



Effects of Low Dose Aspirin on Caspase 3, TNF- α and Apoptotic Index Levels in Preclampsia Maternal Serum-Induced Placental Trophoblast Cell Line In Vitro

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Abstract : Preeclampsia is a major cause in maternal mortality and morbidity. Placental apoptosis is found to be increased in preeclampsia and it is associated with caspase-8 activation on extrinsic pathway, followed by caspase-3 activation which is responsible as executioner caspase to generate apoptosis. Trophoblasts express tumor necrosis factor (TNF) that contain a cytoplasmic “death domain” that mediates apoptotic signals. Low dose aspirin is widely used in preventing preeclampsia reducing apoptosis induced by H₂O₂, and reduce ability of caspase-3 activation. This study aims to examine the difference between apoptotic index, TNF- α and caspase-3 expression in trophoblast cells induced by normal and preeclampsia maternal serum, and effects of aspirin on apoptotic index, TNF- α and caspase-3 in preeclampsia and normal-induced trophoblast. **Methods:** This was experimental study in laboratory. Apoptotic index was measured by *Annexin V-Fluorescent Staining* and expression of TNF- α and caspase-3 were performed by ELISA. **Results:** It was found that expression of TNF- α and caspase-3, and apoptotic index in preeclampsia serum-induced trophoblast cells were higher than normal and controls. Low dose aspirin of 0,5 mM showed significant decreased expression of TNF- α , caspase-3 and apoptotic index ($p < 0.05$) which equal normal and controls. **Conclusion:** Apoptotic index, expression of TNF- α and caspase-3 are affected by serum and aspirin dosage.

Keywords : Aspirin, Apoptotic Index, Caspase 3, TNF- α , Preeclampsia, Trophoblast.

Introduction

Preeclampsia is a major cause of maternal mortality and morbidity. World Health Organization (WHO) reported more than 500.000 maternal mortality was caused by preeclampsia. In 2002, preeclampsia occurs in 5-8% or 6, 6 million pregnant women. Preeclampsia also causes 15% premature birth in industrial

countries.¹According to *Survei Demografi dan Kesehatan Indonesia (SKDI)* in 2007, preeclampsia contributes to 24% of maternal mortality in Indonesia, makes it second cause of maternal death in Indonesia.^{2,3}In Dr. Hasan Sadikin Hospital, Bandung, preeclampsia was recorded in 4,0-10,4% cases and eclampsia in 2,3-4,3% cases in 2008 -2010. It conclude that preeclampsia and eclampsia contributed to 10,4% of maternal mortality in RSHS.⁴⁻⁶

It has been documented that trophoblast were unable to invade spiral artery in preeclampsia, causing spiral artery to dilate inadequately. Failure of trophoblast invasion inhibits decidualization, leading to poor placental blood supply in maternal vessels which further generates placental ischemia and apoptosis.⁷ Increased apoptosis has been documented in preeclampsia, which causes distribution of syncytiotrophoblast microparticles. *In vitro* study, the presence of such microparticles causes disturbance in endothelium, resulting in inflammatory response. Recent studies showed circulating substances in maternal serum, including fetal neutrophil and monocyte, increase in preeclampsia.⁸ Incomplete apoptosis of trophoblasts proposed causing degeneration of placenta in preeclampsia.⁹ Abnormal transformation of spiral artery is considered the likely cause of local ischemia, thrombosis and infarct.¹⁰

Caspase, one of *cysteine aspartate specific proteases*, is a protein which play role to execute apoptosis. Excessive apoptosis occurs in preeclampsia due to caspase-8 activation in extrinsic pathway, followed by caspase-3 activation as executioner which promotes apoptosis in trophoblast cells. Extrinsic pathway (death of cell signal), begin with death of cell signal or necrosis receptor containing *cytoplasmic domain* that mediates apoptotic signals, one of which is TNF receptor type-1 associated with Fas protein (CD95). Fas forms FasL upon its binding to its ligand. FADD (Fas -associated death domain) binds to death receptor, and procaspase-8 afterward. Procaspase-8 is cleaved to its active form, caspase 8, which later activates procaspase and caspase-3 as an executioner.^{10,12}

Aspirin is widely used to prevent preeclampsia. Recent studies showed prophylaxis effects of aspirin reduced apoptosis by 10-19% in high-risk pregnancy. Chen *et al.*¹³ reported that low dose aspirin from 1×10^{-10} mol/L to 1×10^{-8} mol/L, decreased apoptosis which was induced by H_2O_2 . Aspirin is also reported to reduce caspase-3 activation in *hepatocellular carcinoma G2 (HEpG2)* cell line.¹⁴ However, effect of low dose aspirin in inhibiting caspase (3, 8, and 9), and trophoblast cells apoptosis pathways, remains unclear. This study aims to measure expression of apoptotic gene (caspase-3), housekeeping gene (β -actin), and random marker (TNF- α) in preeclampsia maternal *in vitro* using cell line.

Experimental

Cell Culture

Cell was isolated from placenta obtained after delivery. Cell culture was performed consisting of two steps: thawing placental trophoblast line cell, and passage. Thawing began with growing placental trophoblast line cell into tissue culture flask (25 cm²) containing RPMI 1640 medium supplemented with 10% (v/v) FBS (30°, 56°C) and antibiotic-antimycotic (1% Penicillin G-Streptomycin Solution Stabilised and 1% Fungizone Amphotericin B), incubated at 37°C atmosphere 5% (v/v) CO₂. Culture medium was removed 2-3 times a day. Cells were passaged every 7 days or after reached 90% confluence. Placental trophoblast cell line was passaged by initially washing monolayer using PBS three times. Trypsinization was done by releasing cell monolayer from tissue culture flask wall with addition of 0,05% Trypsin-EDTA, incubated for 30' at 37°C. Trypsin-EDTA was removed. Effects of trypsin were neutralized by equal volume of complete medium into cell suspension and aliquoted to tissue culture flasks.¹⁸ Cell viability was measured by Trypan Blue Staining.

Preparation of Placental Trophoblast Line Cell Culture in Serum

Placental trophoblast cell line cultured was supplemented to new medium containing RPMI 1640 supplemented with 10% serum normal maternal or preeclampsia and antibiotic-antimycotic (1% Penicillin G-Streptomycin Solution Stabilised and 1% Fungizone Amphotericin B). Cell line was incubated for 24 hours 37°C with 5% CO₂ (v/v).^{8,18,19}

Measurement of Expression Levels of Caspase-3, and TNF- α

6x10⁵cell/ml containing 10% serum both maternal normal and preeclampsia were removed to 24-well plate 3,5ml for each well, incubated at 37°C with 5% CO₂ (v/v) until confluent.^{30,31} There were five treatments: normal serum in addition of FBS (normal 1), preeclampsia serum in addition of FBS (preeclampsia 1), normal serum (normal 2), preeclampsia serum (preeclampsia 2), and FBS as control. Various doses of aspirin (0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, and 5 mM), were aliquoted to each well, incubated for 0, 24 and 48 hours at 37°C with 5% CO₂ (v/v). Each well was washed with PBS pH 7.4 once for 5 min. Examination of caspase-3, caspase-8, caspase-9, β -actin, and TNF- α were performed with ELISA.^{8,18,19} Data was analyzed with Analysis of Variance (ANOVA).

Examination of Apoptotic Index

6x10⁵cell/ml containing 10% both maternal normal and preeclampsia serum were removed to 24-well plate 3,5ml for each well, incubated at 37°C with 5% CO₂ (v/v).^{21,22} Wells were washed 3-4 times with PBS 37°C to remove culture medium and unattached cells. Various doses of aspirin (0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, and 5 mM), were aliquoted to each well, incubated for 0, 24 and 48 hours at 37°C with 5% CO₂ (v/v). Examination of apoptotic index was performed *Annexin V-Fluorescein Staining and flow cytometry*.^{8,18} Data was analyzed with Analysis of Variance (ANOVA).

Ethical Approval

Written informed consent was obtained from all participants. The ethical reviews boards of the Health Research Ethics Committee, Faculty of Medicine Padjadjaran University and Dr. Hasan Sadikin Hospital, Indonesia, approved this study.

Results and Discussion

It has been documented that trophoblast were unable to invade spiral artery in preeclampsia causing spiral artery to dilate inadequately.⁸ Results showed there is difference of serum protein electrophoresis between normal pregnancy and preeclampsia, α -2 protein, protein beta and ratio A/G. Serum was therefore added to modify cells characteristic, including trophoblast. Neale *et al.* reported that H8 cell line derived from maternal trophoblast in first trimester, can turn to preeclampsia in addition of serum.¹⁹

The result of present study showed quantity of apoptosis in preeclampsia serum-induced trophoblast cells after incubation for 48 hours was higher than normal serum-induced cells (Figure 1-2). Apoptosis in preeclampsia serum-induced trophoblast decreased, following increased low dose aspirin. A significant decreased apoptotic cells equal normal serum-induced trophoblast was obtained after exposure to 0.5 mM. Both maternal and fetal apoptotic cells obtained from normal pregnancy, play major role in trophoblast invasion and adhesion, spiral artery transformation, trophoblast differentiation, and paternal immune tolerance expressed by trophoblast cells. Placental apoptosis and syncytiotrophoblast necrosis in preeclampsia causes distribution of syncytiotrophoblast microparticles. The presence of such microparticles causes endothelial dysfunction and induces inflammatory response in *in vitro* study of compromising pregnant women.¹⁵ Incomplete apoptosis of trophoblast causes placenta degeneration in preeclampsia. However, underlying mechanism of elevated apoptosis in preeclampsia remains unknown. Necrosis receptor in extrinsic pathway containing *cytoplasmic domain* is found mediating apoptosis signals, one of which is TNF receptor type-1 which is associated with Fas protein (CD95).

In this study, levels of TNF- α and caspase-3 were higher in preeclampsia serum-induced trophoblast than that in normal (Figure 3-4). Expression of TNF- α and caspase-3 in preeclampsia serum-induced trophoblast decreased, following increased low dose of aspirin. Levels of TNF- α and caspase-3 significantly decreased ($p < 0.05$) in aspirin exposure of 0.5 mM. TNF- α , a pyrogenic cytokine, inhibits pathogens growth at its low level by activating cellular immune system that directly kills parasite despite its activity is weakened. Excess apoptosis generate macrophage from tissue remodelling which is responsible in phagocytosis, to undergo inflammation causing incapacity to activate IL-10 and TGF- β . TNF- α generated during inflammation, is unable to activate three main anti-apoptosis proteins (FLIP's, Bcl-2 and Bcl-x). Thus, Fas signal interact with FasL signals which further activates Fas pathway in trophoblast. Activated Fas pathway causes no selection and

inhibition of cells to undergo apoptosis.¹⁶ Caspase is one of *Cysteine Aspartate Specific Proteases* protein, which is responsible in executing apoptosis. Caspase-3 is an effector caspase that stimulates proteolytic activation during apoptosis. Increased levels of caspase-3 is positively correlated to increased trophoblast apoptosis.⁸

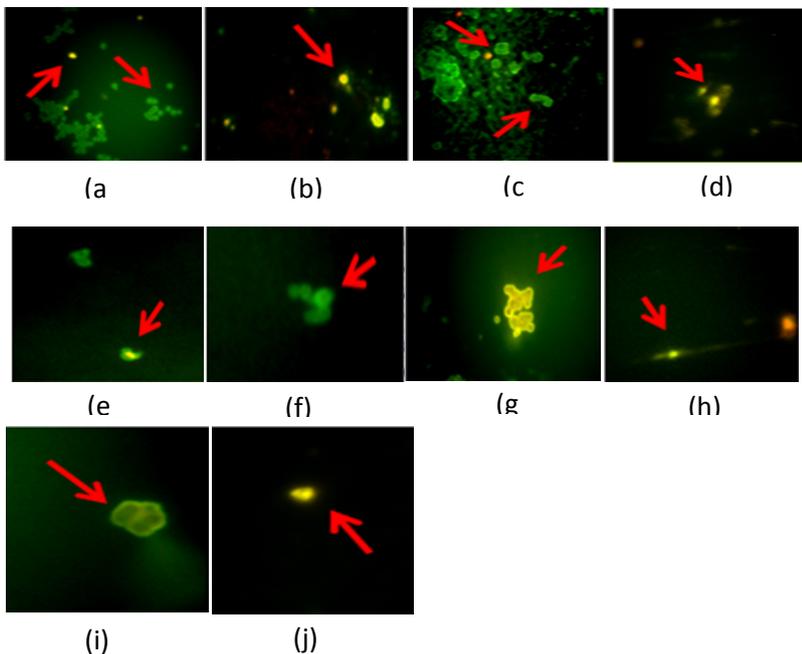


Figure 1. Apoptosis in preeclampsia serum-induced trophoblast cells and normal serum-induced cells after incubation for 48 hours. Living cells were colored in fluorescent (light yellow) whereas death cells were not colored (green). Apoptosis in preeclampsia serum-induced trophoblast decreased, following increased low dose aspirin. (a) DMEM+PBS+Preeclampsia serum (b) DMEM+PBS+Preeclampsia+ Aspirin 0,5 mM (c) DMEM+Preeclampsia serum (d) DMEM=Preeclampsia serum+Aspirin 0,5 Mm (e) DMEM+FBS (f) DMEM+FBS+DMSO (control) (f) DMEM+PBS+Normal serum (g) DMEM+PBS+ Normal serum+Aspirin 0,5 mm (i) DMEM+Normal serum (j) DMEM+Normal serum+Aspirin 0,5 mm

Figure 1. Measurement of apoptotic index of normal and preeclampsia serum-induced trophoblast cells after exposure low dose of aspirin

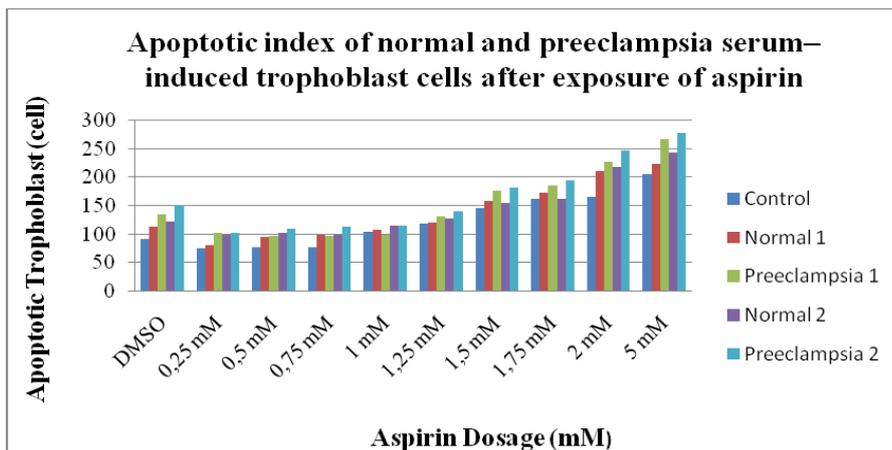


Figure 2. Apoptosis in preeclampsia serum-induced trophoblast cells and normal serum-induced cells after incubation for 48 hours. Note: Control = FBS; Normal 1 = Normal serum+FBS; Preeclampsia 1 = Preeclampsia serum+FBS; Normal 2 = Normal serum; Preeclampsia 2 = Preeclampsia serum

Figure2. Effects of low-dose aspirin to apoptotic index of normal and preeclampsia serum-induced trophoblast cells.

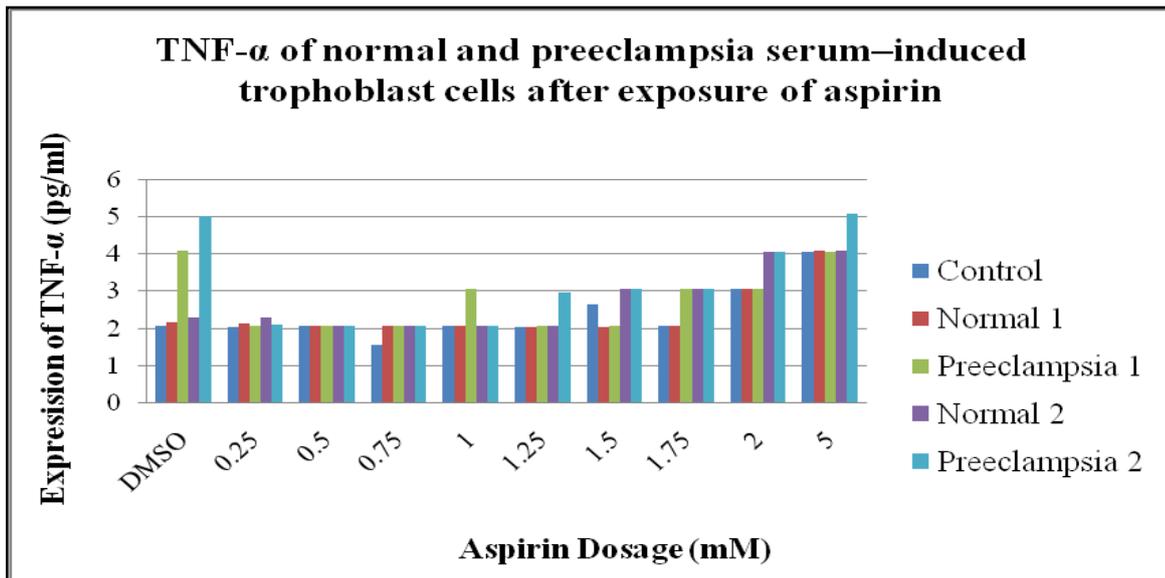


Figure 3. Levels of TNF- α in normal and preeclampsia serum-induced trophoblast cells. Note: Control = FBS; Normal 1 = Normal serum+FBS; Preeclampsia 1 = Preeclampsia serum+FBS; Normal 2 = Normal serum; Preeclampsia 2 = Preeclampsia serum

Figure 3. Effects of low-dose aspirin to TNF- α of normal and preeclampsia serum-induced trophoblast cells

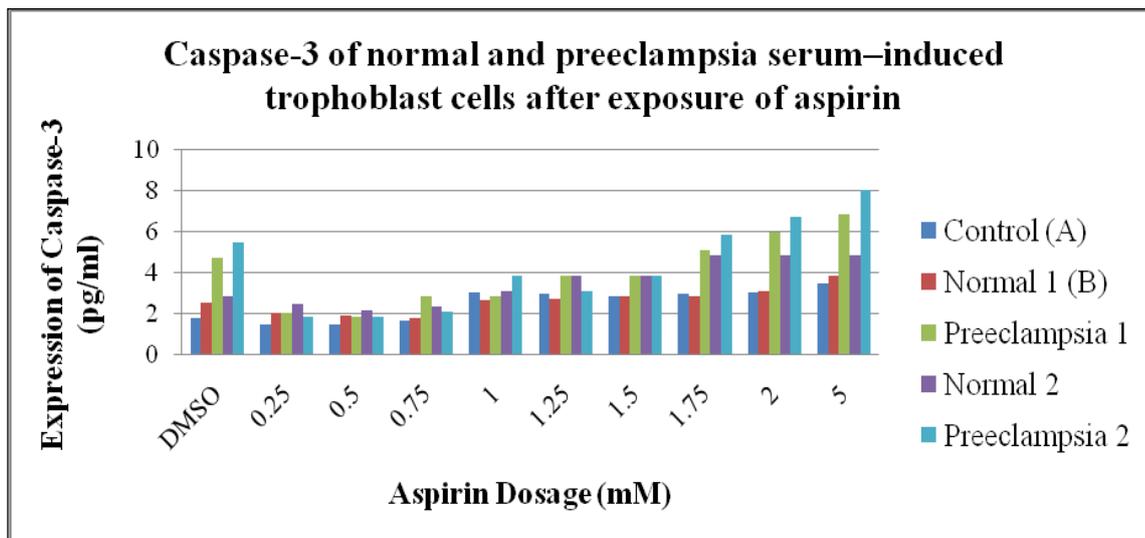


Figure 4. Levels of caspase-3. Levels of caspase-3 were higher in preeclampsia serum-induced trophoblast than that in normal. Note: Control = FBS; Normal 1 = Normal serum+FBS; Preeclampsia 1 = Preeclampsia serum+FBS; Normal 2 = Normal serum; Preeclampsia 2 = Preeclampsia serum

Figure 4. Effects of low-dose aspirin to Caspase-3 in normal and preeclampsia serum-induced trophoblast cells

Aspirin is widely recommended as prophylaxis medicine to prevent preeclampsia. It has been reported that prophylaxis effects in aspirin exposure for preclampsic patients reduce 10-19% mostly in high risk pregnancy. Prophylaxis effects are higher if it is given before 16 weeks of pregnancy. Early treatment of aspirin in preeclampsia is suggested to increase placentation by suppressing pathological inflammation during placentation.¹⁷ Low dose aspirin is known to inhibit thromboxan synthesis. Thromboxan is vasoconstrictor which plays major role in causing hypertension. It has also been shown that low dose aspirin inhibits lipid peroxide and placental prostaglandin H which is responsible in preeclampsia pathogenesis. Chen *et al.* Reported that low dose aspirin from 1×10^{-10} mol/L to 1×10^{-8} mol/L decreased apoptosis and phosphorylation of p38 MAPK

induced by H₂O₂ in BAEC. High dose of aspirin from 1x10⁻⁷ mol/L to 1x10⁻⁴ mol/L induced alteration of apoptosis in BAEC and stimulates phosphorylation of p-38 MAPK which its induced levels are associated with dosage.⁶

Conclusion

There is difference of apoptotic index, caspase-3, and TNF- α expression levels in normal and preeclampsia maternal serum-induced trophoblast cells. Aspirin affects apoptotic index, caspase-3, and TNF- α expression levels in preeclampsia maternal serum-induced placental trophoblast. Apoptotic index, levels of TNF- α and caspase-3 decreased after aspirin exposure of 0,5 mM.

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