

Original Article

Effects of *Theobroma cacao* on heat shock protein 90 and asymmetric dimethylarginine of endothelial cells under the influence of plasma of pre-eclamptic patients

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المخلص

أهداف البحث: أجريت هذه الدراسة للتحقق من الدور التنظيمي لمستخلص الإيثانول من فول ثيوبروما كاكاو على بروتين الصدمة الحرارية 90 وديمثيل أرجنين غير المتماثل للخلايا البطانية الناجمة من بلازما مريضات تسمم-الحمل.

طرق البحث: تم الحصول على الخلايا البطانية من السرة البشرية. في المرحلة المتموجة، وتم تقسيم الخلايا إلى مجموعات؛ مجموعة التحكم (من غير علاج)، مجموعة الخلايا البطانية التي تعرضت إلى 2٪ من بلازما مريضات تسمم-الحمل، مجموعة الخلايا البطانية التي تعرضت إلى 2٪ من بلازما مريضات تسمم-الحمل وعولجت بمستخلص الإيثانول من ثيوبروما كاكاو بجرعات مختلفة (25؛ 50؛ 100). كما تم تحليل مستوى بروتين الصدمة الحرارية 90 بواسطة فحص الإنزيمات المناعية الماصة. تم تحليل التعرض لديمثيل أرجنين غير المتماثل باستخدام الكيمياء الخلوية المناعية.

النتائج: كان هناك انخفاض لمستوى بروتين الصدمة الحرارية 90 في المجموعة التي تعرضت للبلازما المأخوذة من مريضات تسمم-الحمل. وقد تم تثبيط هذا الانخفاض بشكل ملحوظ بمستخلص ثيوبروما كاكاو في الجرعات 50 و 100. عملت البلازما المأخوذة من مريضات تسمم-الحمل على زيادة كبيرة في ديمثيل أرجنين غير المتماثل بالمقارنة مع مجموعة التحكم. تم تثبيط هذه الزيادة بشكل ملحوظ بإعطاء مستخلص ثيوبروما كاكاو الذي تم إعطاؤه بجرعتين أعلى.

الاستنتاجات: عمل مستخلص فول ثيوبروما كاكاو على حماية الخلايا البطانية، التي تعرضت لبلازما مريضات تسمم-الحمل بزيادة بروتين الصدمة الحرارية 90 وتقليل استخراج ديمثيل أرجنين غير المتماثل.

الكلمات المفتاحية: ديمثيل أرجنين غير المتماثل؛ مستخلص الإيثانول؛ بروتين الصدمة الحرارية 90؛ في المختبر؛ بروتين الإجهاد

Abstract

Objectives: This study was conducted to investigate the modulatory role of an ethanol extract of *Theobroma cacao* beans on heat shock protein 90 (HSP90) and asymmetric dimethylarginine (ADMA) levels of endothelial cells under the influence of plasma of pre-eclamptic patients.

Methods: The endothelial cells were obtained from a human umbilicus. In the confluent phase, the cells were subdivided into groups: the control group (no treatment), the endothelial cell group that was exposed to 2% pre-eclamptic patients' plasma, and the endothelial cell group that was exposed to 2% pre-eclamptic patients' plasma and treated with the ethanol extract of *T. cacao* at various doses (25, 50, or 100 ppm). Analysis of HSP90 levels was carried out by an enzyme-linked immunosorbent assay. Quantification of ADMA was conducted by immunocytochemistry.

Results: There was a decreased HSP90 level in the group exposed to the pre-eclamptic patients' plasma. This decrease was significantly attenuated by the extract of *T. cacao* at the doses of 50 and 100 ppm. The pre-eclamptic patients' plasma significantly increased

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ADMA level as compared with the control group. This increase was significantly attenuated by the administration of the *T. cacao* extract at the two highest doses.

Conclusions: The extract of *T. cacao* beans protected the endothelial cells that were exposed to pre-eclamptic patients' plasma by increasing HSP90 levels and reducing ADMA levels.

Keywords: ADMA; Ethanol extract; HSP90; *In vitro*; Stress protein

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Introduction

Pre-eclampsia is a syndrome that develops in pregnant women in the form of hypertension and proteinuria after 20 weeks of pregnancy. This syndrome affects 3–5% of pregnancies and occurs due to a placental abnormality. Because it affects only pregnant women, this disorder can lead to complications, which may happen before or after childbirth to the mother or the child. Pre-eclampsia is dangerous, and early detection efforts and the best treatment of this disorder are still not satisfactory.^{1,2} The final effect of this disorder is expressed as the incomplete invasion degree of the trophoblast and endothelial cell dysfunction, which trigger placental dysregulation.³

Heat shock proteins (HSPs) are expressed by all cells. These proteins have diverse functions; one of their functions is to assist the cells in the defence against stress. Some HSPs are constitutively expressed, and others are adaptively expressed in response to specific injuries.⁴ Heat shock protein 90 (HSP90) is the most abundant chaperone, estimated at approximately 1–2% of total cellular protein.⁵ Research has proved that the increase in Hsp90 levels occurs in red blood cells of pre-eclamptic patients' umbilical cord compared to normotensive pregnant women.⁶ In pre-eclampsia, a decrease in endothelial cell viability is associated with an increase in HSP90 expression.⁷ Other studies have revealed that this heat shock protein is down-regulated in peripheral blood of pre-eclamptic patients with or without foetal growth restriction.⁸

Nitric oxide (NO) is also involved in the development of pre-eclampsia. Anomalous regulation of NO metabolism is caused by down-regulation of NO synthase owing to competitive inhibition by asymmetric dimethylarginine (ADMA).⁹ The latter is an endogenous competitive inhibitor of NO synthase and plays a role in endothelial dysfunction.¹⁰ Various studies have confirmed that ADMA levels are increased in pre-eclamptic patients^{11–17} although the results of some studies are inconsistent.^{18,19} In fact, the ADMA level is an indicator of the risk of pre-eclampsia in pregnancy.²⁰ Considering that endothelial NO synthase (eNOS) interacts with HSP90 as a regulatory protein,²¹ changes in NO regulation should alter HSP90 expression. Until now, no studies have proved HSP90 regulation or its

relation to ADMA in endothelial cells of pre-eclamptic patients. Moreover, no studies have shown an influence of an herbal ingredient on HSP90 and ADMA in endothelial cells of pre-eclamptic patients.

Theobroma cacao is a plant currently used in beverages and foods by people all over the world. *T. cacao* contains a flavonoid that can protect blood vessels. Some researchers have stated that *T. cacao* can inhibit atherosclerosis through modulation of oxidative stress and inflammation and normalisation of blood pressure and blood lipid levels.²² Several studies have suggested that *T. cacao* can inhibit the up-regulation of vascular factors in pre-eclampsia, including IL-6, sVCAM-1, and ET-1.²³ One *in silico* study proved that *T. cacao* ingredients can interact with eNOS; this effect suggests that *T. cacao* will also affect HSP90.²⁴ In addition, flavanols from cacao may interfere with various disruptors of NOS activity, such as NADPH oxidase and ADMA.²⁵ As far as we know, no studies have revealed a protective effect of *T. cacao* on endothelial cells in pre-eclampsia through HSP90 modulation and ADMA down-regulation. Therefore, in this study, we implemented exposure of endothelial cells to plasma of pre-eclamptic patients and evaluated the effects of a *T. cacao* extract on HSP90 and ADMA.

Materials and Methods

Isolation and culture of human umbilical vein endothelial cells (HUVECs)

This study was conducted *in vitro*. HUVECs that reached the confluent state were subdivided into five groups: the control group (no treatment), endothelial-cell group that was exposed to 2% pre-eclamptic patients' plasma, and an endothelial-cell group that was exposed to 2% pre-eclamptic patients' plasma and treated with an ethanol extract of *T. cacao* at various doses (25, 50, or 100 ppm). Isolation and cultivation of HUVECs were carried out according to methods detailed in other studies.^{23,24,26}

Isolation of plasma of pre-eclamptic patients

This procedure was carried out according to the method described elsewhere.^{23,24,26} This procedure was approved by the Health Research Ethical Committee, Faculty of Medicine, Brawijaya University, Malang, Indonesia. Pre-eclampsia plasma concentration of 2% is the optimal dose that is capable of eliciting a response: a decrease in the diameter of relaxed blood vessels according to another study.²⁷

Extraction

T. cacao beans were obtained from PTPN XII JI, Rajawali 49, Surabaya, East Java, Indonesia. Beans obtained in dry form were the voucher provided by the Coffee and Cacao Research Center, Jember, East Java, Indonesia. Extraction was carried out by pulverizing the cocoa beans by 400 mesh grinding. Next, 300 g of the powdered cacao beans was added to n-hexane (1:3, w/v) and incubated for 48 h at room temperature. Then, the extract was filtered through Whatman paper (size 40). The precipitate was oven-dried at a

temperature of 45–50°C for 3–4 h. Finally, maceration was carried out with 70% ethanol (1:3) for 2 × 24 h. This process separated polyphenolics. These polyphenolic compounds were dried in the vacuum oven for 8–9 h at 45–50°C. These procedures generated 14.7 g of extract.

HSP90 analysis

Analysis of HSP90 levels was carried out in a HUVECs medium. Analysis was carried out by an enzyme-linked immunosorbent assay (ELISA) technique. The ELISA kit was purchased from Biologend Inc., catalogue #430507, USA. Analytical procedures were performed in accordance with the sequence of procedures stated in the kit's manual.

ADMA analysis

For immunohistochemical detection of ADMA, tissue slides were deparaffinised, hydrated, and washed with PBS. Before ADMA staining, endogenous peroxidase activity was inhibited with 3% H₂O₂ in methanol. Slides were incubated for 30 min with a 1:200 dilution of an anti-ADMA antibody (BD Transduction Laboratories, Franklin Lakes, NJ) and signals were revealed using an immunoperoxidase kit (Vector Laboratories, Burlingame, CA). Control slices were stained with a secondary antibody only. All the slices were counterstained with hematoxylin (Fisher Scientific). Immunofluorescent detection of ADMA was also conducted to confirm the immunohistochemical staining and to quantify ADMA fluorescent staining. Tissue slices were deparaffinised, hydrated, and pre-treated with 0.1% trypsin for 30 min at 37°C for antigen retrieval purposes. After that, the slices were blocked and next treated with a 1:200 dilution of the anti-ADMA antibody and a 1:200 dilution of an anti-rabbit biotin-conjugated secondary antibody (Invitrogen, Carlsbad, CA). Image analysis was performed under a light microscope (400× magnification) in a blinded fashion by two independent observers. Data are presented as percentages of ADMA level in cells.

Ethics

This study's protocol passed the ethical review and was approved by the Health Research Ethical Committee, Faculty of Medicine, Brawijaya University, Malang, Indonesia.

Statistical analysis

HSP90 and ADMA data are shown as means ± standard deviation. Differences between treatment groups were analysed by one-way ANOVA in the SPSS 17.0 statistical software. If there was significance of some data in the ANOVA, then a Least Significant Difference test was carried out. A difference with $p < 0.05$ was defined as statistically significant.

Results

Figure 1 shows HSP90 levels in endothelial cell media of various cell groups. The HSP90 level was significantly lower in the pre-eclampsia plasma group than in the no-treatment control group ($p < 0.05$). Among the doses of

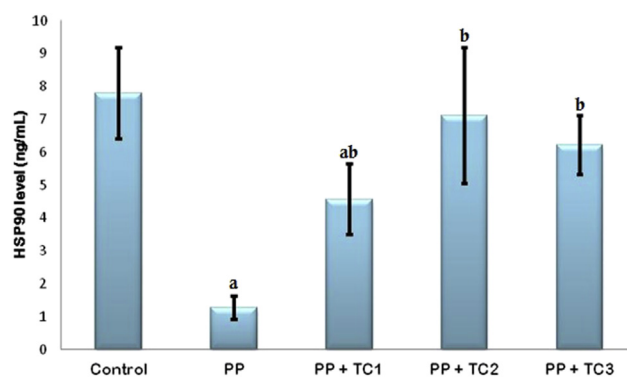


Figure 1: HSP90 levels in HUVECs culture medium of various cell groups. Note: Data are presented as mean ± standard deviation; ^a $p < 0.05$ as compared to the control group; ^b $p < 0.05$ as compared with the HUVEC group that was exposed to pre-eclampsia plasma (PP); Hsp90: heat shock protein 90; ng/ml: nanograms/milliliter.

25, 50, and 100 ppm *T. cacao* bean extract, only the two highest doses significantly prevented the decrease in the HSP90 level ($p < 0.05$); the dose of 50 and 100 ppm caused a level comparable to that in the no-treatment control group ($p > 0.05$). There was no difference in HSP90 levels between the dose of 50 and 100 ppm ($p > 0.05$).

ADMA amounts in endothelial cells in various treatment groups are presented in Figures 2 and 3. ADMA level was significantly higher in the pre-eclampsia plasma group than in the no-treatment control group ($p < 0.05$). The increase in ADMA level was lowered significantly after administration of the *T. cacao* bean extract at the doses of 50 or 100 ppm. This decrease made ADMA levels equivalent to those in the no-treatment control group ($p > 0.05$).

Discussion

HSP90 is a cell chaperone that participates in regulation of the conformation of some proteins to protect signal transduction and cell proliferation and to prevent

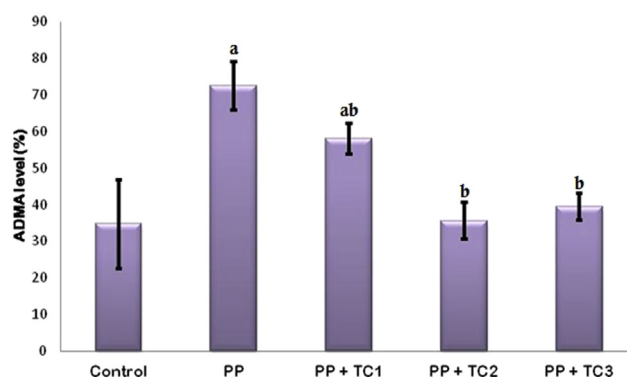


Figure 2: ADMA level in various groups of HUVECs. Note: Data are presented as mean ± standard deviation; ^a $p < 0.05$ as compared to the control group; ^b $p < 0.05$ as compared to HUVECs that were exposed to pre-eclampsia plasma (PP); ADMA: asymmetric dimethylarginine; %: percentage of cells that contain ADMA.

apoptosis.²⁸ In the present study, HSP90 levels decreased significantly in endothelial cells that were exposed to pre-eclamptic patients' plasma in comparison with the control group (no treatment). This finding indicated that pre-eclamptic patients' plasma contained various factors such as down-regulators of HSP90 expression. In other words, the endothelial cell protection mechanism involving HSP90 was weakened by the pre-eclampsia plasma exposure. Furthermore, this decrease disturbed eNOS coupling activity because the association of eNOS with HSP90 is required for eNOS coupling activity. Our *in vitro* study is an extension of previous findings that HSP90 is down-regulated in the peripheral blood of pre-eclamptic patients with or without foetal growth restriction.⁸ Another study revealed a decrease in HSP90 expression in HUVECs of pre-eclamptic patients.²⁹ The findings of this study contradict some previous findings.⁷

According to one study, HSP90 forms a complex with eNOS to maintain NO levels.³⁰ In the present study, we found that the ADMA amount was significantly higher in the endothelial-cell group that was exposed to pre-eclamptic patients' plasma than in the no-treatment control group. Our finding is indicative of two points. Firstly, either ADMA up-regulation occurred or there was ADMA addition from the pre-eclampsia plasma to the endothelial-cell culture medium resulting in the increase of ADMA levels. Secondly, a loss of endothelial-cell protection, which was marked by the decrease in HSP90 levels, happened at least due to the increased ADMA expression. Some studies have proved that ADMA is present in blood and correlates with the severity of pre-eclampsia.^{31,32}

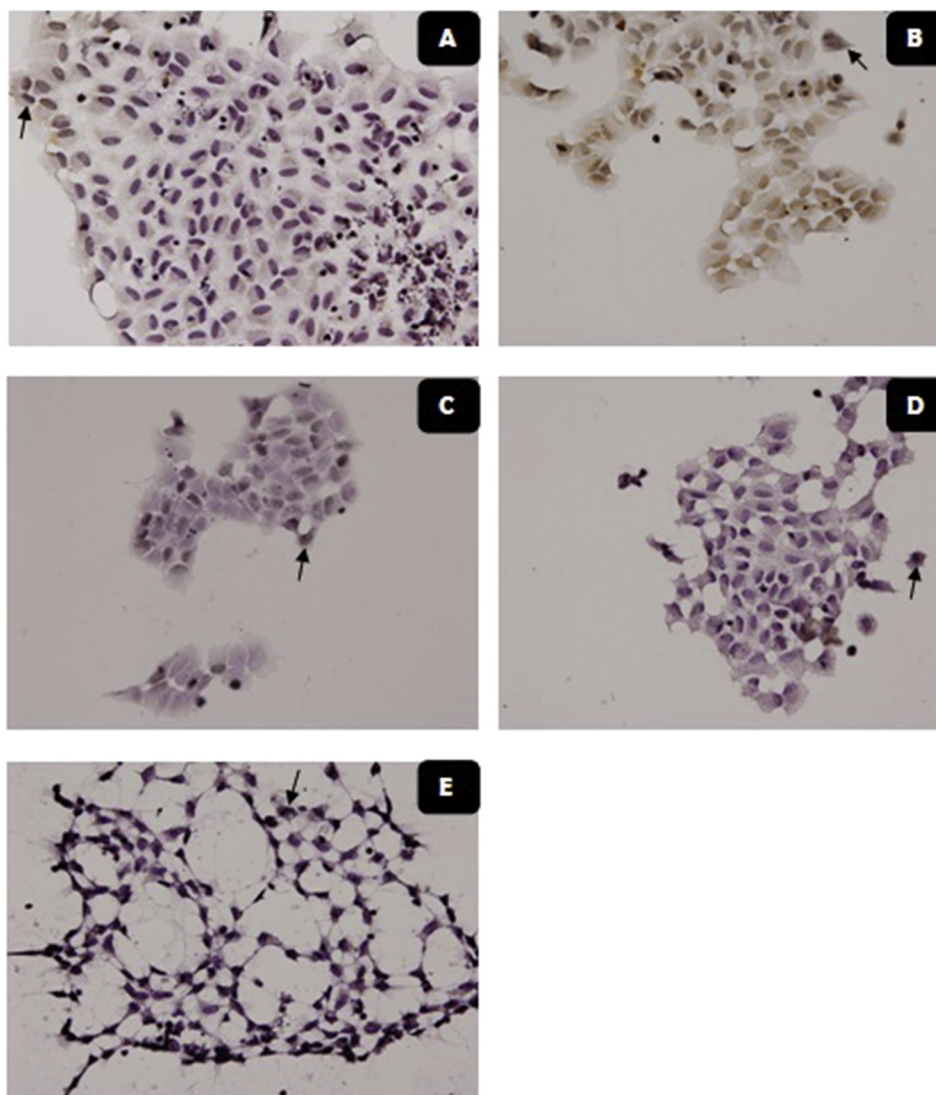


Figure 3: Immunohistochemical analysis for detection of ADMA levels in HUVECs. The arrow indicates an endothelial cell in which ADMA is distributed. ADMA presence in immunocytochemical results is indicated by brown staining in the nucleus and cytoplasm. Cells that do not contain ADMA are indicated by a purple colour. The image was captured at 400 \times magnification by means of a light microscope. The no-treatment control group (A), the endothelial-cell group that was exposed to 2% pre-eclamptic patients' plasma (B), and the endothelial-cell group that was exposed to 2% pre-eclamptic patients' plasma and was treated with an ethanol extract of *T. cacao* at various doses: 25 ppm (C), 50 ppm (D), and 100 ppm (E).

In the present study, the two highest doses of the *T. cacao* extract prevented the decrease in the HSP90 expression and increased the ADMA concentration to levels comparable to those in the no-treatment control group. This result indicated that active ingredients of *T. cacao* can attenuate the changes in HSP90 and ADMA levels induced by pre-eclampsia plasma in endothelial cells. Our study extends other findings that the action of *T. cacao* does not involve only NO stimulation. HSP90 modulation in this study led to inhibition of ADMA expression.³³ The amount of produced ADMA is dependent on the extent of methylation of arginine in protein and the protein turnover rate.³³ One study showed that epicatechin of *T. cacao* can induce phosphorylation or activation of HSP90,³⁴ and accordingly, HSP90 expression increased after administration of the two highest doses of the *T. cacao* extract here. A flavanol from *T. cacao* can suppress ADMA activity.²⁵

Conclusion

The extract of *T. cacao* beans protected endothelial cells that were exposed to pre-eclamptic patients' plasma by up-regulating HSP90 and reducing ADMA levels.

Authors' contributions

AK, SCWB, and NN: conception and design of the study and analysis and interpretation of the data. AK: collection and compilation of data and drafting of the manuscript. SCWB and NN: critical revision of the article for important intellectual content. All the co-authors have declared their approval of the final manuscript and serve as its guarantors.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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