An evaluation of soyghurt potential on tumor necrosis factor-α and soluble endoglin levels in preclampsia maternal serum-induced placental trophoblast cell in vitro

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Abstract

Soyghurt is soy milk that has been fermented by probiotic bacteria i.e. Lactobacillus bulgaricus ATCC 11842, which can enhance the immune system (immunomodulator). In vitro laboratory analytical study has been conducted on soyghurt as immunosupressant to decrease of TNF-α and sEng levels as molecular marker in preeclampsia pregnancy. The study used experimental method with 4 stages (i.e making of soybean milk and soyghurt, making of growth and pH curve, trophoblast primary cell culture, and determination of TNF-α and sEng levels) and analyzed by ANOVA followed by Duncan’s multiple range test (DMRT). Results showed that the level of TNF-α on preeclampsia trophoblast cell with soyghurt had decreased from 34.79 pg/ml to 25.87 pg/ml after incubation for 24 h, but more decreased from 36.16 pg/ml to 24.98 pg/ml after 48 h. The level of sEng on preeclampsia trophoblast cell with soyghurt had decreased from 31.23 pg/ml to 24.16 pg/ml after 24 h and more decreased from 31.76 pg/ml to 23.65 pg/ml after 48 h. Decreased levels of TNF-α and sEng were very significant, with p <0.001. In conclusion, soyghurt has the ability as an immunomodulator that decrease the level of TNF-α and sEng in which the greatest result was obtained in concentration of 80% and 48 h incubation time.

Introduction

Maternal mortality was 321 per 100.000 living birth in 2007 according to Health Office, West Java. Preeclampsia and eclampsia are the second causes in maternal deaths after hemorrhage (Gurnadi et al., 2015). Maternal deaths due to hemorrhage was 40-60%, whilst preeclampsia and eclampsia were 20-30%. For two years (2006-2007), preeclampsia occurrence was 13.62% cases in Hasan Sadikin Hospital Bandung (Gunardi et al., 2016).

Etiology of preeclampsia remains unclear. This disease is therefore considered as disease of theory. Trophoblast invasion has been proposed as the major cause that promotes disturbance in spiral artery remodelling. Trophoblast invasion occurs due to immunology maladaptation. Poor trophoblast invasion causes endovascular trophoblast to layer decidual blood vessel, yet unable to reach myometrium that later diminish blood vessel. Diminished arteriole miometrium causes disturbance in placental blood circulation. Bad perfusion and hypoxia trigger release of placental debris, resulting in inflammation and other pathological events (Pramatirta et al., 2015).

Inflammatory mediators, angiogenics, antiangiogenics and placental metabolism are further present that lead to endothelial damage. Endothelial damage stimulates trombocyte to undergo adhesion, agegregation, and release. Both these events are terminated as indicated by decreased vasodilators, such as prostacyclin and nitrite oxide/endothelium-derived relaxing factor and increased vasoconstrictors, such as tromboxan and endothelin, and TNF-α (Pramatirta et al., 2015).

There are angiogenic and antiangiogenic factors during placentation. In preeclampsia, angiogenic imbalance worsen hypoxia and placental implantation. There are two antiangiogenics excessively produced in preeclamptic patients, soluble Endoglin (sEng) and soluble Fms-like tyrosine kinase (SFlt-1) (Gurnadi et al., 2015; Pramatirta et al., 2015; Gunardi et al., 2016).
One of the promising strategies in balancing angiogens and antiogens, as well as act as immunomodulator, is by administration of sojghurt. Previous study showed sojghurt increases immunity to pneumonia by decreasing the adhesion ability and number of Klebsiella pneumonia (Fauziah et al., 2013). In that study, Hep-2 cell line were grown in medium supplemented with sojghurt 10-100%. Sojghurt is a synbiotic in fermented soymilk using Lactobacillus sp. and Streptococcus thermophilus. Lactic acid bacteria is a bacteria group that possess many beneficial effects for the hosts by enhancing the growth and immunity against diseases. Sojghurt is also a health drink for those who suffer lactose intolerance by decreasing lactose to 25%. Other components such as saponin and soyprotein also have antioxidant properties. Yoghurt contain isoflavon known to reduce the risk of degenerative disease such as osteoporosis and cancer (Fauziah et al., 2015).

Trophoblast cells are derived from nine months placental trophoblast in the end of trimester. In previous in vitro study, trophoblast H8 cell line derived from first trimester, were disturbed after treated with serum which equal with common maternal disturbance (Neale et al., 2003). Serum is a blood plasma without fibrinogen composed of protein including electrolyte, antibody, antigen, hormone and exogenous substances. Antibody contained in serum is known to generate inflammation (Gunardi et al., 2016). This study aimed to observe effect of sojghurt on level of TNF-α and soluble Endoglin (sEng) in vitro on nine months trophoblast cell induced by maternal preeclampsia serum.

Materials and Methods

The research was conducted from October 2015 - June 2016 in Department of Obstetrics and Gynecology, Hasan Sadikin Hospital, Faculty of Medicine, Padjadjaran University, Laboratory of Cell Culture and Laboratory of Microbiology, Padjadjaran University. Blood samples were carried from normal and preeclamptic pregnant women. Informed consent were obtained from research subjects.

Preparation of sojghurt

Soymilk was made of yellow soybean. A 300 g soybean was washed and soaked in 5 L water mixed with sodium bicarbonate (NaHCO₃) of 0.25 – 0.5% for 12-24 h. Soybean was washed and peeled. Soybean was mixed with 2.5 L of hot water (80°C - 100°C) and crushed with a blender -for 7 min to obtain pasta form. Pasta was filtered, and added to 125 g sugar, sterilised at 121°C 1 atm (15 lbs) for 10 min. Sojghurt was made of soymilk using L. bulgaricus cultured in MRS. L. bulgaricus of 100 mL was inoculated in soymilk medium, then incubated in shaker bath incubator for 24 h at 37-40°C 125 rpm (Fauziah et al., 2013).

Growth and pH curve of L. bulgaricus ATCC 11842 in sojghurt

Growth rate of L. bulgaricus was observed to obtain optimum age of inoculum to be suitable in assessement of proliferation and pH of cells. Starter of 10 mL was added into soymilk of90 mL. Culture was then batch fermented in scale of 100 mL at 37°C for 24 min and the growth was measured every 2 hour. Bacteria was counted directly with total plate count. Growth curve was made by plotting colony towards time (Fauziah et al., 2015).

Measurement of IC₅₀ (inhibitor concentration 50%) in various concentration of sojghurt on trophoblast cells

Samples in serial concentration (10%-100%) in trophoblast cells were used to determine cytotoxicity toward cells. Positive control was aquadest and DPPH, and negative control was aquadest™ and trophoblast without sojghurt. Absorbance was recorded at 517 nm wavelength at hour 24, and IC₅₀ of each sample was then calculated. Negative control was used as standard in IC₅₀ assessement (Gunardi et al., 2016).

Cell culture

Trophoblast cell was carried from maternal placenta of 34-42 weeks of pregnancy. AmnioMax was used as selective medium for placenta. Cells were passaged for 7 times using RPMI 1640. Primary culture of trophoblast was supplemented into new medium containing RPMI 1640 supplemented with 10% of normal or preeclampsia serum, antibiotic-antimikotic (1% Penicillin G-Streptomycin Solution Stabilised dan 1% Fungizone Amphotericin B). Cells were then incubated for 24 h at 37°C in atmosphere of 5% CO₂ (v/v) to reach confluence Viability was measured on its antibodi titer (Pramatirta et al., 2016).

Measurement of TNF-α and sEng

Cells of 6x10⁴ cell/ml containing 10% of normal and preeclampsia serum, was placed into 96 well-plate, and incubated at 37°C 5% CO₂ (v/v) until confluent (Figure 1). Wells were washed 3-4 times with PBS 37°C to remove the medium and unattached cells. Sojghurt in various concentration was added in accordance with growth rate and pH, incubated for
24 h at 37°C atmosphere 5% CO₂ (v/v). Each well was washed with PBS pH 7.4 for 5 min. Level of TNF-α and sEng were measured with enzyme-linked immunosorbent assay (ELISA) (Gunardi et al., 2016).

Data analysis
Data were analyzed with ANOVA and Kruskal Wallis test. Data analysis was performed with software SPSS 22.

Results

Growth rate and pH of Lactobacillus bulgaricus ATCC 11842 in soyghurt

Results showed growth rate of L. bulgaricus ATCC 11842 in soyghurt towards time can be seen in Figure 2. As shown in Figure 2, there was correlation between growth rate of L. bulgaricus ATCC 11842 and incubation time. L. bulgaricus ATCC 11842 showed highest growth at 18 h that reached seven-folds from 0 h, and decreased afterwards. These findings confirm that soyghurt is a good medium of L. bulgaricus ATCC 11842 growth. pH optimization of L. bulgaricus ATCC 11842 in soyghurt toward incubation time is presented in Figure 3. As shown in Figure 3, there was correlation between decreased pH L. bulgaricus ATCC 11842 and incubation time. Optimum growth of L. bulgaricus ATCC 11842 was on 18 h with pH of 5.39 which was considered not too acid that do not cause damage on mammalian cell. Mammalian cells are known to have tolerance for pH ±5.0 – 8.8 (Sayuti et al., 2012). LC₅₀ values are presented in Figure 4. In this study, soyghurt were divided in 11 concentrations (0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% dan 100%).

Measurement of IC₅₀ of soyghurt in various concentrations on trophoblast cells

Figure 4 showed measurement of soyghurt in triplicate. At concentration of 81.6% (Amniomax), 90.7% (DMEM) and 87.2% (RPMI 1640), soyghurt showed 50% trophoblast cells. IC₅₀ value less than 81% indicate non-toxicity towards cells.

Effects of soyghurt in various concentration, incubation time, and serums on level of TNF-α

Variables tested in this study were normally distributed both in normal and preeclampsia serum treated with soyghurt in various concentrations incubated for 24 h and 48 h (data are not shown). Effects of soyghurt in various concentration, incubation time, and serums on level of TNF-α is presented in Figure 5. As shown in Figure 4, level of TNF-α decreased with increasing incubation time and increased soyghurt concentration. Soyghurt decreased level of TNF-α (p<0.001). Level of TNF-α in preeclampsia-induced trophoblast cells decreased from 34.79 pg/ml to 25.87 pg/ml after treatment with soyghurt of 80% incubated for 24 h, and lowered after 48 h from 36.16 pg/ml to 24.98 pg/ml.

Effects of soyghurt in various concentration, incubation time, and serums on level of sEng

Effects of soyghurt in various concentration, incubation time, and serums on level of sEng, are presented in Figure 6. Based on Figure 6, soyghurt
significantly decreased sEng level (p<0.001) in concentration-dependent manner. Lower levels of sEng was associated with higher concentration of soyghurt. Level of sEng decreased from 31.23 pg/ml to 24.16 pg/ml at 80% soyghurt incubated for 24 h, and decreased from 31.76 pg/ml to 23.65 pg/ml after 48 h.

Discussion

In this study, soyghurt showed immunomodulatory and antioxidant activities. Level of TNF-α and sEng in preeclampsia-induced cells decreased which was comparable to that of normal cells. Trophoblast invasion occurred in preeclampsia due to immunology maladaptation, reduce uteroplacental perfusion that causes placental ischemia. Antiangiogenics such as soluble Endoglin (sEng) are produced excessively (Keogh et al., 2007; Pramatirta et al., 2015). Inflammatory mediators, angiogenics, antiangiogenics and placental metabolism leads to endothelial damage. Endothelial damage stimulates trombocyte to undergo adhesion, agegregation, and release. Both these events are terminated as indicated by decreased vasodilators, such as prostacyclin and nitrite oxide/endothelium-derived relaxing factor and increased vasoconstrictors, such as tromboxan and endothelin, and TNF-α (Yuan et al., 2005; Gurnadi et al., 2015).

To the best of our best knowledge, there is no studies that observe potential of probiotics and antioxidant in soyghurt on preeclampsia. Probiotics are orally ingested. Bacteria are usually killed by gastric acid, HCl, yet probiotics can be digested into colon. Probiotics play roles in producing essential nutrient and antibiotic that kills pathogens, and inhibits pathogen adhesion on colon receptor. Probiotics stimulate immunoglobulin E (IgE) release to againsts pathogens in digestive tract, and also neutralize toxin produced by pathogens that results in reduced free radicals in the body (Fauziah et al., 2015; Herawati et al., 2015).

Probiotics also plays role as antigen carrier, and bind to targeted tissue which activate macrophage to stimulate immune system. Probiotics stimulate cells to increase the producing IgA, IgM, and IgG, as well as specific sIgA both in blood serum that transport nutrient to all tissues, and colon lumen, and also modulate immune response towards inflammation and balancing vasoconstrictors (Herawati et al., 2015).

*Lactobacillus bulgaricus* was used in this study due to its ability to produce lactic acid with sufficient pH which is considered safe to the body. *L. bulgaricus* has high lipolitic among probiotics, as indicated by more favorable taste compared to other soymilks (De Preter et al., 2007). The presence of isoflavonoid in soybeans has been long known to possess antioxidant properties. Many health researches reported potential of soy-based products in treating degenerative diseases Isoflavones in soybeans has many beneficial effects on lipid profile repair, protection of LDL against oxdation, enhance
antioxidant activity in liver, anti-inflammatory, and kills pathogens. Anti-inflammatory mechanism of isoflavones underlies on inhibition of arachidonate acid metabolism, prostaglandin synthesis, histamine release, or radical scavenging activity. Cells are therefore protected from negative effects, resulting in enhanced viability by balancing the molecules in the body. Beneficial effects of probiotics and plants are digested into cells through blood circulation (Kumar et al., 2002; Lammersfeld et al., 2009).

Soyghurt is a fermented soymilk using probiotic, Lactobacillus bulgaricus (Fauziah et al., 2013). L.bulgaricus is the common probiotic used in fermentation, other than Lactobacillus acidophilus or Bifidobacterium. Soymilk can be a substrate for Lactobacillus bulgaricus that increases nutrient content in soymilk (Kaboosi, 2011). Lactobacillus bulgaricus produces secondary metabolite, lactic acid with higher pH than L. acidophilus, which is considered safe for cells (Isolauri et al., 2001; Pretzer et al., 2005; Fauziah et al., 2015). Soyghurt possess ability to enhance immune system (immunomodulator) due to metabolite secreted by probiotics, strong antioxidant, and do not contain lactose (Sanjaya et al., 2007). Moreover, soybean is known to contain isoflavones that plays role in serum lipid repair, protection of LDL on oxidation, increase activity of certain antioxidant enzymes in liver and kills pathogens, and also prevent inflammation. Referring to previous study, L.bulgaricus has highest lipolitic content among Lactobacillus that can enhance nutrient content, mainly in dairy product (Tambebak and Bhutada, 2010; Fauziah et al., 2013).

**Conclusion**

Soyghurt has the ability as an immunomodulator because can decrease the level of TNF-α and sEng. The highest treatment for decrease TNF-α and sEng was obtained in 80% concentration of soyghurt and 48 h incubated time.

**References**


