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Germinated-soy milk in supressing inflammation and oxidative stress in blood plasma and breast milk of lactating mothers

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Abstract

Current study aimed to determine the potency of germinated-soy milk (GSM) on Superoxide Dismutase (SOD) activity, and Malondialdehyde (MDA) and C-Reactive Protein (C-RP) levels in the blood plasma and skim milk of lactating mothers. Subjects were 50 lactating mothers, 20-35 years old, having newborns up to 6 months old, and willing to sign informed consent. Subjects were divided into two groups consisting of 25 women. Group I was treated with GSM, while group II was given placebo for two consecutive months. Samples of blood plasma and mother's breast milk were taken 3 times at different times: 0, 1 and 2 months after intervention. Blood samples were taken intravenously using venoject tubes containing EDTA. The blood plasma and skim milk were used as test samples for activities of SOD, as well as the MDA and C-RP levels. Differences were considered significant at $P<0.05$. Results, GSM increased SOD activity in the blood plasma $(P = 0.043)$, and also in skim milk $(P = 0.56)$. On the other hand, level of MDA decreased in the blood plasma $(P = 9.65E\text{-}06)$, and in skim milk $(P = 0.047)$, and also the CRP reduced in the blood plasma $(P = 0.0015)$, and in skim milk $(P = 0.77)$. GSM could be used to reduce oxidative stress and suppress inflammatory levels of lactating mothers.

Introduction

Oxidative stress that reduces activity of antioxidants happens along pregnancy up to lactating period (Kasapović *et al.*, 2005), and is followed with an increase of free radicals and reactive oxygen species (ROS) formation (Perrone *et al.*, 2010). Mother's breast milk contains several bioactive compounds including immunologic factors, enzymes, growth factors, and hormones (Oveisi *et al.*, 2010). During prenatal period, the baby gets his/her nutrition from the mother through placenta and when the baby is born mother's breast milk is given.

Mother's breast milk, the main component of baby's food, is a natural compound and safe to be consumed as it contains various nutritional compounds which are important for their growth and development. Mother's breast milk contains several types of enzymes brought by blood to the mammary glands (Savić *et al.*, 2005). Mother's breast milk therefore contains antioxidant enzymes i.e. Superoxide Dismutase (SOD) and living cells which have their roles in antioxidant and immune system of the baby. According to Kasapović *et al.* (2005) antioxidant enzymes in breast milk had a tertiary structure, which differentiated it from other antioxidant enzymes. The antioxidants in milk were more hydrophobic than the antioxidants that were not in breast milk. Moreover, the antioxidants in breast milk were also less sensitive to proteolysis and denaturant along the baby's digestive system. This meant that in breast-fed infants, the protein in it, including SOD, remained intact, did not undergo proteolysis or denaturation in the gastrointestinal tract, so that the plasma levels of SOD was high, as well as their activities. SOD have a role in detoxifying free radicals (Flores-Mateo *et al.*, 2009), like malondialdehyde (MDA), an end product of lipid peroxidation (Garratt *et al.*, 2011). Immature condition of baby's immune system is supported by his/her mother through mother's breast milk as indicated by a significant relationship in the level of blood plasma between mother and her baby (Zarban *et al.*, 2009).

Antioxidant status of the baby is strongly dependent on the mother's antioxidant status. Food intake rich in antioxidant is therefore a prerequisite for lactating mothers, mainly to increase levels in the mother's breast milk as well as in the blood plasma.

Winarsi *et al.* (2009 and 2010) recently reported that the germinated-soy milk (GSM) was able

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to improve antioxidant and immune status, and inflammation of women with metabolic syndrome (Winarsi and Purwanto, 2010a), and even induce secretion of hormone insulin of women with type-2 diabetes mellitus (T2DM) (Winarsi and Purwanto, 2010b). These patients were also facing oxidative stress just as the lactating mothers. To overcome oxidative stress, a food product which is antioxidant rich such as GSM, should be consumed.

The GSM contains 39.1 ppm isoflavones which is bound to milk protein. The isoflavone level is higher than that of non germinated one 26.7 ppm (Winarsi *et al.,* 2010). Isoflavones are known as potential antioxidants (Hu *et al*., 2004; Winarsi *et al*., 2005; Winarsi *et al.*, 2010), so these compounds are able to improve antioxidant and immune status of women with oxidative stress. Isoflavones are also proven as anti-inflammatory (Hall *et al*., 2005; Winarsi *et al*., 2010). However, there is no available data about GSM and its effect on antioxidant and anti-inflammation status in the blood plasma and mother's breast milk of lactating mothers. This study aimed to determine effect of GSM in increasing SOD activity, reducing MDA and CRP levels in the blood plasma and mother's breast milk.

Materials and Methods

Current study used a Randomized Clinical Trial with double blinded approach. The research was approved by the ethics committee of the Medical Faculty of Diponegoro University, Semarang, Indonesia.

Preparation of germinated-soy milk

Germinated-soy milk was initially prepared by germinating the seeds overnight. Next, the germinated seeds were cleaned from their husks, blended, and sieved. Sugar, salt, milk powder and pandanus leaves was added into GSM. To prepare the placebo, the same ingredients were used except the germinated-soy (Winarsi, 2013).

Subject researchs

As many as 50 lactating mothers, at the age of 20-35 years old, having newborn baby up to 6 months old, healthy, living in Purwokerto, and willing to sign Informed consent (Table 1) as research subjects. They come from several of integrated healthcare in the region Purwokerto, Central Java, Indonesia.

Grouping research subjects and intervention

Subjects were divided into two groups consisting of 25 people each. The first group was treated with germinated-soy milk, while the second group had the placebo. Intervention was carried out in the morning, at 6:00 to 10:00 a.m, for two consecutive months at the amount of 125-150 mL.day^{-1}.person^{-1}.

Table 1. Profile of research subjects

	Germinated-soy milk*	Placebo*
N (persons)	25	25
Mother's age (years)	27.16±0.78	29.11±1.27
Baby's age (month)	3.07±0.32	2.57±0.3
BMI (kg/m^2)	22.77±0.56	22.21±0.61

*Data were representative of mean + SE; n=25 replications.

Blood plasma and breast milk collection

Samples of mother's blood plasma and breast milk, were taken three times, started at the baseline, followed by one and two months after intervention. Taking sample was conducted in the morning, between 6:00 to 10:00 a.m. As much as two mL of blood was taken intravenously using venoject tube containing EDTA, and then centrifuged at 3,000 rpm for 10 min. Plasma obtained from this step was used as a sample test.

Breast milk samples of 3 mL were taken manually (done by the research subjects themselves) in the morning between 06:00 to 10:00 a.m, and then centrifuged at 3,000 rpm for 10 min at 4ºC. After centrifugation, lipid layer on the milk surface was then removed, whereas the skimmed milk fraction obtained was used as a sample test.

Testing of blood plasma and breast milk for SOD enzyme activity, MDA and CRP levels

Testing of SOD activity using the SOD activity assay kit, Bio Vision. Blood plasma was dilluted 4 times, while skimmed milk was only once. Briefly, each of 20 µL sample solutions (blood plasma and skim milk) was taken and placed in the available wells and also in blank well 2, then 20 µL H_2O added to blank well 1 and 3. Following to this, 200 µL WST working solution was added into each well, and 20 µL dilution buffer for blank wells 2 and 3. Finally, 20 µL enzyme working solution was added to each well sample and blank well 1, and stirred homogenously. Plates were then incubated in 37^{o}C for 20 minutes. Then read under ELISA reader at 450 nm wavelength. The SOD activity was calculated as follows:

$$\text{SOD Activity (inhibition rate \%)} = \frac{(A_{blank1} - A_{blank3}) - (A_{sample} - A_{blank2})}{(A_{blank1} - A_{blank3})} \times 100$$

The MDA level in both blood plasma and skim milk was measured as written in the Malondialdehyde assay kit Northwest as follows. TBA reagent was reconstituted in 10.5 mL aqua bidest. As much as ten µL reagent BHT was added to microcentrifuge vial followed by either 250 µL calibrator or sample, 250 µL acid reagent, 250 µL TBA reagent, and vortexed vigorously for five seconds, incubated for 60 minutes at 60°C, then centrifuged at 10,000 x g for 2-3 minutes. Absorbances were read at the wavelength of 532 nm.

The C-RP level in both blood plasma and breast milk of lactating mothers was measured following the instruction in the High Sensitivity Enzyme Immuno assay kit for the Quantitative Determination of C-Reactive Protein Concentration in Human Serum Diagnostic Automation, Inc. The steps were blood plasma was diluted for 100 times, but the skim milk only for 50 times. Ten µl of the CRP standard was poured into a patterned plate, and then added with diluted sample, and control. Of each well, 100 µl reagent CRP enzyme conjugate was also added, left for 30 minutes to allow the solution to mix up, incubated for 45 minutes at room temperature, 18-25°C. Solution was then thrown away and washed 5 times using aqua bidest. Dried completely by putting tissue paper to absorb the rest solution. One hundred µl TMB solution was then added into each well, and mixed gently for 5 seconds, and incubated for 20 minutes at room temperature. Finally, 100 µl stop reaction was added into each well, mixed gently for 30 seconds to allow change in colour from blue to yellow then its absorbance read at 450 nm for 15 minutes.

Statistical analysis

The result was expressed as mean ± SE. Differences were considered significant at *P<0.05*.

Results

At the baseline, the SOD activity in blood plasma was 158.51%, and increased from 202.17 to 336.42% *(P=0.04)* after taking GSM for 2 consecutive months. However, that in the placebo group did not indicate any difference *(P=0.38)*. Its activity in the mother's breast milk was high, 441.88% and even higher (from 360.42 to 699.31%) after consuming GSM for 2 consecutive months though this change was not significantly different *(P=0.56)*. A similar trend was shown by the placebo's group *(P=0.81)* (Figure 1).

The MDA level baseline in blood plasma of lactating mothers was noted at 23.6 µM and gradually decreased to 12.94 µM *(p<0.001)* after consuming GSM for 1 month it further reduced to 12.66 µM *(P<0.001)* for 2 month consumption, but in the placebo group, did not show any change *(P=0.54)*. Meanwhile, the MDA level in mother's breast milk was initially at the level of 62.2 µM. A decrease from 62.01 to 48.85 µM *(P=0.047)* was recorded for subjects who consumed GSM for 2 months but showed opposite result in the placebo group *(P=0.55)* (Figure 2).

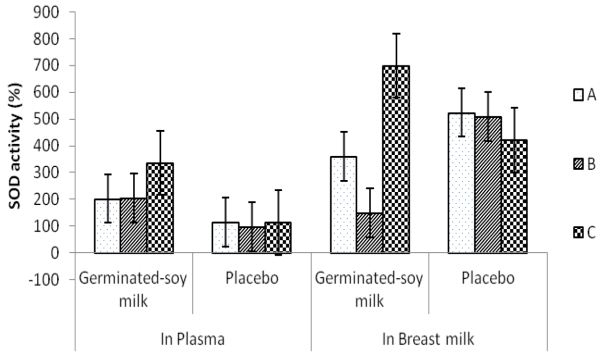

Figure 1. Effect of germinated-soy milk on SOD activity in blood plasma and skim milk of lactating mothers

Notes: A, group consuming germinated-soy milk or placebo in blood plasma or skim milk of lactating mothers at baseline time; B, group consuming germinated-soy milk or placebo in blood plasma or skim milk of lactating mothers at 1 month after intervention; C, group consuming germinated-soy milk or placebo in blood plasma or skim milk of lactating mothers at 2 month after intervention; n = 25; P < 0.05

The C-RP levels in plasma also decreased from 9.35 to 2.38 pg/mL *(P = 0.0015)*. The CRP levels in breast milk was low (0.09 mg/L) but showed a decrease from 0.099 to 0.046 mg/L *(P = 0.77)* in the group who consumed GSM for 2 months (Figure 3).

Discussions

Activity of SOD enzyme in blood plasma and skim milk of lactating mothers

The increased of plasma SOD activity in this research may be related with the content of bioactive compounds in GSM. According Winarsi *et al.* (2010) besides containing protein, lipid and carbohydrate, as much as 39.1 ppm isoflavones was also found in GSM. The isoflavone was higher than that in soy milk which was only 26.7 ppm. Among those isoflavones, glycitein – a type of isoflavone with highest antioxidant potency (Hubert *et al.*, 2008), was noted at the level of 7.6 ppm. It was then assumed that glycitein has the potential as highest antioxidant compared to other type of isoflavone. It is suggested that glycitein played significant role in increasing the SOD activity.

In addition due to the tertiary structure and

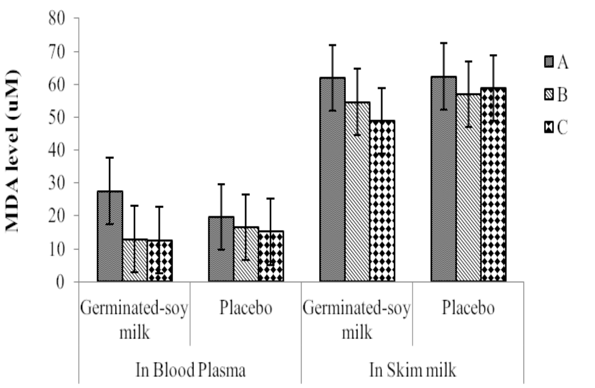

Figure 2. Effect of germinated-soy milk on MDA level in blood plasma and skim milk of lactating mothers

Notes: A, group consuming germinated-soy milk or placebo in blood plasma or skim milk of lactating mothers at baseline time; B, group consuming germinated-soy milk or placebo in blood plasma or skim milk of lactating mothers at 1 month after intervention; C, group consuming germinated-soy milk or placebo in blood plasma or breast milk of lactating mothers at 2 month after intervention; n = 25; $P < 0.05$

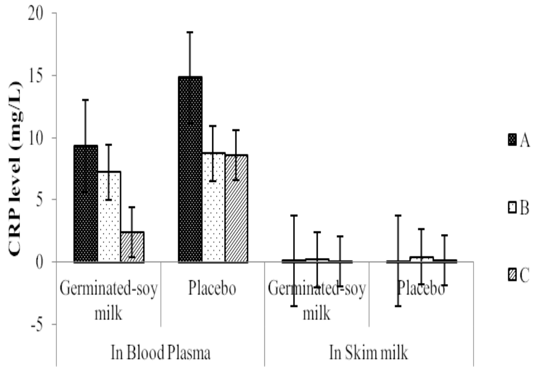

Figure 3. Effect of germinated-soy milk on C-RP level in blood plasma and skim milk of lactating mothers

Notes: A, group consuming germinated-soy milk or placebo in blood plasma or skim milk of lactating mothers at baseline time; B, group consuming germinated-soy milk or placebo in blood plasma or skim milk of lactating mothers at 1 month after intervention; C, group consuming germinated-soy milk or placebo in blood plasma or skim milk of lactating mothers at 2 month after intervention; n = 25; $P < 0.05$

hydrophobic, antioxidant enzymes in the breast milk is not easily denatured, and intact in the gastrointestinal tract, so that the plasma SOD levels was high; Ezaki *et al.* (2008) added that breast milk contained Cu 45.1 mg/dl, and Zn 487 mg/dl, the minerals needed in SOD activity. Zinc has a structural stabilizing role, while Cu directly Involved in the catalytic activity. Therefore it was reasonable, if the activity of SOD in plasma increased markedly after subjects consume the GSM.

Isoflavone could increase SOD levels through several mechanisms, i.e. increasing production of SOD enzyme, leading to an increase of its level and its activity as well. Isoflavones could also suppress acitivity of free radicals by binding it as well as its amplification. Alternatively, it begins with oxidation of isoflavones to isoflavone radicals, but when they react to another type of isoflavone they become stable.

Casein coming from cow's milk or soy milk is a common type of protein that is containing in daily diet. According to Gilani *et al.* (2012) both types of milk are classified as high quality protein foods with different characteristic, including its composition in amino acids, peptides and its bioactive nitrogen non protein compounds. Jahan-Mihan (2011) added that composition of amino acids are similar even when the levels are different.

Amino acids contained in protein of GSM are also believed to contribute in the synthesis of protein including SOD enzyme. There are 18 different types of amino acids to form the SOD, all these amino acids are present in soy seeds protein, and might also possibly be present in the germinated-soy. It is therefore believed that if the presence of GSM in daily diet the synthesis of SOD enzyme. A combination role between isoflavones and amino acids contained in GSM produces synergism in a good antioxidant, leading to increase of SOD activity in the blood plasma of lactating mothers.

Both colostrum and non-colostrum of mother's breast milk are important for the nutrition of the newborn baby as well as improving the antioxidant system. The concentration and level of antioxidant activity in the mother's breast milk, determine degree of cell's protection to peroxidation. To explain the protection effect of various factors including the antioxidative one, it is believed that this protection appears due to the synergistic role of those various components. This may be related to the presence of large and small components in mother's breast milk and the expression of their antioxidative effect. For example, the presence of metal transition ions which are able to suppress pro-oxidative, or the presence of either enzyme or non enzyme components which eliminate formation of free radicals. Mother's breast milk contains various enzymes which are bound and transferred by blood to the mammary glands, so the SOD enzyme is also available in the mammary cells and mother's breast milk.

Non significant increase of SOD activity in mother's breast milk as noted in this study after consuming GSM might be due to high SOD level in the subjects blood at the early phase of research, which caused only a small difference. Another possibility might be due to an imbalance condition between level of required antioxidant and its intake, leads to a sudden unavailable antioxidant during the transition period in lactating mothers. In this situation, metabolism will happen faster than normal then leave huge amount of free radicals.

Mother's breast milk contains several types of bioactive components like protein, immunoglobulin, nitrogen non-protein, lipid, vitamin, minerals and polymorphonuclear (PMN) cells which include leukocytes, macrophages, T lymphocytes, B lymphocytes, plasma cells, and epithellial cells. The last compounds are resistant to free radicals, which leads to formation of more free radicals in mother's breast milk and finally the available SOD could not be to eliminate them.

The MDA level of the blood plasma and skim milk of lactating mothers

Initially, the MDA levels was high. Bjelakovic *et al.* (2012) stated that abundant level of MDA reflects the high level of lipid peroxidation in mother's breast milk. During lactating period increase of polyamine oxidase (PAO) activity is parallel with the decrease of diamine oxidase (DAO) activity.

According to Ballard *et al.* (2013) PAO exists in all types of tissues in vertebrates and biological liquid like mother's breast milk. The PAO is the major enzyme within the polyamine catabolic pathway. This enzyme catalyzes oxidative deamination of bioactive polyamines like spermine (Sp), spermidine (Spd) and putrescine (PUT) contained in mother's breast milk. Whereas diamine oxidase, another type of enzyme which is also found in mother's breast milk, contains Cu, catalyzes and produces γ-Aminobutyric acid (GABA) or malondialdehyde (MDA). Malondialdehyde is an end product in biodegradation of polyamine and toxic to the cells, its existence in the cell must therefore be eliminated.

Some researchers claim polyamines play a significant role in maturation process in the function of gastrointestinal tract during the neonatal. The amine compound also protect food allergies in suckling babies by decreasing mucosal permeability to antigenic proteins (Zarban *et al.*, 2007; Møller *et al.*, 2011). Polyamine oxidase catalyzes the oxidative deamination of Sp and Spd, to produce the PUT and aminoaldehydes, amonia (NH_3) and H_2O_2. The production of H_2O_2 is raised upon amine degradation which correlated with oxidative burst and cell's death. The amino aldehydes and H_2O_2 are potentially toxic agents which are able to induce oxidative stress. During the first month of the lactating period, the PAO activity tends to increase the MDA level.

Decreased levels of MDA in the plasma and breast milk of lactating mothers supports the findings of Bjelakovic *et al.* (2012), may be related to the role of enzymes in it. Putrescine is a substrate for DAO, its level in the mother's breast milk is very low, however, it still varies during the first post-partum. Diamine oxidase is also known as histamine degradating enzyme produced by placenta in an abundant level. Diamine oxidase performs a role as metabolic barrier to prevent excessive entry of bioactive histamine from the placenta into the maternal or fetal circulation. The highest DAO activity in colostrum as well as decreased DAO activity in mature human milk was noted in 30th day of lactation. Malondialdehyde in mother's breast milk comes from blood and excreted through mammary glands in the colostrum before parturition (Olayaki *et al*., 2008). High level of MDA at early phase of lactation but progressively decrease represent a change of dynamic metabolic activity through regulation of endocrines.

Inspite of physiological change, baby's age of 1.95+0.81 month old, it might also due to consumption of GSM rich in isoflavone. Though this study did not measure activities of PAO and DAO, but significant decrease of MDA level noted in the subjects treated with GSM for 2 consecutive months. It might attract a conclusion if GSM contains such components which were able to reduce their level.

The C-RP level in blood plasma and skim milk of lactating mothers

Synthesis of CRP is regulated by the IL-6 level of the hepatic cells and IL-1 of the macrophage, and leukocytes which are activated in the vascular wall. IL-1 is manifestated in inflammation reaction (like fever, increase of CRP level, leukocytosis, and leukocyte infiltration surround the inflammation area). Whereas the IL-6 shows significant role in responding in an acute phase by increasing the synthesis of CRP. Its level in the blood plasma is still higher than that of its normal one (0.03 - 4.94 mg/L).

C-Reactive Protein is produced by cells as an abnormal protein of blood because of inflammation. Its concentration, increases after having an infection caused by inflammation or tissues abnormality within six hours. Its concentration in blood plasma, however, could increase twice in every hour, up to maximum level after 50 hours (Kask, 2010). C-Reactive Protein is the most sensitive marker as its level might increase up to 1000 times before infection happens. The current study however, noted if GSM given to the subjects had reduced cytokine inflammation of lactating mothers. This product was able to reduce the level of CRP up to 74.34%.

The lunacyn peptides, a result of hydrolysis of germinated-soy protein has potential to be used as antioxidant as well as anti- inflammation. Anti-inflammation character of peptides related with its composition, structure and hydrophobocity. For example the following amino acids like Tyr, Tryp, Met, Lys, Cys, and His show their activity as antioxidant (Wang and De Mejia, 2005). Those amino acids together with their aromatic group will donate their proton to the electron defficient radicals. These amino acids could repair their radical-scavenging characters through several patterns. Antioxidant activity of His peptides related with donating hydrogen, trapped lipid peroxide radicals by chelating metal ions of the imidazole. Conversely, the SH group contained in cysteine take an independent role as antioxidants which directly interact with radicals (Qian *et al*., 2008). According to Rajapakse *et al.* (2005) apart from suitable amino acids, the sequence of peptides is also plays a significant role in its activity as antioxidant and anti-inflammation. Peptides which are bound to specific structures of other peptides also strongly affect their capacity as antioxidants. For example, an amino acid which binds to dipeptides show a higher antioxidant character than if it binds to different peptides (Negre-Salvayre *et al.*, 2008). Conformation of peptides, however, shows a double character, i.e. synergistics and/or antagonistics which depend on antioxidant activity of free amino acids (Hernandez-Ledesma *et al*., 2005). So far, there is no information about mechanism of antioxidant peptides, several researchers, however, assumed that lunacyn peptide could suppress lipid peroxidation, scavengers of free radicals, and chelators of transition metal ions. These peptides, therefore, protect and keep the cells safe from ROS effects.

Peptides which have the potential to be used as antioxidant and anti-inflammation contains 5-16 amino acids (Sefatie *et al.*, 2013). Antioxidant peptides derived from foods are safe and healthy, low in molecular weight, cheap, high activity, and easy to be absorbed as compared with enzymatic antioxidants. These peptides antioxidants have better characters as they are more stable in any situation and may not cause immunity disorders. Soy peptides have been reported by Moure *et al.* (2005) to have molecular weight < 10 kDa. It is clear that the soy peptides have both antioxidants and anti- inflammation. In this study, the CRP in mother's breast milk was very low (0.09 mg/L) and getting lower after taking GSM for 2 consecutive months to become 0.046 mg/L, but statistically did not different *(P=0.77)*.

Conclusion, germinated-soy milk is a functional drink rich in antioxidant and able to suppress oxidative stress as well as cytokine inflammation of lactating mothers. Its potential as an antioxidant and anti-inflammation were shown by the presence of isoflavones, protein, amino acids, and lunacyn peptides of the GSM, through an increasing the SOD, and parallely reducing the MDA and C-RP levels in blood plasma and mother's breast milk. The GSM might be produced massively and so could be consumed by lactating mothers anywhere .

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