Global Medical and Health Communication

Online submission: http://ejournal.unisba.ac.id/index.php/gmhc
DOI: https://doi.org/10.29313/gmhc.v7i1.3245

GMHC. 2019;7(1):64–70
pISSN 2301-9123 | eISSN 2460-5441

RESEARCH ARTICLE

Soyghurt Supernatant on Mouse Embryonic Fibroblast (MEF) Cell

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Abstract

Yogurt is a functional food developed with various modifications in the fermentation process. Replacing animal milk into soymilk as raw material is one approach. Yogurt has a good effect on human health. Probiotic and bioactive compounds in yogurt can inhibit cell proliferation and stimulate apoptosis on the cancer cell line. However, there is no report about the effect of yogurt on a normal cell. This research was conducted to examine the impact of soyghurt supernatant intervention toward the viability of mouse embryonic fibroblast (MEF) cell. The is an in vitro study using MEF cell isolated from 10th days gestational age mice embryo conducted at Microbiology Laboratorium and Cell Culture and Cytogenetic Laboratory, Universitas Padjadjaran, Bandung in November 2018–January 2019. Soyghurt made from soymilk fermented by *Lactobacillus bulgaricus* ATCC 11842. The number of the bacterial colony calculated by total plate count (TPC) method and pH calculated by pH meter. Soyghurt supernatant was made from soyghurt and then intervened into MEF cells by 1–20% concentration. The cell viability showed in the 50% inhibitory concentration (IC_{50}) analysis. The intervention of soyghurt supernatant at 1–20% concentration showed there was no proliferation inhibition until 50% population (IC_{50}). However, from the morphology analysis, there was MEF cell morphology alteration on the group given soyghurt supernatant with >12.5% concentration. Counter mechanism effect from soymilk fermentation by probiotic could be the driver for this result. In conclusion, soyghurt supernatant intervention at 1–20% concentration did not have a cytotoxic effect on MEF cell, but enhancement of soyghurt supernatant concentration can increase cytotoxic potential.

Key words: Cell viability, MEF cell, soyghurt supernatant

Supernatan *Soyghurt* pada Sel *Mouse Embryonic Fibroblast* (MEF)

Abstrak

Yoghurt merupakan *functional food* yang dikembangkan dengan berbagai modifikasi dalam proses pembuatannya. Mengganti susu hewan dengan susu kedelai sebagai bahan baku adalah salah satunya. Yoghurt memiliki efek yang baik bagi kesehatan manusia. Senyawa probiotik dan bioaktif pada yoghurt dapat menginhibisi proliferasi sel dan menstimulasi apoptosis pada sel lini kanker. Akan tetapi, tidak terdapat laporan mengenai efek yoghurt pada sel normal. Penelitian ini dilakukan untuk menguji pengaruh intervensi supernatan *soyghurt* terhadap viabilitas sel *mouse embryonic fibroblast* (MEF). Ini adalah penelitian *in vitro* menggunakan sel MEF yang diisolasi dari embrio tikus hari ke-10 usia kebuntingan yang dilakukan di Laboratorium Mikrobiologi dan Laboratorium Kultur Sel dan Sitogenetika, Universitas Padjadjaran, Bandung pada November 2018–Januari 2019. *Soyghurt* dibuat dari susu kedelai yang difermentasi oleh *Lactobacillus bulgaricus* ATCC 11842. Jumlah koloni bakteri dihitung dengan metode *total plate count* (TPC) dan pH diukur dengan pH meter. Supernatan *soyghurt* dibuat dari *soyghurt* dan kemudian diintervensi ke dalam sel MEF dengan konsentrasi 1–20%. Viabilitas sel ditunjukkan dalam analisis penghambatan 50% (IC_{50}). Pemberian supernatan *soyghurt* konsentrasi 1–20% menunjukkan tidak terdapat inhibisi proliferasi 50% (IC_{50}). Namun, dari analisis morfologi, terdapat perubahan morfologi sel MEF pada kelompok yang diberi supernatan *soyghurt* dengan konsentrasi >12,5%. Efek mekanisme yang saling meniadakan dari fermentasi susu kedelai dengan probiotik diduga menjadi mekanisme hasil dari penelitian ini. Simpulan, intervensi supernatan *soyghurt* pada konsentrasi 1–20% tidak memiliki efek sitotoksik pada sel MEF, namun peningkatan konsentrasi supernatan *soyghurt* dapat meningkatkan potensi sitotoksik.

Kata kunci: Sel MEF, supernatan *soyghurt*, viabilitas sel

Received: 28 November 2017; Revised: 8 May 2019; Accepted: 10 May 2019; Published: 10 May 2019

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Introduction

Soybean is well known as a based plant food source for daily consumption. Besides protein, soy also contains fiber, iron, and lower level of free fatty acid. With technology development in food and dairy product, soybean is not only consumed as tofu and tempeh but also used as a raw material for fermented products. One of the fermented products produced from soy is soyghurt. The fermentation itself involved lactic acid bacteria, such as *Lactobacillus* sp. and *Streptococcus thermophilus*.[1]

Yogurt and probiotic have existed since Greece era. This processed food is used to be known as a functional food, that does not only contain a nutritional compound needed by a human but also have a beneficial effect on human health.[2,3] Some modification has been developed in the yogurt production process such as combined with additional natural resources as a raw material.[1] In this research, soybean used as a raw material in the soyghurt production process.

Fermentation of soymilk has excellent benefits for human health. Not only serves as an alternative type of beverage that can be consumed daily, but also able to be as an anticancer.[4] Probiotic as one of the critical components of soyghurt can inhibit proliferation and cytotoxic effect on the cancer cell line.[5] Another essential element for soyghurt is soybean, which contains phenolic compounds such as isoflavone, flavonoid, saponin, and quinone. These compounds also have a cytotoxic effect on the cancer cell line.[6]

Many studies have examined the effect of probiotic on the cancer cell line. However, there are no reports on the impact of yogurt on normal cell viability. A typical cell that often used in in vitro studies is the mouse embryonic fibroblast (MEF) cells. The MEF cells are the primary cell isolated from mouse embryonic. The MEF cell was used in many studies because these cells are easy to separate and fast to proliferate. Several studies that examined the cytotoxic effects of plant extracts on cancer cell line using MEF cells as a comparison of healthy cells.[7–9] This research was conducted to examine the impact of soyghurt supernatant intervention toward the viability of mouse embryonic fibroblast (MEF) cell.

Methods

This study has used yellow soybean (*Glycine max*) that cultivated from Tasikmalaya, West Java, Indonesia. The production of soyghurt from yellow soybean conducted in Microbiology Laboratorium and Cell Culture and Cytogenetic Laboratory, Universitas Padjadjaran, Bandung, Indonesia. The process of manufacturing, according to the previous research with some modification.[1]

First, soymilk made from yellow soybean. As much as 300 grams soybean washed and soaked in 5 L water that mixed with 0.25–0.5% sodium bicarbonate ($NaHCO_3$) for 24 hours. Soybean then washed and peeled, and crushed with blender for 5 minutes then mixed with 2.5 L of hot water (80°C) until becoming a puree. Puree then filtered and added 125 grams sugar, sterilized at 121°C atm for 10 minutes. After that, 100 mL *Lactobacillus bulgaricus* ATCC 11842 was inoculated in soymilk then incubated in an incubator for 48 hours at 37–40°C.[1]

After incubation, an entire colony of bacteria from soyghurt calculated with total plate count (TPC) method.[10] The 1 mL of soyghurt diluted in NaCl (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}).[7] Diluted soyghurt was cultured in the De Man, Rogosa, and Sharpe (MRS) agar. After 48 hours, the colony bacteria calculated. The pH of soyghurt examined by pH meter.[1,2] The bacterial colonies number obtained from this soyghurt in according to *Standar Nasional Indonesia*/SNI (Indonesian National Standards) 01-2981-1992. The characteristics of soyghurt produced include the colonies amounts were 2×10^8 CFU/mL, and acidity (pH) measured was 5.39.

Soyghurt supernatant made from soyghurt. Soyghurt centrifuged with 1,500 rpm T 4°C for 10 minutes. The supernatant harvested, then filtered using a 0.2 μm syringe filter (Sigma-Aldrich).[11] After obtained the soyghurt supernatant, then phytochemical screening was conducted for further analysis about a compound that contained in soyghurt supernatant.

The MEF cell was isolated from mice embryo on the 10th day of gestational age according to previous protocol.[12] On the 10th gestational days, the pregnant mouse sacrificed, and the embryo was taken and washed with phosphate-buffered saline (Sigma-Aldrich). Head and visceral organ separated from the fetus. The embryo tissue was enumerated and added 0.25% trypsin-EDTA (Thermo Fisher Scientific) for 10 minutes until it becomes a single cell. Then, the cell was cultured in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific) culture medium containing 1% antibiotic solution (Sigma-Aldrich)

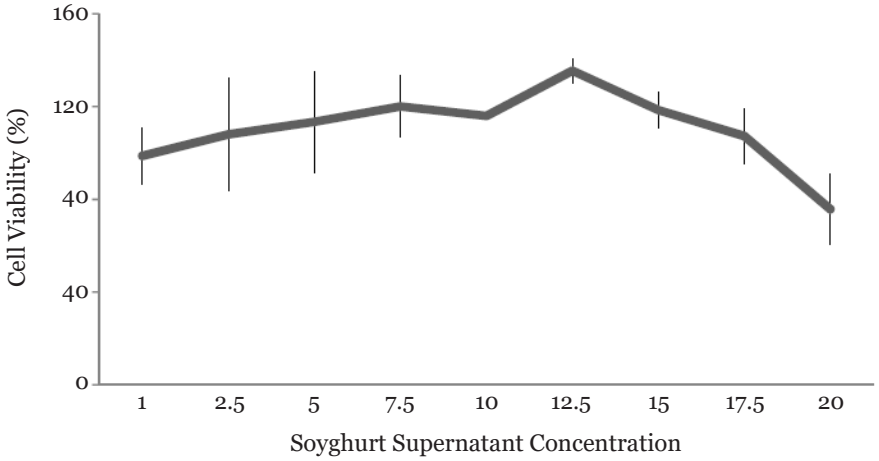

Figure 1 IC_{50} of Soyghurt Supernatant Intervention on MEF Cell
Data presented as mean±SD for three replication, soyghurt supernatant in X-axis, and relative cell viability in Y-axis

and 10% fetal bovine serum (Sigma-Aldrich). Then, soyghurt supernatant supplemented to MEF cells. From other study shown that bacteria supernatant with 10% concentration can inhibit the proliferation of cancer cell line.[13] Based on that result, the soyghurt supernatant concentration of 1% to 20% v/v chosen.

The cells were plated 96-well plate (5×10^4) for MTT assay examination. After confluence, cell was treated by soyghurt supernatant with various concentration (0%, 1%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, and 20% v/v), then incubated in incubator CO_2 T 37°C. After 24 hours, the viability cell examined using MTT assay method. Medium from the cell was taken out. MTT reagent 100 µL (5 mg/mL) added, then incubated for 2–4 hours in incubator CO_2 T 37°C until formazan crystal formed. After that, 200 µL MTT solvent (DMSO) added and formazan crystal as dissolved and the absorbance read at OD=450–550 nm. Measurements conducted and the concentration required for a 50% inhibition of viability (IC_{50}) analyzed graphically. The standard graph plotted by taking the concentration of soyghurt supernatant in the X-axis and relative cell viability in Y-axis.[14,15]

The procedure of this study approved by the Health Research Ethics Committee of Faculty of Medicine Universitas Padjadjaran, Bandung with letter number: 1268/UN6.KEP/EC/2018.

Table Results of Yoghurt Supernatant Phytochemical Screening

Name of Compound	Results
Flavonoid	–
Saponin	+
Polyphenol	–
Alkaloid	–
Sesquiterpenes/monoterpenes	+
Titerpenoid	–
Quinon	+
Tanin	–

Results

Results of soyghurt supernatant phytochemical analysis contained saponins, sesquiterpenes/monoterpenes, and quinones (Table).

Figure 1 shows the intervention of the soyghurt supernatant at a 1–20% concentration has not reached a 50% inhibition of cell proliferation (IC_{50}). However, when the dose increased, it showed a slight inhibition that is close to 20% proliferation of MEF cell.

Figure 2 showed morphological changing on MEF cell. The cells become dense, then shrinks, and fragmentation occurred at >12.5% soyghurt supernatant concentration compared to the control group. The morphological anomaly also showed in 1% concentration.

Discussion

In this study, animal milk substituted by soymilk

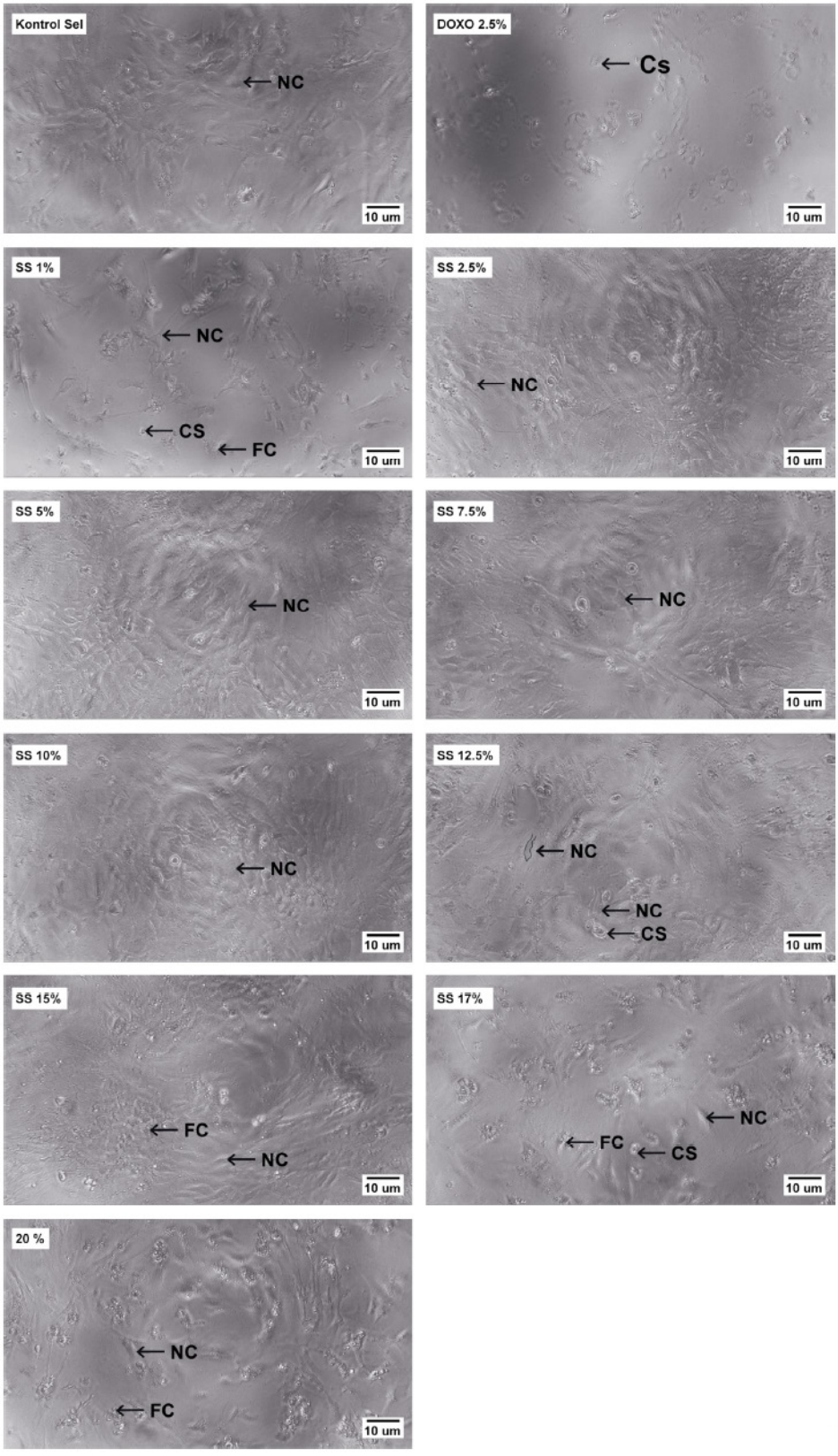

Figure 2 MEF Cell Morphology After Soyghurt Supernatant Intervention
Observations using an Olympus inverted microscope (40× magnification). SS: soyghurt supernatant; NC: normal cell; CS: cell shrinkage; and FC: fragmented cell

for fermentation. Soymilk has content that can be fermented by lactic acid bacteria for the bacterial growth process. This fermentation process produces higher antioxidant amount compared to unfermented soymilk.[16]

The probiotics used in the fermentation process produced the nutritional components needed for human health, such as proteins, vitamins, and antioxidant compounds.[4] In this study, a phytochemical screening test performed on the soyghurt supernatant, which used as an intervention in MEF cells. The result shows that the supernatant of soyghurt contains saponins, sesquiterpenes/monoterpenes, and quinones (Table). Saponins are a glycosides group found in plants and usually used to produce detergents.[17,18]

The density of the MEF cell observed after intervention with an increase in the concentration of soyghurt supernatant. The IC_{50} (Figure 1) results showed that MEF cells did not experience the inhibition of proliferation up to 50% by soyghurt supernatant treatment with a concentration of 1–20% v/v. The different result directly obtained in the intervention of probiotics in cancer cells line, which showed proliferation inhibition and apoptosis. The case mechanisms include intrinsic and extrinsic apoptosis activation.[17,18] Previous studies showed that treatment on 100 µL doses of bacterial supernatant prevents cytokine-induced apoptosis in intestinal epithelial cells. Whereas in other research demonstrated that *Lactobacillus* supernatant doses >100 µL bacteria could inhibit proliferation of human colonic carcinoma cell line HT-29.[13] Similar results showed from soybean extract treatment to cancer cells line.[6] In this study, it found that there was no proliferation inhibition until 50% population after intervention with soyghurt supernatant. We hypothesize that there is a counter mechanism presence caused by the combination of probiotics and soybeans. Our hypotheses that soyghurt has a mutually repealing mechanism that does not effect on MEF cell viability.

However, we observed that there were morphological changes at MEF cells with >12.5% concentration v/v. This result suspected because of the content of saponin in the soyghurt supernatant. Saponin is a glycosidic found in plants and has a detergent-like effect that can lyse cells walls such as blood cells and fungi. The active saponin ingredients are equivalent to Triton X that can be extracted from several plants and used as material to lyse fungi.[17,18] Moreover, we also suspect that cell morphological changes occurred because of quinone presence in the supernatant that has an antitumor effect.

Other studies report that the effect of saponins treatment in 4T1 cancer cells depends on the number of the dose. At low doses, the saponin compounds produced from ginseng will not inhibit cell viability, migration, and cell invasion, but in higher doses, it will inhibit cancer cell metastasis.[19] Different effects appear in neural cells; according to other studies, saponin has been shown to have the ability to support nerve cell embryo proliferation. In 3–4 days, with a dose of 100 ppm saponin will increase the number of nerve cells (42.89±5.90) compare to without saponin (19.22±6.67).[20] The difference in results depends on the characteristics of gene expression that interact with saponin compounds. This result showed that saponins treatment on endometrial cells could inhibit *miR-21-5p* gene and also have an apoptotic effect,[21] whereas in healthy neural cells, saponin support cell proliferation.[20] In this study showed that saponin which contained in soyghurt supernatant did not have a cytotoxic effect to MEF cells.

Beside saponins, the compounds that found in soyghurt are sesquiterpenes. Sesquiterpenes are terpenoid C15 composed of three isoprene units. Naturally, sesquiterpenes obtained in the hydrocarbon compound such as lactose and alcohol.[22] The structure of sesquiterpene that dominantly used in tumor treatment is a molecule with a ring structure, contain oxygen and carbonyl groups. This structure is called alpha-methylene-gamma-lactone, or shortly called lactone sesquiterpenes. The structure of sesquiterpenes causes chemical changes that initiate damage to target cells or foreign microorganisms. In cancer cells, the protein activity will increase the sensitivity of the lactone sesquiterpenes to alkylate the membrane cell. This alkylation process will cause cell damage. Besides that, sesquiterpenes can regulate gene expression to activate and deactivate transcription factors on cancer cells.[23] Protein changes only useful in the cancer cell, while in healthy cells, the alkylation of cell walls will not appear. Furthermore, it will not trigger cell damage.

Other research showed that phenolic compound could stimulate the proliferation of cells, especially fibroblast cells. In the study accomplished by Nurulita et al.[24] demonstrated that apple extract containing phenolic compound

had a protective effect on NIH3T3 cells after H_2O_2 induction. The similar discovery also found in the banana stem sap treatment in the wound healing process. The results have shown that flavonoid and derivatives contents can stimulate fibroblast cells proliferation in the wound healing process.[25–27]

Conclusions

Soyghurt supernatant intervention at 1–20% concentration did not have a cytotoxic effect on MEF cell, but enhancement of soyghurt supernatant concentration can increase cytotoxic potential. For this reason, further research required for a complete understanding of the bioactive content of soyghurt and its mechanism regarding cell viability.

Conflict of Interest

The authors declare no conflict of interests.

Acknowledgements

The author's gratitude to Universitas Padjadjaran for funding and facilities support to this research. Authors also grateful to research technician in Microbiology Laboratory and Cell Culture and Cytogenetic Laboratory, Universitas Padjadjaran, for supporting this research.

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