KOMISI ETIK PENELITIAN KESEHATAN

FAKULTAS KEDOKTERAN
UNIVERSITAS WIJAYA KUSUMA SURABAYA

KETERANGAN KELAIKAN ETIK
"ETHICAL CLEARENCE"

No. 5 /SLE/FK/UWKS/2022

KOMISI ETIK PENELITIAN KESEHATAN

PENELITIAN BERJUDUL:
Moringa oleifera decrease in IL-6, IL-17, and STAT-3 in high glucose human trophoblast cell culture

PENELITI UTAMA:
Dr. dr. Harry Kurniawan Gondo, SpOG (K)

UNIT / LEMBAGA / TEMPAT PENELITIAN:
Lab. Faal/ Physiology Fakultas Kedokteran Universitas Brawijaya Malang dan Salah satu Rumah Sakit Swasta Surabaya

MENYATAKAN:
" LAIK ETIK "

Surabaya, 11 Januari 2022

Mengetahui,
Dekan

Ketua Unit,

Prof. Dr. Suhartati, dr. MS

Dr. Erny, dr., Sp.A (K)

SPER Publications and Solutions Pvt. Ltd.

Original Article

Moringa oleifera decrease in IL-6, IL-17, and STAT-3 in high glucose human trophoblast cell culture

Harry Kurniawan Gondo[1*]

[1] Department of Obstetric and Gynecologic, Faculty of Medicine, Wijaya Kusuma University of Surabaya, Indonesia.

Correspondence: Harry Kurniawan Gondo, Department of Obstetric and Gynecologic, Faculty of Medicine, Wijaya Kusuma University of Surabaya, Indonesia. harry.gondo@uwks.ac.id

ABSTRACT

Preeclampsia (PE) was still the main cause of maternal death in the world. Many efforts to prevent this however some studies report that PE had a high incidence mainly in rural areas. Hence, one needs to solve this problem using herbal remedies. Recently, Moringa oleifera (MO) was reported to have health benefits as antioxidative properties. Its main bioactive isothiocyanate (ITC) which had been reported to have anti-inflammatory properties. However, it was not known its potency to prevent or treat PE. We conducted a study using human trophoblast cell culture. Trophoblasts are isolated from placental tissue. To induce PE, we use high glucose to induce inflammation. After confluent, cells were grouped into 2 treatment groups, namely (1) normal glucose (5 mM as negative control) and high glucose exposure of 33 mM (positive control), each group was divided into 2 subgroups, (a) without isothiocyanate treatment and (b) isothiocyanate treatment dose of 0.1; 0,2; 0.4 and 0.8 mg/ml. Next, each treatment is cultured in a 5% CO2 incubator at, a temperature of 37 °C for 3 days. The supernatant was collected and assay its cytokine using ELISA. The result shows that pro-inflammation cytokines such as IL-6, IL17, and STAT3 were significantly decreased and anti-inflammation cytokine IL-10 was significantly increased compared to the control group. Hence we concluded that the ITC of Moringa oleifera had alleviated inflammation in the PE model human trophoblast cell culture.

Keywords: IL6, IL17, STAT3, Trophoblast cell, Isothiocyanate, Moringa oleifera

Introduction

Until now preeclampsia was still the prime cause of the mother's death in the world. More than 25 percent of maternal deaths in Indonesia were caused by PE [1]. In molecular studies, pre-eclampsia is strongly associated with the inadequacy of trophoblast invasion and the failure of spiral artery remodeling that would be the cause of maternal and fetal death [2].

Trophoblast cell invasion towards the uterine wall is an important process in pregnancy. Trophoblast cells will convert the spiral artery of the uterus (remodeling spiral artery) into blood vessels that have low resistance and large blood flow in supplying the placenta to support the growth and development of the fetus. Invasion of trophoblast extravillous cells will change the extracellular matrix (ECM) so that the blood vessels of the uterine spiralis artery are able to circulate towards the intervillous space to drain the mother's blood as fetal nutrition [3].

In normal pregnancy, IL-6 plays an important role in the preconception, implantation, and development phases of the placenta. Interleukin-6 along with other cytokines and growth factors have a role in controlling placental morphogenesis and coordinating the proliferation of trophoblast cells [4]. The il-6 activity begins with the bonding of IL-6 with the surface receptors IL-6 (IL-6R) and GP 130 (Glucoprotein 130) which will further activate Janus Kinase (JAK), the Signal Transducers and Activators of Transcription 3 (STAT3) pathway, the Mitogen-Activated Protein Kinase (MAPK) pathway and will be passed on as a signal to the nucleus to induce transcription in certain target genes [5]. However, in PE, there was immune dominance of T Helper 1 cells (Th1) against Th2 in early

Access this article online

Website: www.japer.in	**E-ISSN:** 2249-3379

How to cite this article: Gondo HK. Moringa oleifera decrease in IL-6, IL-17, and STAT-3 in high glucose human trophoblast cell culture. J Adv Pharm Edu Res. 2022;12(4):86-90. https://doi.org/10.51847/Scc6BhYAWA

This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-Non Commercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

pregnancy. The dominance of the Th1 immune system in PE will be followed by an increase in several proinflammatory cytokine mediators, one of which is the cytokine Interleukin-6 (IL-6) which increases in the serum blood, amniotic fluid, and placenta. Besides Th1, an increase in Interleukin-6 (IL-6) and trophoblast apoptosis was strongly associated with PE pathomechanism. Increased IL-6 will act as a pro-inflammatory mediator with immunological dominance Th1. Changes in the pro-inflammatory environment result in changes in the characteristics of trophoblast cells that were originally resistant to Fas-ligand (FasL, apoptosis mediator) to be susceptible to apoptosis. IL-6 along with TGF beta 1 (Transforming Growth Factor – beta 1) through the STAT3 pathway stimulates increased secretion of IL-17. Increased levels of IL-17 will further induce endothelial tissue apoptosis by activating caspase–3 and increasing the BAX/BCL2 ratio [6].

Several efforts for PE prevention and treatment are made, ranging from medical treatment, dietary arrangements, and improved lifestyle with regular exercise or with the use of traditional plants as medicines. The use of the local plant as traditional medicine has been known for a long time by Indonesian people. One type of plant used in traditional medicine is Moringa oleifera (MO) [7, 8]. Recently, MO was popular as a supplement food for pregnancy. Its leaves are rich in antioxidants and are reported to have anti-inflammatory properties [9, 10]. However, its anti-inflammatory activities in the inflammation process in PE were never known yet. Hence, we conduct this study intending to know the effect of MO isothiocyanate on inflammation of human trophoblast cell culture.

Materials and Methods

Time and location

This research was conducted in the Laboratory of Physiology Faculty of Medicine Universitas Brawijaya Malang Indonesia and the umbilical cord was taken from a private hospital in Surabaya Indonesia. Normal placental tissue obtains from caesarian surgery delivery and normal delivery. Normal placental tissue is obtained with the patient's consent.

Trophoblast cell isolation and culture work procedure

The placenta is taken in a transport medium to keep its trophoblast cells alive. Previously the base of the 6-well culture plate was coated with a glass cover and dripped with ± 0.5-1 ml of gelatin (0.2%) and incubated for ± 30-60 minutes. Placental tissue was washed using sterile PBS-A (PBS-A) pH 7.4 containing pen-strep antibiotics in a petri dish until it is free of blood. The tissue is cut into small pieces ± 2 mm3 and rinsed with a sterile PBSA pH 7.4 containing pen-strep, then pickled and centrifuged at 2500 rpm for 10 minutes. The supernatant is discarded and pellet I is suspended with 5 mL medium-free serum culture (M-199 + pen strep), pickled, and centrifuged at 2500 rpm for 10 minutes. The supernatant is removed and pellet II is suspended with a culture medium containing serum (M-199 + pen-strep + 10% FBS), taken as much as ± 500L pieces of tissue are inserted on the 6 well culture plate and incubated in a 5% CO2 incubator, temperature 37°C for 30 minutes. Added 1.5 ml medium M-199 containing 10% FBS and then incubated in a 5% CO2 incubator, temperature 37°C. Replacement of the culture medium is carried out after 24 hours with M-199 + 10% FBS then replanted in the CO2 incubator at 5%, s uhu 37 °C for 3 days then harvested.

To take trophoblast cells from the placenta, this part is important because the placenta is made up of many cells. To get trophoblast cells from the placenta, the human placenta is taken all. The part of the placenta taken is the basal part of the placenta, where the surface of the placenta meets the uterine wall (maternal-fetal interface surface). Placental tissue is separated from blood vessels, fibrous fingers, and amniotic membranes in a blunt manner, where blunt parts of the scalpel can be used. Trophoblasts are isolated from placental tissue. Where 1 gram of placenta a term isolated and cultured will be obtained from about 2.5 million trophoblast cells. Fibrous tissue and blood vessels are removed, placental tissue is washed then the tissue is chopped. The tissue suspension is incubated with 0.2 % mg/ml Collagenase type I (Sigma) for 45 minutes, 37OC with shaking. Incubation is stopped by adding culture media (*Dulbecco's Modified Eagle Medium, DMEM*/F12 (1:1) Added with 15 mmol/l Hydroxypiperazineethansuphonic acid, HEPES, 14 mmol/l NaHCO3, 33 μmol/l biotins, 17 μmol/l D-pantothenate and 10% FBS). The cell suspension is rotated at 1500 rpm for 7 minutes then the supernatant is discarded. Pellets containing trophoblast cells are resuscitated with a culture medium then the cell is rotated at 1500 rpm for 7 minutes. Pellets are re-suspended with culture media.

Isothiocyanate and glucose administration treatment

Moringa isothiocyanate (ITC) was purchased from Sigma Aldrich (Prod No 476013). Administration of glucose as an experimental model of GDM events. Primary cultures of trophoblast cells that have been confluent after 3 days are grouped into 2 treatment groups, namely (1) negative control without adolescence, positive control by glucose administration, treatment control 1; 2; 3, and 4 with the treatment of ITC therapy doses of 0.1; 0,2; 0.4 and 0.8 mg/ml. Furthermore, each treatment is cultured in a CO2 incubator at 5%, temperature of 37 °C for 3 days.

Observation of the number of cells expressing levels of IL-6, IL-10, IL-17, and STAT 3 By ELISA method

Measurement of cytokine levels IL-6, IL-10, IL-17, and STAT3 by ELISA method. The quantity of IL-17 in trophoblast cells is seen through the abundance of Anti-IL-17 per Rhoda mine

(orange) color in binding to IL-17 cells. trophoblasts bound to anti-trophoblasts labeled FITC dye (green). The method used is double staining Immunofluoresen with a reading of 3 airy with a magnification of 1000x the view of the placenta. Olympus 7.00 immunoflow software using FX 81 type Konvocal Microscope analyzer.

In stat3 expression, The quantity of STAT3 in blas trophosal cells is seen through the number of Anti-STAT3 colors Rhoda mine (orange) in binding to STAT3 trophoblast cells bound to anti-trophos blas labeled FITC dye (green). The method used is double staining Immunofluoresen with a reading of 3 airy with a magnification of 1000x the view of the placenta. Olympus 7.00 immunoflow software uses fx 81 type konfocal microscope measuring instruments. Each specific microplate well containing monoclonal anti-IL-17 and STAT3 antibodies added 100 L diluent RD1A then continued with the addition of 100 L standard solution each specific microplate well containing monoclonal anti-IL-17 antibodies and STAT3 added 100 L diluent RD1A then followed by the addition of 100 L standard solution or sample to each well. Standard solution using wells A1-H1 and A2-H2. Other wells are used for samples. The microplate is covered with an adhesive strip. Microplates are incubated for 2 hours at room temperature. The contents of the well are then discarded and washed with a washing buffer 4 times. Washing was done by filling each well with a 300 ml washing buffer using a multi-channel pipette. In the last wash, any remaining washing buffers are discarded by inspiring or pouring. The microplate is turned over and dried by wiping it with clean suction paper. Into each well is added 200 L conjugate IL-17 and STAT3 then the microplate is covered with adhesive strips and incubated for 2 hours at room temperature. The microplate is then washed again with aspiration, then added back 200 L substrate to each well, incubated for 20 minutes at room temperature, and protected from sunlight. Finally, 50 L of stop solution is added to each well. The optical density of each well is read with a micro ELISA reader within 30 minutes at a wavelength of 450 nm.

Results and Discussion

Based on the results of IL-6 and IL-10 levels showed that ITC at a dose of 0.2 mg/day significantly reduce the level of interleukin-6 (IL-6) **(Figure 1)**. Furthermore, ITC dose 0.4 mg/day/kg BB significantly increase the level of interleukin-10 (IL-10) **(Figure 2)**.

Table 1. Average Effect of ITC Therapy On Levels of interleukin-6 (IL-6) and interleukin-10 (IL-10)

Treatment Group	N	Mean	
		interleukin-6 (IL-6)	interleukin-10 (IL-10)
P-	5	9,88	472,22
P+	5	17,80	114,5
P1	5	13,58	121,77
P2	5	10,15	241,31
P3	5	10	431,16
P4	5	8	463,28

As seen in **Table 1**, in control group (P-), an average value was 9.88 and in control positive group, its average value was 17,70. Trophoblast cell culture given glucose 33 mM causing an effect of increasing IL-6 levels in trophoblast cell cultures by 17.80. In each treatment is given a different dose of ITC. In P1 there was a decrease in the average il-6 level of 13.58 followed by P2 il-6 levels decreased by 10.15. In P3 il-6 levels decreased to 10 and there was a decrease in IL-6 levels at P4 by 8. P4 has the lowest average number and is close to the negative control **(Figure 1)**. IL-6 is a pro-inflammatory cytokine associated with the immune system in pre-eclampsia pregnancy. In pre-eclampsia, there is maladapted at the site of implantation which contributes to the subsequent disruption of trophoblast cell invasion in the formation of the decidua spiral artery which decreases the blood flow of the uteroplacental artery thus making the placenta ischemic and hypoxia. Increased levels of antiangiogenic factors, placental debris, ROS, and pro-inflammatory cytokines result in endothelial cell activation and vascular damage leading to proteinuria and hypertension which are the main symptoms of this pre-eclampsia.

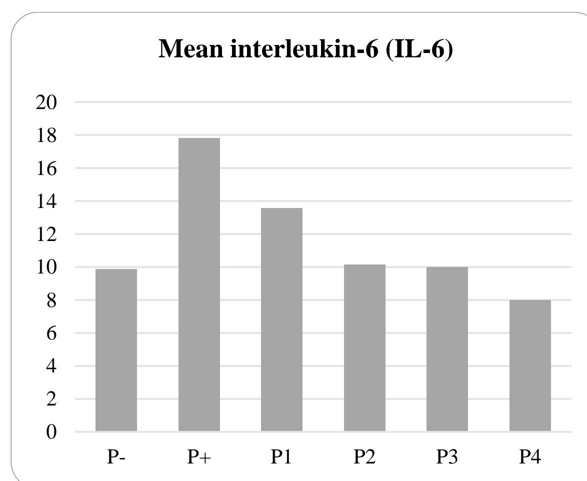

Figure 1. The average effect of ITC administration at IL-6 levels

As an anti-inflammation cytokine, IL-10 had a significant increase after ITC treatment. In negative treatment obtained an average of 472.22 levels of IL-10 is still normal, this is because the negative treatment of trophoblast cell culture has not been induced by alloxan. In positive treatment, trophoblast cell cultures began to be given glucose of 33 mM, causing a decreased effect of 114.5. In P1 there was an increase in the average il-10 level of 121.77 followed by P2 which increased by 241.31. In P3 there was an increase of 431.16, and there was an increase back in P4 of 463.28, close to the negative control **(Figure 2)**. This is because IL-10 is a cytokine secreted by monocytes, which has a pleiotropic effect on the immune system and inflammation. IL-10 is known for its ability to inhibit the activity and function of effectors of T cells, monocytes, and macrophages. IL-10 is the main anti-inflammatory cytokine in the natural and adaptive immune response to stop excessive inflammatory response through the activation of macrophages and T cells. This cytokine is a local and systemic inflammatory mediator and can be

produced by the body in large quantities so that it is easily detected in serum.

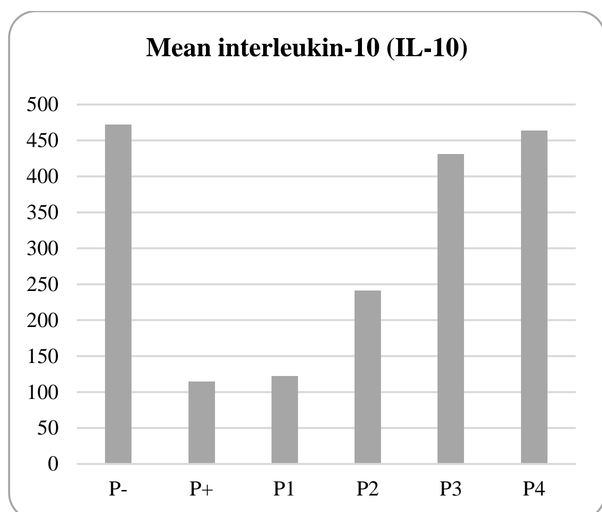

Figure 2. The average effect of ITC administration at IL-10 levels

Interleukin-6 (IL-6) is an anti-inflammatory cytokine, while interleukin10 (IL-10), interleukin 17 (IL-17), and STAT-3 are pro-inflammatory cytokines, so it is obtained that in the negative treatment of IL-17 amounted to 294.14 while STAT-3 amounted to 5.59. In the positive treatment of trophoblast cell culture began to be given glucose of 33 mM, resulting in an average of IL-17 of 609.00 while STAT-3 of 15.66. Each treatment of IL-17 and STAT-3 ITC is given different doses. On IL-17, P1; P2; P3; P4 has an average of 559.90; 378,69; 305,36; 298.99 while on STAT-3 13.68; 11,76; 10,55; 6.93. This can be seen in **Table 2** below.

Table 2. Average Effect of ITC Therapy On Interleukin-17 (IL-17) and STAT-3 Levels

Kelompok Perlakuan	N	Mean	
		interleukin-17 (IL-17)	STAT-3
P-	5	294,14	5,59
P+	5	609,00	15,66
P1	5	559,90	13,68
P2	5	378,69	11,76
P3	5	305,36	10,55
P4	5	298,99	6,93

The treatment with ITC with different doses showed a significant decrease of IL-17 at each dose, where the lowest levels of IL-17 were obtained at the use of ITC doses of 0.4 ng trophoblast culture cells, namely in treatment 4 of 298.99.same as STAT-3, the use of ITC doses of 0.4 ng trophoblast culture cells, namely in treatment 4 of 298.99, In the event that by giving ITC was significantly reduce IL-17 **(Figure 3)** and STAT-3 **(Figure 4)**.

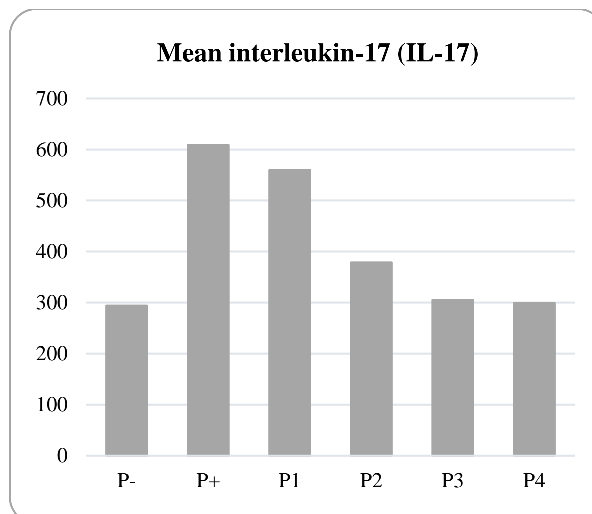

Figure 3. The average effect of ITC administration at IL-17 levels

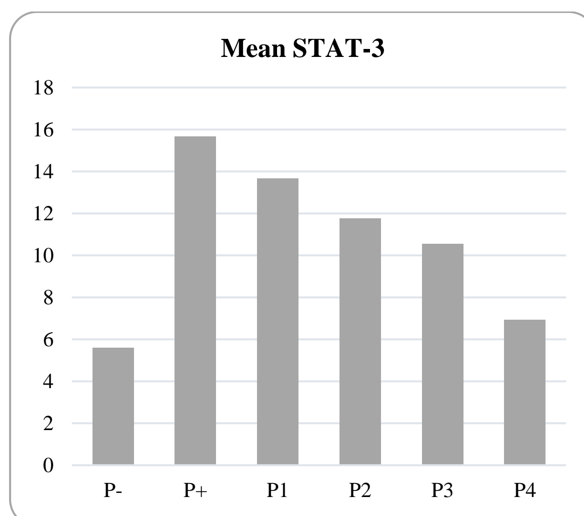

Figure 4. The average effect of ITC administration at STAT3 levels

Conclusion

ITC has prominent anti-oxidant, anti-inflammatory, anti-microbial, neuroprotective and cardioprotective activity. Some ITCs s such as Sulforaphane (SFN), Allyl Isothiocyanate are reported to have anti-platelet and antithrombotic activity. Isopropyl and 2-butyl isothiocyanate are the main constituents and 2-methyl-butyl Isothiocyanate is a small constituent of oil. Moringa Isothiocyanate is a solid compound at room temperature with rhamnose sugars that help give MIC its atypical stability. Of the MIC, 4-(α-L-Rhamnosyloxy)-benzyl isothiocyanate (MIC-1), is dominant and exists only in moringa seeds. Moringa seed extract enriched with ITC showed anti-inflammatory, and antioxidant, activity, relieving attenuated colitis ulcerative and insulin resistance in mice fed a high-fat diet.

Acknowledgments: None

Conflict of interest: None

Financial support: This research was fund by University Grant of Universitas Wijaya Kusuma Surabaya Indonesia.

Ethics statement: This research was conducted according ethical principles in medical research involving human subject in as mention in Helsinki declaration.

References

1. Pragitara CF, Etika R, Herawati L. Risks of preterm birth and low Apgar score among preeclamptic women. Indones J Med Health. 2020;1(1):6-17.
2. Gathiram P, Moodley J. Pre-eclampsia: its pathogenesis and pathophysiolgy. Cardiovas J Afr. 2016;27(2):71-8. doi:10.5830/CVJA-2016-009
3. Hammer A. Immunological Regulation of Trophoblast Invasion. J Reprod Immunol. 2011;90(1):21-8.
4. Bowen R. Salivary Gland and Saliva. 2012. Available from: http://vivo.colostate.edu/ hbooks/pathphys/digestion/pregastric/salivary.html (13 Maret 2012)
5. Prins JR, Lopes NG, Robertson SA. Interleukin 6 in Pregnancy and Gestational Disorders. J Reprod Immunol. 2012;95(1-2):1-14.
6. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro-and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta (BBA)-Mol Cell Res. 2011;1813(5):878-88.
7. Aziz N, Wal A, Wal P, Pal RS. Preparation and evaluation of the polyherbal powder: the nature's pharmacy for the treatment of diabetes mellitus and its complications. Pharmacophore. 2019;10(1):60-70.
8. Gondo HK. Moringa oleifera decrease blood sugar level and blood pressure in pregnant diabetic rats. J Adv Pharm Educ Res. 2021;11(2):89.
9. Illiandri O, Widjojanto E, Mintaroem K. Moringa oleifera Increase Memory Functions in Protein Energy Malnutrition Rat Model. J Kedokt Brawijaya. 2010;26(1):28-31.
10. Jaiswal M, Divers J, Dabelea D, Isom S, Bell RA, Martin CL, et al. Prevalence of and risk factors for diabetic peripheral neuropathy in youth with type 1 and type 2 diabetes: SEARCH for Diabetes in Youth Study. Diabetes care. 2017;40(9):1226-32.doi:10.2337/dc17-0179