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Homocysteine, Folic Acid, Vitamin B6, Vitamin B12, and Biochemical Parameters of Bone Metabolism in Female Patients with Systemic Lupus Erythematosus

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Abstract

Objectives: Premature osteoporosis is one of the long term complications of Systemic Lupus Erythematosus (SLE). Recent studies showed that increased level of homocysteine was detected in SLE patients and it was associated with deterioration of bone health. The aim of this study was to determine the association between homocysteine and biochemical parameters of bone metabolism in SLE patients.

Subjects and methods: Thirty-nine female patients who fulfilled American College of Rheumatology 1997 criteria for SLE under 50 years old and twelve healthy female as control group were studied. Various laboratory parameters including serum homocysteine, folic acid, vitamin B6, vitamin B12, bCTx, osteocalcin, MDA, and RANKL were measured.

Results: This study found that significantly higher levels of homocysteine were found in SLE patients (p=0.010). There was also a significantly higher level of MDA and RANKL in SLE patient (p=0.042, p=0.030). Whereas, the folic acid, vitamin B6, vitamin B12, bCTX, and osteocalcin levels were not statistically different between SLE patients and control group. High homocysteine level was significantly associated with increased levels of bCTx (p=0.000, r=0.943), MDA (p=0.002, r=0.731), and RANKL (p=0.000, r=0.758). High level of homocysteine associated with decreased levels of osteocalcin (p=0.000, r=-0.771), folic acid (p=0.000, r=-0.734), vitamin B6 (p=0.046, r=-0.332). But an insignificant relationship was found between serum homocysteine and vitamin B12 (p=0.080, r=-0.284).

Conclusion: Bone diminution in SLE seems to be attributable by homocysteine that influence bone formation and bone resorption process.

Keywords: SLE; Homocysteine; Folic acid; B6; B12; bCTx; Osteocalcin; MDA; RANKL; Osteoporosis

Introduction

Systemic Lupus Erythematosus (SLE) is a systemic autoimmune disease which can cause multiple organ damage [1]. Studies carried out in different countries suggest significant variation in the survival rate of patients with SLE. Previous study reported that five-year survival rates of SLE patients in Cipto Mangukusumo hospital Indonesia was 88% from 108 SLE patients within 1990-2002 [2]. The high rates of morbidity and mortality of SLE patients is caused by the disease process or its complications. One of the long term complications in SLE is a high prevalence of premature osteoporosis. The incidence of osteoporosis in SLE patients occurs at an early onset, at an average age of 39 years [3]. It caused by prolong systemic glucocorticoids usage, chronic inflammatory processes, and metabolic factors such as homocysteine [4]. Homocysteine is a metabolic factor that associated with inflammatory diseases. Low levels of folic acid, vitamin B12, and vitamin B6 could be the reason for high homocysteine levels, because both are intrinsically involved in the metabolism of homocysteine [5,6]. Recent studies found that high levels of homocysteine was associated with decreased bone mass density and an early onset of osteoporosis [7,8]. Experimental data suggest that high homocysteine levels affect both osteoclasts and osteoblasts activity [9]. And their activities can be seen by measuring the chemical markers of bone metabolism.

The other mechanism of osteoporosis in SLE patient is stress oxidative. In an inflammatory condition, the activation of macrophage cause oxidation of lipid which is lead to the production of free radical and stress oxidative [10]. The condition of stress oxidative can be measured by calculating the level of malondialdehyde (MDA) which is the end product of lipid peroxidation. Free radicals such as reactive oxygen species (ROS) can stimulate osteoclastogenesis by causing receptor activator of NF-κB ligand (RANKL) expression on osteoblast. This RANKL expression can activate the precursor of osteoclast, macrophage-colony stimulating factor (M-CSF) to differentiate [11].

The aim of this study was to determine the association between homocysteine and biochemical parameters of bone metabolism in patients with SLE. Moreover, serum folic acid, vitamin B6, vitamin

B12, MDA, and RANKL level were studied to investigate the correlation with high levels of homocysteine.

Subjects and Methods

Subjects

Thirty-nine female with SLE meeting the American College of Rheumatology 1997 criteria for SLE, aged 16-47 years, SLEDAI score >5, disease duration 2 months-96 months, average consumption of steroids (Methylprednisolone <50 mg/day), and use at least one type of immunosupresant agent were studied after giving informed consent. SLE patients who are pregnant or breast-feeding, anemia, cancer, impaired kidney function, using hormone replacement therapy, consume folic acid and vitamin B supplements, anticonvulsants agent, carbamazepine, phenytoin, xanthopterin, tamoxifen, and theophylline were excluded from the study. Subjects were obtained from Rheumatology Clinic of Dr. Saiful Anwar Hospital, Malang, Indonesia. Twelve healthy, age matched premenopausal women (medical students, resident, and healthy blood donors from the same geographical area) were studied as a control group after giving informed consent. This study used an observational analytic cross sectional approach and it was conducted in March 2012 to October 2012.

Blood sample collection

5 cc of venous blood samples were collected in test tube with aseptic precautions. After 1 h of collections, sample was centrifuged at 3000 rpm for 5 min. Serum was separated and collected in polythene tube with cork. The sera with no sign of hemolysis was stored in tubes at temperature of -70°C for further analysis.

Biochemical analysis

Various laboratory parameters including serum homocysteine, folic acid, vitamin B6, vitamin B12, bCTx, osteocalcin, and RANKL were measured by enzyme-linked immunosorbent assay (ELISA) method. The following parameters were measured by commercial ELISA Kit: homocysteine (NovaTeinBio Human ELISA Kit Total Hcy), folic acid (NovaTeinBio Human ELISA Kit Folic Acid), vitamin B6 (NovaTeinBio Human ELISA Kit Vit B6), vitamin B12 (Vit B12 NovaTeinBio Human ELISA Kit), bCTx (Cusabio bCTx Human ELISA Kit), osteocalcin (NovaTeinBio Human ELISA Kit Osteocalcin), and RANKL (NovaTeinBio Human ELISA Kit RANKL). The level of MDA was measured by Thiobarbituric Acid Reactive Substance (TBARS) method.

Statistical analysis

Statistical analysis was performed by SPSS for Windows version 16. Numerical variables were reported in terms of mean and standard deviation. In this analysis, variables showing p-value less than 0.05 were considered to be statistically significant respectively. Pearson correlation test was used to test the correlation.

Results

For every patient, data were recorded on age, SLE disease activity index (SLEDAI) scores, disease duration, glucocorticoid dose, immunosuppressant agent, and current clinical manifestations (Table 1).

Age (y.o.)	30.49 ± 8.46 (16-47)
SLEDAI score	12.85 ± 5.67 (6-26)
Disease duration (months)	26.44 ± 24.92 (2-96)
Percentage of patients receiving glucocorticoid treatment	100%
Current glucocorticoid dose (mg/day)	26,87 ± 17,96 (4-48)
Percentage of patients receiving Immunosuppresant	
Methotrexate	7.69%
Azathioprin	10.25%
Chloroquin	12.82%
Mycophenolate mofetil	12.82%
Cyclophosphamid	5.13%
Percentage of clinical manifestation	
Malar rash	46.15%
Oral ulcers	30.77%
Arthritis	43.59%
Pleuritis or pericarditis	17.95%
Renal disorder	48.72%
Neurologic disorder	2.56%
Hematologic disorder	7.69%
Immunologic disorder	100%

Table 1: Clinical characteristics of the SLE patients studied (n=39).

The mean age was 30 years with a range from 16 to 47 years and the mean disease duration was 26 months with a range from 2 to 96 months. At the time of the study most SLE patients had only mild disease activity as indicated by SLEDAI 6 – 26. All of the SLE patients were recieved corticosteroid therapy with the mean glucocorticoid dose was 26 mg/day with a range 4 – 48 mg/day. Nineteen SLE patients were received immunosuppresant agents. The most frequent disease manifestations were renal disorder, malar rash, and arthritis.

The mean levels of homocysteine, bCTx, osteocalcin, folic acid, vitamin B6, vitamin B12, RANKL, and MDA in SLE patient and control group were described in Table 2.

This study found that significantly higher levels of homocysteine were found in SLE patients (p=0.010). There was also a significantly higher level of MDA and RANKL in SLE patient (p=0.042, p=0.030). But folic acid, vitamin B6, vitamin B12, bCTX, and osteocalcin levels were not statistically different between SLE patients and control group.

The correlation between parameters was observed only in SLE patients. From the correlation analysis we found that there were positive and significant correlation was observed between serum level of homocysteine and bCTx (p=0.000, r=0.943). Another positive and significant correlations were also found between serum level of homocysteine and MDA (p=0.002, r=0.731). And this level of MDA has significant and positive correlation with RANKL (p=0.000, r=0.758).

Parameters	SLE (Mean ± SD) n= 39	Control (Mean ± SD) n= 12	Significance
Homocysteine (µmol/L)	13.52 ± 4.15	7.54 ± 2.27	0.010*
bCTx (pg/mL)	1445 ± 363.48	1057.03 ± 301.99	0.100 NS
Osteocalcin (ng/mL)	14.50 ± 5.66	10.25 ± 2.93	0.260 NS
Folic acid (nmol/L)	5.00 ± 1.55	6.36 ± 1.55	0.150 NS
Vitamin B6 (nmol/L)	7.87 ± 4.06	6.06 ± 1.00	0.106 NS
Vitamin B12 (pmol/L)	189.28 ± 81.12	165.50 ± 50.56	0.170 NS
MDA	0.067 ± 0.019	0.03 ± 0.001	0.042*
RANKL	14.63 ± 6.29	1.58 ± 1.32	0.030*
p<0.05 was significant, NS: Not Significant			

Table 2: The level of homocysteine, folic acid, vitamin B6, B12, biochemical bone formation and resorption markers.

Negative and significant correlations were observed between homocysteine and osteocalcin (p=0.000, r=-0.771), homocysteine and folic acid (p=0.000, r=-0.734), and homocysteine and vitamin B6 (p=0.046, r=-0.332). But an insignificant relationship was found between serum homocysteine and vitamin B12 (p=0.080, r=-0.284) (Table 3).

Parameters	r value	p value
bCTX	0.943	0.000*
Osteocalcin	-0.771	0.000*
Folic acid	-0.734	0.000*
Vitamin B6	-0.332	0.046*
Vitamin B12	-0.284	0.080NS
MDA	0.731	0.002*
r: correlation coefficient, p<0.05 was significant, NS: Not Significant		

Table 3: Correlation of homocysteine and bCTx, osteocalcin, folic acid, vitamin B6, and vitamin B12 in SLE Patient.

Discussion

Our finding suggest that levels of serum homocysteine are higher in SLE patients than in age-matched healthy female. Elevated levels of homocysteine in patients with SLE also reported in several researches [12-14]. Homocysteine are regulated by a number of coenzymes and cofactors that required for homocysteine metabolism, such as folic acid, vitamin B6, and vitamin B12. Increased homocysteine levels in SLE patients are caused by a chronic inflammation and abnormal immune response leading to decrease of several vitamins. In SLE patients, the inflammatory process triggered by the presence of autoantibodies that induce further inflammatory reaction resulted in tissue damage [4]. Activation of immune cells, particularly macrophages, will produce Reactive Oxygen Species (ROS) which cause the oxidation of folic acid, vitamin B6, and B12 [15]. In addition, the inflammatory process also increase the proliferation of immune cells that would lead to an increased turnover of folic acid, vitamin B6, and B12 [16]. Both are thought to be the cause of decreased levels of folic acid, vitamin B6, and B12 in inflammatory conditions. In this study, there are no significantly different of folic acid, vitamin B6, vitamin B12 level between SLE patients and control group. But the need of these vitamins is increase in SLE patients because increase of turnover and homocysteine metabolism [17].

Several studies showed that high levels of homocystein were associated with decreased bone mass density and early onset of osteoporosis [7]. Other studies mentioned that high levels of homocystiene and low levels of folic acid were associated with decreased bone mass density, whereas levels of vitamin B6 and vitamin B12 had no significant effect [8]. Elshorbagy et al. reported that decrease of vitamin B12 and folate levels contribute to increased osteoclast activity which was characterized by high levels of biochemical markers of bone resorption, whereas there was no effect of vitamin B6 [18]. In our study, a positive and significant correlation was observed between serum homocysteine and bCTx, whereas a negative and significant relationship was found between serum homocysteine and osteocalcin. It means that homocysteine contributes to the increase of bone resorption and decrease of bone formation process.

Recent studies tried to understand the mechanisms regarding the role of homocyteine, folic acid, vitamin B6, and vitamin B12 on bone metabolism. Homocysteine auto-oxidation results in increased production of intracellular ROS and stimulates p38 MAPK activation which influence the differentiation of osteoclast precursor cells [16]. Homocysteine also induces activation of RANK, a receptor for RANKL, which is a key element in the process of osteoclast differentiation [19]. There was positive and significant correlation between homocysteine level and MDA in this study. And this MDA level has positive and significant correlation with RANKL. Our result implicated that homocysteine cause an increase production of intracellular ROS that influences osteoclast differentiation through ROS-RANKL pathway.

In this study we also found a negative correlation between homocysteine and osteocalcin. It indicates that the high level of homocysteine cause decrease of osteoblast activity. Kim et al. reported that homocysteine induces apoptosis of human bone marrow stromal cells via caspase-dependent pathway [20]. The intrinsic apoptotic signals derived from DNA damage and ROS production also induced by accumulation of homocysteine [21]. In a study conducted by Park et al., reported that homocysteine induces osteoblast cell apoptosis through endoplasmic reticulum stress [22]. Other studies shown that homocysteine weaken collagen crosslink's and in large amounts it can interfere bone remodeling process [6,23].

The levels of osteocalcin were slightly more in SLE patients. Serum OC is considered a specific marker of osteoblast function, as its levels have been shown to correlate with bone formation rates. However, since it is also released from bone matrix during bone resorption, it reflects the overall turnover of bone and is considered as a bone turnover marker. In a higher activity of bone resorption such as in SLE patient, our body responds by increasing osteoclast activity to balance bone remodeling process [24]. Some studies report that there were increasing serum osteocalcin levels in postmenopausal women with osteoporosis [25-27]. So, this theory is explaining the slightly increasing of OC level in our study. Besides that, OC has a high affinity for calcium and has a compact a helical conformation. The

carboxyglutamic acid (Gla) residues of OC are capable of binding to bone matrix hydroxyapatite, thus leading to bone mineralization. Osteoporotic patient may have a decreased rate of bone mineralization due to the reduction in hydroxyapatite crystal formation. In this condition, free OC may be present in the circulation, thus explaining the increased serum OC concentration in osteoporotic patient [28].

Elevated level of homocysteine in SLE patients also produce high levels of inflammatory mediators which induces thrombosis of small blood vessels, and a low level of other angiogenic factors. It causes cellular necrosis of a number of osteocytes, followed by localized to bone demineralization. Ongoing inflammation in these localized areas will diminish vascular supply on the osteoblast precursors that available to replace bone demineralization. The longer it will experience a loss of bone fracture in the weakened bone [29]. That is why osteoporosis occurs earlier in patients with SLE.

Osteoporosis in SLE patient also influenced by several kinds of drugs, such as glucocorticoids and immunosuppressants. All the subjects who contributed in this study are consuming glucocorticoids with average dose 26.87 mg/day. Approximately half of the patients who contribute to this research using immunosuppressant drugs, such as methotrexate, azathioprin, chloroquine, cyclophosphamide and mycophenolat mofetil. Among those immunosuppressants, methotrexate has the influence in decreasing bone mineral density. Study of Raghu Nadhanan et al. found that low dose of methotrexate can increase the risk of osteopenia [30]. But, other immunosuppressants such as Azathioprin, Chloroquine, Cyclophosphamide and Mycophenolat mofetil have no interference in bone mass density [31-33].

To diagnose osteoporosis we can use several methods, such as biochemical markers of bone turnover including bone formation and bone resorption. Of all the biochemical markers of bone turnover, the ones most commonly used in clinical practice are bone-specific alkaline phosphatase (BSAP), osteocalcin (OC), N-telopeptide of collagen cross-links (NTx), and C-telopeptide of collagen cross-links (CTx) [34]. Plain radiographic findings can suggest the presence of osteopenia, bone loss, or if a fracture is suspected, but they cannot be used to diagnose osteoporosis. Dual-energy X-ray Absorptiometry (DXA) is currently the criterion standard for the evaluation of bone mineral density (BMD). Compared to imaging techniques, assays for biochemical markers of bone turnover are safe, cheap, easily performed, and can detect early changes in bone metabolism [35]. In this study, we use bCTX and RANKL as bone resorption marker and Osteocalcin as bone formation marker. But, for further study we suggest to do BMD measurement to confirm the correlation.

Conclusions

Our results implicate several clinical consequences. Firstly, the high level of homocysteine was found in SLE patients. Secondly, homocysteine influences bone formation and bone resorption process which lead to bone diminution in SLE patients. In our study, we cannot conclude that giving supplementation of folic acid, B6, B12, and other antioxidant could prevent osteoporosis due to insignificant result.

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