Total Production of H2o2 in Complete Hydatidiform Mole Cell-Culture with 17-Beta Estradiol Exposure

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***Abstract*—B**ackground: The pathomechanisms of gestational trophoblastic disease have not yet been able to be extensively understood. It is associated with oxidative damage related to the production of reactive oxygen species (ROS). Estrogen, as well as all of its metabolites, can produce ROS. Therefore it is necessary to analyze how far the involvement of estrogen, in this case 17β estradiol, to the pathological mechanisms of gestational trophoblastic disease by the measurement of total ROS level.

Objective: To determine the role of 17β estradiol on the total production of ROS in hydatidiform mole tissue culture.

Methods: The study was carried out experimentally in the Laboratory of Cell/Tissue Culture, Central Laboratory of Biomedics and Laboratory of Physiology, Medical Faculty University of Brawijaya, Malang by using Randomized Control Group Design. One group of hydatidiform mole tissue culture supplemented with various doses of estrogen (17β estradiol) compared to a group without supplementation.

Results: There were highly significant differences in ROS levels between treatment groups with 17 beta-estradiol 5 g/ml and 17 beta-estradiol 40 g/ml ($p = 0.000$), between treatment groups of 17 beta-estradiol 10 g/ml and 17 beta-estradiol 40 g/ml ($p = 0.000$) and between treatment groups of 17 beta-estradiol 20 g/ml and treatment groups of 17 beta-estradiol 40 g/ml ($p = 0.000$).

Conclusion: The exposure of 17β estradiol significantly affected the total production of H2O2 in hydatidiform mole cell culture in the dose 20 g/ml and 40 g/ml, while it did not occur significantly in the dose 5 g/ml, 10 g/ml, and control group.

Synopsis: The exposure of 17β estradiol significantly affected the total production of H2O2 in hydatidiform mole cell culture in various doses.

***Keywords*—**ROS, hydatidiform mole, 17-Beta Estradiol.

I. Introduction

GESTATIONAL trophoblastic diseases/ GTD is a group of disease arising from placental trophoblastic tissue after both normal and abnormal fertilization. World Health Organization (WHO) classified GTD into hydatidiform mole (complete and partial), invasive mole, choriocarsinoma, placental site trophoblastic tumor, and unclassified miscellaneous trophoblastic lesions. Complete hydatidiform mole (CHM) is a rare variant and is characterized by placental chorionic villi lesions that are widened, edematous and vesicular, accompanied by trophoblastic proliferation in various degrees.

There are wide variations in GTD distribution worldwide, with higher frequencies found in some parts of Asia, the Middle East and Africa where Turkey holds the middle rank among those countries [1]. Ozalp et al. reported that the incidence of HM was 1.87 per 1000 deliveries in Turkey [2]. In contrast, the incidence of HM was 11.0 per 1000 deliveries in Sanliurfa, Southeast Anatolia [3].

Most patients with hydatidiform mole will recover completely after a thorough management although approximately 15-20% of the tumors will transform into malignant gestational trophoblastic tumors (GTT). Gestational trophoblastic tumor is a group of malignant diseases that are associated with chorialis villi, especially its trophoblast cells, occuring after pregnancy and are mostly preceded by a hydatidiform mole [4].

Reactive Oxygen Species are produced during normal metabolic and physiological process. However, in certain circumstances, an increase in oxidants and a decrease in antioxidants productions can not be prevented, leading to oxidants/antioxidants imbalance shifts into an oxidative condition. As a consequence, the process of oxidative stress is involved in many pathological conditions [5]. The disruption of oxidants/antioxidants balance are thought to be the underlying factors that causes oxidative damage to cellular molecules such as DNA [6]. Alkaline comet assay is widely used to examine DNA damage in vivo. This test is simple, rapid and sensitive, therefore very useful and has been widely used to estimate the endogenous DNA damage in various conditions, e.g. after a treatment with genotoxic agents [5], as a response to the ingestion of food additives [7], to test the effect of extensive antioxidants consumptions on DNA damage, etc [8].

The cells in our body are in a balance. The balance between cell proliferation, differentiation and apoptosis is one of the main regulator of the invasive behavior or tumorigenic activity on trophoblast. The underlying pathological mechanisms of gestational trophoblastic disease has not been explored extensively although there is a thought that this mechanism is associated with oxidative damage related to the

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production of reactive oxygen species [9]. Malignant trophoblastic disease (gestational trophoblastic tumors) can cause mild to severe clinical symptoms that may result in death. The apearance of the clinical symptoms depends on the tumor invasive ability and its metastases. Therefore, early detection and proper handling is required for the treatment of this disease. Efforts to prevent a mole from becoming a malignant trophoblastic disease is deemed necessary given the danger it possesses, although even now its pathophysiology is still unclear and very little research has been done.

Conducting a research on molar trophoblast cells is impossible in humans. Therefore, in vitro trophoblast cell culture media that mimic the actual conditions is necessary for allowing different treatments to be given as desired. Because there were no studies on the role of estrogen in trophoblast cell transformation, we were interested in doing an experimental research on the effects of estrogen (17β estradiol) on the total ROS production in hydatidiform mole tissue culture.

II. METHODS

The experimental research was carried out in a laboratory using Randomized Group Control Design. The experimental design used was Complete Randomized Design (CRD) with one treatment group i.e. group of hydatidiform mole tissue culture supplemented with various doses of estrogen (17β estradiol) and without supplementation.

The study used a hydatidiform mole tissue sample from hydatidiform mole suction curretage results. The tissue cultures were divided into 5 groups.

Group I : without added estrogen (17βestradiol).

Group II : with a physiological dose of estrogen added (10 nm 7βestradiol).

Group III : with a low dose of estrogen added (5nm17βestradiol).

Group IV : with a moderate dose of estrogen added (20 nm 17βestradiol).

Group V : with a high-dose of estrogen added (40 nm 17βestradiol).

Each culture was then undergoing a colorimetri examination to measure the total production of Reactive Oxygen Species. The culture medium ROS levels were measured using the Colorimetric Hydrogen Peroxide Kit (Assay Design). The r ROS measurement results were used for observation and data analysis. The data were analyzed using ANOVA test followed by Tukey multiple comparison test and regression test. The statistical test was considered significant if $p < 0.05$.

III. RESULTS

TABLE I
THE MEAN NUMBERS OF TOTAL ROS PRODUCTION

	N	Mean	Std. Deviation	Std. Error	Minimum	Maximum
Control	5	.5386	.05288	.02365	.50	.63
5 ug/ml 17 beta estradiol	5	.5404	.06203	.02774	.45	.60
10 ug/ml 17 beta estradiol	5	.6222	.08113	.03628	.51	.73
20 ug/ml 17 beta estradiol	5	.6878	.15031	.06722	.43	.80
40 ug/ml 17 beta estradiol	5	.9810	.11378	.05089	.82	1.14
Total	25	.6740	.18947	.03789	.43	1.14

TABLE II
THE SUMMARY OF ANOVA TEST RESULTS

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.667	4	.167	17.085	.000
Within Groups	.195	20	.010		
Total	.862	24			

TABEL III
TUKEY'S MULTIPLE COMPARISONS TEST FOR TOTAL ROS PRODUCTION IN HYDATIDIFORM MOLE TISSUE CULTURE

(I) Groups	(J) Groups	Mean Difference (I-J)	Std. Error	Sig.
Control	17 beta estradiol 5 ug/ml	-.00180	.06246	.977
	17 beta estradiol 10 ug/ml	-.08360	.06246	.196
	17 beta estradiol 20 ug/ml	-.14920*	.06246	.027
	17 beta estradiol 40 ug/ml	-.44240*	.06246	.000
17 beta estradiol 5 ug/ml	Control	.00180	.06246	.977
	17 beta estradiol 10 ug/ml	-.08180	.06246	.205
	17 beta estradiol 20 ug/ml	-.14740*	.06246	.029
	17 beta estradiol 40 ug/ml	-.44060*	.06246	.000
17 beta estradiol 10 ug/ml	Control	.08360	.06246	.196
	17 beta estradiol 5 ug/ml	.08180	.06246	.205
	17 beta estradiol 20 ug/ml	-.06560	.06246	.306
	17 beta estradiol 40 ug/ml	-.35880*	.06246	.000
17 beta estradiol 20 ug/ml	Control	.14920*	.06246	.027
	17 beta estradiol 5 ug/ml	.14740*	.06246	.029
	17 beta estradiol 10 ug/ml	.06560	.06246	.306
	17 beta estradiol 40 ug/ml	-.29320*	.06246	.000
17 beta estradiol 40 ug/ml	Control	.44240*	.06246	.000
	17 beta estradiol 5 ug/ml	.44060*	.06246	.000
	17 beta estradiol 10 ug/ml	.35880*	.06246	.000
	17 beta estradiol 20 ug/ml	.29320*	.06246	.000

*. The mean difference is significant at the 0.05 level.

This study used numerical variables to look for one factor which was the difference of the total production of ROS in hydatidiform mole tissue culture treated with different doses of 17β estradiol. To prove the hypothesis of the study, One Way ANOVA test was used to compare the mean of five sample groups: the control group, 5μg/ml 17β estradiol treatment group, 10μg/ml 17β estradiol treatment group, 20μg/ml 17β estradiol treatment groups and 40μg/ml 17β estradiol treatment group.

Furthermore, the research results on the total production of ROS in hydatidiform mole tissue culture as seen on the attachment were then processed and analyzed using Oneway ANOVA (Analysis of Variance) to detect any differences in the influence of the various administered dose of 17β estradiol on the total production of ROS in hydatidiform mole tissue culture. The analysis technique used was chosen to find out the effect of various doses of 17β estradiol treatments on the production of ROS. If H0 conclusion was rejected according to One Way ANOVA test results, the analysis would then be followed by a multiple comparison test, Least Significant Difference/LSD.

The hypothesis was determined as follows: H0 was accepted if the significance value obtained > alpha of 0.05 while rejected if the significance value obtained < alpha of 0.05. H0 of this study was that there was no difference in total ROS production in hydatidiform mole tissue culture with 17β estradiol exposure. On the other hand, the H1 of this study was that there was a difference in total ROS production in hydatidiform mole tissue culture with 17β estradiol exposure.

The ANOVA test results for the total ROS production in hydatidiform mole tissue culture for each treatment dose of 17β estradiol were presented in Table 1 and Table 2.

The comparative test for six mean levels of ROS using the F test (ANOVA) showed that there was a profound (very significant) difference between the mean levels of ROS in the control group and the treatment groups of 17 beta-estradiol in different sizes. This was indicated by the value of Sig (p) = $0.000 < \alpha = 0.05$.

H0 was rejected and therefore, there was a difference in mean levels of ROS among the six sample groups. A multiple comparison test was then performed using Least Significant Difference/LSD test.

A. Multiple Comparisons

The next step is to process the data using post hoc methods for multiple comparisons with Tukey test (Tukey's Test) as a multiple comparison test that had a high enough sensitivity in testing the differences between treatments in multiple comparisons. In this method, multiple comparisons would be performed on the total production of ROS in hydatidiform mole tissue culture between each varying dose of 17β estradiol treatment. Therefore, the difference between the

varying dose of 17β estradiol and the total production of ROS in hydatidiform mole tissue culture could be seen from the results of Tukey's test in Table 3.

IV. DISCUSSION

The results of the statistical analyses showed that ROS production in hydatidiform mole tissue culture different significantly at each treatment dose of 17β estradiol ($p = 0.000 < 0.05$). Therefore, the average difference between each treatment group tested in this study differed quite a lot, so that statistically there were significant differences. The effect of 17β estradiol on the production of ROS in hydatidiform mole tissue culture was not seen where at doses of 5 ug and 10 ug. There was no significant difference compared to the control group. This was due to the result that the doses of 5 ug and 10 ug which were low and physiological doses did not affect ROS production. The production of ROS in hydatidiform mole tissue culture increased again when given a dose of 17β estradiol higher than 20 ug and 40 ug. Apparently, the 40 ug dose causes the highest ROS production in hydatidiform mole tissue culture. Therefore, based on the descriptive assessments according to the average production of ROS in hydatidiform mole tissue culture, it could be said that the 17β estradiol treatments rendered different effects or influences in increasing the production of ROS in hydatidiform mole tissue culture when compared to the control group.

The existence of significant differences in ROS production in hydatidiform mole tissue culture exposed to 17β estradiol was as influenced by the presence of 17β estradiol that caused an increase in ROS concentrations in a dose of 20 ug and 40 ug. Trophoblast cells in normal and pathological histology are just the same as both express estrogen receptors [10]. Within molar trophoblast cells, the expression of estrogen receptor alpha (ER-a) was located in the villous cytotrophoblast (CT). Western Blot analysis showed that estrogen beta receptors within chorionic villi (CV) is limited only to the syncytiotrophoblast (ST)[11]. The binding of 17 β estradiol to estrogen receptors causes signal transduction. Oxidative metabolism of estrogen through the cathecol pathway which involves the cytochrome P-450 enzymes catalyzes the oxidative metabolism of estradiol. Estrogen metabolites have carcinogenic properties: estrogen 3,4-quinone causes an unstable bond with adenine and guanine in DNA which causes depurination as well as mutations both in vitro and in vivo. The reduction of estrogen quinone to go back as catechol hydroquinone triggered the production of reactive oxygen species (ROS) and further causing oxidative damage to lipids and DNA due to estrogen exposure.

V.CONCLUSION

According to the result of this study, we conclude that there was a significant rise of ROS level on the increased dose of 17β estradiol exposed to the hydatide mole culture. It was shown by the more significant increase of total ROS level on the dose 20 ug and 40 ug of 17β estradiol exposed than those on the control, dose 5 ug and 10 ug of 17β estradiol exposed.

This study could be the potential base for the next research, especially regarding to the DNA damage on the hydatide mole culture as well another variable increasing ROS level on the hydatide mole culture.

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