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RESEARCH ARTICLE

Pomegranate Fruit extract Administration in mice induced by Formaldehyde to Folliculogenesis Observation and Caspase-3 Expression

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ABSTRACT:

General population exposure of formaldehyde can occur via inhalation, ingestion due to food and water also dermal contact. Recent studies suggested formaldehyde exposure induce oxidative stress in reproductive system and cause menstrual cycle disorder. Pomegranate is commonly known in Indonesia, often use as a traditional herbal, extract of all part of fruit appear to have protective effect to the damage in the body including the down regulation of apoptosis mechanism due to ellagic acid compound. Oxidative stress was induced by formaldehyde (140 mg/kg/ day p.o) for 12 days consecutively. Mice were treated with pomegranate fruit extract in 100, 150, and 200 mg/kg/BW/day p.o for 12 days after exposure of formaldehyde. On day 25th, mice were euthanized thenovary tissue were isolated and processed for immunochemistry staining withcaspase-3 antibody and *hematoxylin eosin* staining. *Caspase-3* expression was significantly different in groupswhich treated by pomegranate fruit extract. The number of follicle including primary, secondary, tertiary, and de Graaf follicle is not different in each group. In conclusion pomegranate fruit extract in maximum dose of 200 mg.kg/day per day appears todownregulate the expression of *caspase-3* but ineffective in number of follicle in formaldehyde mice model.

KEYWORDS: pomegranate, ellagic acid, folliculogenesis, caspase-3, toxicity

INTRODUCTION:

Formaldehyde is commonly produced as an aqueous solution called formalin, used in the production of abrasive materials, woods, insulation, foundry binders, brake lining made from phenol resins, surface coating, molding compounds, laminates, wood adhesive, explosive, and in small amount of formaldehyde as a preservation and embalming of biological specimens (1). Formaldehyde is released to water from the discharges of both treated and untreated industrial wastewater from its production and from its use in the manufacture of formaldehyde containing resins (*Integrated Risk Information System* 2010).

This condition could cause the exposure of formaldehyde in the air, water, food, also cosmetics entering human body and cause damaged in organ and cells. Previous study found that formaldehyde could make damage in folliculogenesis, caspases activation, and DNA damage (2, 3)

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Caspase-3 is a member of caspase family contain cysteinyl aspartate requiring protease is a key factor of apoptosis process. Under normal condition, caspase is an inactive form in cytosol, activated during stress condition, DNA damage, mitochondrial damage, and infection (4). Our body need caspase-3 expression in an adequate expression, abundant of caspase-3 expression could cause degenerative disease and lack of caspase-3 could cause cancer (5). Ellagic acid is most potent antioxidant compound of pomegranate, Previous study found ellagic acid has antioxidant, antiinflammation,

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and antiapoptotic effect (6,7). EA could increase endogen antioxidant through cells to prevent oxidative stress. This potential effect however could decrease apoptosis effect due to oxidative stress(8)

MATERIAL AND METHODS:

Chemicals:

Formaldehyde were obtained from Sigma Aldrich. Pomegranate were obtained from Semarang, Central Java and extracted in GadjahMada University, Jogjakarta, Indonesia.

Animal Maintenance:

Female *Mus musculus* were used for the study age 8-10 weeks 20-25 gram weighed. The study were approved by ethical committee of University of Airlangga. Mice were acclimatized for one week and maintained in 20-25°C on 13/11 photo period with ad libitum supply for standard mice feed and drinking water. Vaginal swab were conducted before administration of formaldehyde to maintain the menstrual cycle.

Administration of formaldehyde and pomegranate fruit extract:

Mice were divided in 4 groups (n=11), each group were treated with formaldehyde 10% 140 mg/kg body weight each day for 12 days and continue treated with pomegranate extract fruit for 12 days. Control group were treated with aquabidest with CmcNa 0,5%, each three groups were treated with different dose of pomegranate extract (100 mg/kg BW, 150 mg/kg BW, 200 mg/kg BW) dissolved in 0,5% CmcNa as suspension, treatment was started from the next day after administration of formaldehyde. Pomegranate fruit extract was given by gavage feeding at 24 h interval for 12 consecutive days. One group of control was given only vehicle and aquabidest. All groups of mice were sacrificed on day 13 of administration of pomegranate fruit extract. Ovaries were excised immediately after scarifying the animal and washed in normal saline. Ovaries of animal were collected in buffer formaldehyde 10%.

Histology:

Post mortem samples of ovarian were fixed in 10% buffered formalin, processed and embedded in paraffin. Sections were stained for hematoxylin and eosin (HE) and caspase-3 were determined by immunohistochemistry staining.

Statistical analysis:

Statistical analysis was performed using R program software with One-way Analysis of Variance (ANOVA) and Kruskal-Wallis test followed by post hoc test. Values are expressed as mean \pm SD or median, maximum, minimum depend on test of normality. A

value $p < 0,05$ was taken as statistically significant from each group.

RESULT:

Ellagic acid downregulates expression and activation of caspase-3. The expression of caspase-3 were down regulated in mice ovarium exposed by formaldehyde and treated with pomegranate fruit extract as compared to control group treated with aquabidest only. Decreased in expression of caspase-3 was observed approximately down from 4,400; 1,345; 1,000; 0,060 with a dose of 100 mg/ kg to 200 mg/kg body weight

Ellagic acid regulates folliculogenesis:

The protective effect of ellagic acid was analyzed on folliculogenesis process, this process was measured in terms of amount of primary, secondary, tertiary, and de graaf follicle. Ellagic acid increases the number of primary follicle in mice exposure formaldehyde treated with pomegranate fruit extract. This increase however, were not significantly different in different dose. Secondary follicle was increased but has a decrease amount in treatment with dose 150 mg/ kg body weight, tertiary follicle was increase in different dose compare to control group, and there were no de graaf follicle in control and treatment with 100 mg/kg body weight, and there was one follicle de graaf in 150 mg/kg and 200 mg/kg body weight treatment

DISCUSSION:

Caspase-3 expression:

Caspase-3 is a reported to be mainly caspase for apoptotic program of cells (4), recently activation of caspase-3 from precursor forms induced by oxidative stress, DNA cross-link, and increase in voltage dependent anion channel (VDAC).

Over expression of caspase-3 is reported to cause an autoimun disease, reduced of caspase-3 has been shown in cancer cells. Our finding of a high level of caspase-3 in ovarium of formaldehyde exposed mice and their up regulation by ellagic acid treatment suggest antiapoptotic effect of ellagic acid. Ellagic acid induced endogen antioxidant (SOD, CAT, GPX) (7,9), through modified affinity of keap-1, a repressor protein of Nrf-2, then binds to the ARE as a heterodimer, leading to upregulation of antioxidant endogen gene transcription (8,10). Other effect of EA has been reported due to stabilization of mitochondrial outer membrane permeabilization (MOMP) by normalizing bax bcl-2(11).

Primary follicle:

Primary follicle is a growth follicle from primordial follicle, morphologically primary follicle consist of single layer of columnar cells, growth oocyte, and

pellucid zone (12). Recent study showed formaldehyde exposure could reduce the number of primary follicle (13). Data shown in this present study demonstrated that with ellagic acid treatment in different dose every 24 hours could increase the number of primary follicle, although this difference is not significant in statistic analyzed. Previous study showed development of granulosa single layer needs optimal environment(14), formaldehyde exposure however could induce cell injury, affecting granulosa and oocyte. Small follicle has lower defense against changing environment(15)

It makes the number of apoptotic cells do not significant with increasing dose of ellagic acid. In primary follicle, oocyte actively influences the granulosa cells development, this factor including growth differentiation factor (GDF)-9 and bone morphogenic protein (BMP)-15 (12). Granulosa cells however, also induce oocyte development. Formaldehyde exposure could reduce expression of this factor but treatment ellagic acid could reduce this stress oxidative. Besides, cellular response manifestation to injury need a very long time, but in molecular protein has a highly sensitive response to environment changes, in this study, although primary follicle remain low, but caspase-3 response decrease during the increase of pomegranate fruit extract dose.

Secondary follicle:

Next stage of follicle development is secondary follicle, has more than a single layer of columnar cells and development oocyte(12). This present study showed that treatment of pomegranate fruit extract in mice with formaldehyde exposure could make the unstabilized increase in number of secondary follicle. 150 mg/kg body weight treatment group has less follicle than the other. Formaldehyde exposure could increase oxidative stress and make abundant atresia in follicle. EA has a short half life, abundant in body in first 4 hours and become urolithin with reduce its affinity after 4 hours (16). We proposed by high number of atresia, 4 hours effectively half life could not prevent damaged due to formaldehyde exposure. This damage, however could reduce number of FSH receptor, estrogen receptor, and androgen receptor

Tertiary follicle:

Secondary follicle become larger and has antrum. In this study, the number of tertiary follicle remain unstabilized, we found 200 mg/kg body weight has a smallest number of tertiary follicle. Previous study showed that tertiary stages of follicle development has the highest number of physiologic atresia, follicle with good FSH receptor and estrogen production could survive(12). During oxidative stress caused by formaldehyde exposure, FSH receptor and estrogen production. EA treatment however could not cure

damage in cellular stage of follicular development. we therefore expect that the number of follicle in our study remain unstabilized in high number of atresia stages (secondary, tertiary), but can be adequately decrease the molecular response of oxidative stress.

De Graaf follicle:

deGraaf follicle is a follicle that ready to ovulate, has a large and greatest number of granulosa cells. we found that 150 and 200 mg/kg body weight dose of pomegranate fruit extract could increase the number of de Graaf follicle while 0 and 100 mg/kg body weight has null number of de Graaf follicle. previous study suggest that the de Graaf follicle, due to the largest size and the most abundant level of granulosa cells, provided broad defense against stress oxidative (2). it is proved that formaldehyde could induce chronic damage in granulosa cells.

Administration of pomegranate fruit extract in 150 and 200 mg/kg body weight in this research proved could repair the damage in granulosa cells, but it's not statistically different due to the short time of pomegranate fruit extract.

CONCLUSION:

Pomegranate fruit extract could reduce expression of caspase-3 but no effect in cellular response of folliculogenesis. In addition, administration of pomegranate fruit extract twice or three times a day may be important for cellular response due to its half life.

CONFLICT OF INTEREST

The author declared that there is no conflict of interest regarding the publication of this article.

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