration	r = 0.412 p = 0.003**	1	r = 0.207 p = 0.123
IGF-1 difference concentration	r = 0.332 p = 0.015*	r = 0.207 p = 0.123	1

*significant $p \leq 0.05$ (2- tailed), ** significant ≤ 0.01 (2-tailed)

DISCUSSION

The research has proved that the administration of green clover leaves extract in postmenopausal women caused significant difference estrogen concentration towards the controlled-group. The amount of increase of estrogen concentration before and after the administration also differs significancy towards the controlled-group. It has not been reported before that the administration of green clover leaves extract may increase the estrogen concentration in postmenopausal women. It is different with what Gamer-Wizard and friends reported (2006) that the administration of black cohosh in postmenopausal women has no significant effect to increase estrogen, however it is different from the group that be given conjugated estrogen. This might be because the administration of green clover leaves extract in postmenopausal women does not give aromatase inhibition effect or it does not compete to bind SHBG or it also does not increase SHBG production by hepatic cells. The above probabilities are based on the in vivo research that has been reported by Pino and friends (2005). The limitation of this resrach is that it does not conduct SHBG experiment.

The result of the research proved axial-load bearing exercise may increase estrogen concentration in postmenopausal women. This matter proved that physical exercise stressor with middle intensity (60-75% of maximum heart rate) in the research subject can induce the hormonal respond through HPA-axis. The intensity exercise given is optimal for stimulating cortisol increase so it does not cause negative feedback in HPA-axis. The increase of enough cortisol concentration induce androgen production. This result is intensified by the result of cortisol concentration test which showed that there is a significant difference in the controlled-group and physical exercise group after the experiment. The only limitation of this research dose not conduct the cortisol concentration test as a research variable in all research groups. The highest result of estrogen concentration after the combined administration showed the cummulative effect on estrogen concentration from green clover leaves extract consumption and the induction of extragonadal estrogen as result of physical exercise, that it has not been reported before.

This research has proved that axial load-bearing exercise in postmenopausal women is increasing IGF-1 concentration significantly. There is correlation of the estrogen concentration after experiment and estrogen difference concentration with IGF-1 difference concentration. Michael and friends (1993) who have conducted in vivo research on mice reported that there is an assumption that estrogen through hyperplasia effect in anterior pituitary may result in increasing distribution of mRNA from IGFBP-2 and IGF-1 significancy on the pituitary anterior tissue. Based on the researchers' argumentation above, there might be an assumption that the average changes on IGF-1 compound on the physical group in this research is the result on the expression induction effect of IGF-1 by Growth Hormon (GH) through GH receptor and IGF-1 expression induction by estrogen hormone in the pituitary anterior, so IGF-1 is in the increase circulation. The research on mice has shown that physical exercise may increase IGF-1 serum concentration and local IGF-1 concentration in the bones (Oxlund et al. 1988). The result of the experiment intesities the assumption that the increase of average of IGF-1 compund in the physical exercise group, besides as the result of GH induction, it also because estrogen hormone stimulation as the result of physical exercise.

Mihalache and friends (2002) reported their research on nine month-year old mice which has been treated with ovarectomy. The 7% of soy protein given in the mice's food caused the increase of biochemical marker of bone formation activities alkaline phosphatase compared with the controlled –group. Atkinson and friend (2004) in their double –bind, randomized, placebo-controlled trial, also done this research on the research subject of women aged 49-65 years old. The biochemical marker of bone formation in the group that was given isoflavon supplement (from red clover) within a year showed the significant increase with significant value bone-specific alkaline phosphatase p = 0.04, and collagen type 1 N-propeptide p = 0.01 compared with the placebo group. The result of this research has proved that green clover leaves extract has the effect conform with isoflavon effect from soy protein and red clover on the previous research. The association of IGF-1 on the close correlation of estrogen difference concentration and NMid concentration justifies that the increment of NMId concentration in this research is the effect of the increment of estrogen concentration and the increment of of IGF-1 concentration, that intensifies cross-talk mechanism segmentation of estrogen and IGF-1 in osteoblast cell.

CONCLUSION

Green clover leaves extract will more intensify biochemical marker of bone formation N-Mid Osteocalcin and tends to reduce bone biochemical marker of bone resorption C-telopeptide in the combined group. This research has proved that green clover leaves extract consumption show additive effect toward axial load-bearing physical exercise or the green clover leaves extract gives more osteogenic effect on

axial load-bearing physical exercise so that can delay the increment of imbalance bone remodelling process in postmenopausal women

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