Research Report

The effect of 17β Estradiol Exposure on Mutant p53 Expression in Hydatidiform Mole Trophoblast Cell Culture

Perbandingan Ekspresi p53 Mutant pada Kultur Jaringan Plasenta Normal dan Kultur Jaringan Mola Hidatidosa dengan Pemaparan 17β Estradiol

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Abstract

Objectives: To compare the mutant p53 expression in normal trophoblast (N) cell culture with hydatidiform mole trophoblast (HM) cell culture which was exposed to 17β estradiol.

Methods: An experimental study conducted at the Laboratory of Physiology Faculty of Medicine, Brawijaya University Malang using N cell culture and HM cell culture with 17β estradiol exposure. Trophoblast cell culture of normal and hydatidiform mole was divided in 6 groups, such as: 1. Without added 17β estradiol; 2. Added 5 nm 17β estradiol; 3. Added 10 nm 17β estradiol; 4. Added 20 nm 17β estradiol; 5. Added 40 nm 17β estradiol; 6. Added 80 nm 17β estradiol. Then performed immunocytochemistry staining using p53 mutant primary antibody and observed the expression of p53 mutant. Data from observations analized with the ANOVA test and correlation test.

Results: Mutant p53 expression in N cell culture showed no significant differences in each treatment dose of 17β estradiol ($p = 0086 > 0.05$). The dose at 80nm 17β estradiol showed an average of highest mutant p53 expression on N cell culture rather than giving the dose of 17β estradiol on 40 nm, 20 nm, 10nm and 5 nm. While the control group showed a lowest average of mutant p53 expression in N cell culture when compared to the treatment group which was exposed to 17β estradiol. Mutant p53 expression in HM cell culture showed a significant difference at each treatment dose of 17β estradiol ($p = 0.000 < 0.05$). The existence of the effect of 17β estradiol begins when the expression of mutant p53 in HM cell culture becomes higher after being given treatment in the form of 17β estradiol on the dose of 5 nm compared with the expression of 17β estradiol in the control group. Then the expression of mutant p53 in HM cell culture is increasing when given doses of 17β estradiol at 20 nm and 40 nm. At a dose of 40 nm it shows the highest expression of mutant p53. Expression of mutant p53 in HM cell culture decreased when given at doses 80 nm.

Conclusion: Mutant p53 expression in N cell culture exposed to 17β estradiol showed no significant difference. Expression of mutant p53 in HM cell culture which was exposed to 17β estradiol showed a significant difference. Mutant p53 expression in N and HM cell culture which was exposed to 17β estradiol showed significantly different, in which mutant p53 expression in N cell culture is lower than the expression of mutant p53 in HM tissue culture.

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Keyword: p53 mutant, 17β-estradiol, hydatidiform mole

Abstrak

***Tujuan**: Membandingkan ekspresi p53 mutant pada kultur jaringan plasenta normal dengan kultur jaringan mola hidatidosa yang dipapar dengan 17β estradiol.*

***Metode**: Suatu studi eksperimental yang dilakukan di Laboratorium Fisiologi Fakultas Kedokteran Universitas Brawijaya Malang menggunakan kultur jaringan plasenta normal dan mola hidatidosa yang dipapar 17β estradiol, kultur jaringan plasenta normal dan mola hidatidosa dibagi dalam 6 kelompok, yaitu: 1. tanpa ditambahkan 17β estradiol; 2. ditambahkan 5 nm 17β estradiol; 3. ditambahkan 10 nm 17β estradiol; 4. ditambahkan 20 nm 17β estradiol; 5. ditambahkan 40 nm 17 estradiol; 6. ditambahkan 80 nm 17β estradiol. Kemudian dilakukan pengecatan imunositokimia menggu-na-kan antibodi primer p53 mutant dan dilakukan pengamatan eks-presi p53 mutant. Data hasil pengamatan dilakukan analisis data dengan uji ANOVA dan uji korelasi.*

***Hasil**: Ekspresi p53 mutant pada kultur jaringan plasenta normal yang dipapar dengan 17β estradiol tidak menunjukkan adanya perbedaan yang signifikan. Ekspresi p53 mutant pada kultur jaringan mola hidatidosa yang dipapar dengan 17β estradiol menunjukkan adanya perbedaan yang signifikan. Ekspresi p53 mutant pada kultur jaringan plasenta normal dengan kultur jaringan mola hidatidosa yang dipapar dengan 17β estradiol menunjukkan berbeda signifikan, di mana ekspresi p53 mutant pada kultur jaringan plasenta normal lebih rendah daripada ekspresi p53 mutant pada kultur jaringan mola hidatidosa.*

***Kesimpulan**: Terdapat perbedaan ekspresi p53 mutant jaringan plasenta normal dengan mola hidatidosa.*

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***Kata kunci**: p53 mutant, 17β estradiol, mola hidatidosa*

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INTRODUCTION

The placenta is an organ that is vital to support fetal growth and development and successful pregnancy. Trophoblast cells from the placenta proliferate, migrate, and invade the uterus in order to maintain the development of the fetus.[1] Trophoblast cells are only found when a woman is pregnant. When there is a

failure in trophoblastic disease differentiation of the placenta, a form of hydropic degeneration of the chorion resemble bubbles-like chorion called hydatidiform mole.[2]

Hydatidiform mole can occur in all women in the reproductive period, the youngest patient ever reported was 12 years old and the oldest was 57 years old. Most patients will improve after HM treated completely. However, approximately 15 - 20% will undergo transformation into malignant Gestational trophoblastic tumors (GTT). Gestational trophoblastic tumor is a group of malignant diseases associated with villous chorialis and most are preceded by hydatidiform mole.[3]

Incidence of HM in the United States is 1 : 1450 - 1 : 2000 childbirth and Japan is 3 : 2000 childbirths. Epidemiological research in Bandung and Malang in 2002 (population-based study) showed that the incidence of hydatidiform mole, respectively is 1 : 500 and 1 : 400. If in Indonesia there are estimation 22 million births per year, it will happen 44000 - 55000 hydatidiform mole cases per year. When 15% of them become malignant, GTT will occur as many as 6600 - 8250 cases per year.[4]

The process of carcinogenesis HM into GTT is still not clearly understood and there is only a few studies. Several factors are known have role in the process of GTT carcinogenesis, include: DNA ploidy, expression of phospholipids, oncogenes, tumor suppressor genes, nutrition and hormonal status.[5]

Tumor supressor gene is a protein that easily activated when DNA damage occurs. p53 gene is the main tumor supressor because its function in inhibiting cell cycle, differentiation, apoptosis and angiogenesis. In malignant gestational trophoblastic tumor or choriocarcinoma, there is p53 protein mutation that the cell proliferation is not inhibited and the cells continue to proliferate. p53 over expression is important in the pathogenesis of complete mole and choriocarcinoma and related to the aggressive nature of gestational trophoblastic disease. p53 over expression associated with p53-dependent apoptosis to modulate excessive proliferation of trophoblast.[6] p53 gene mutation is important in the pathogenesis of gestational trophoblastic tumor and its progressivity in humans. With the polymerase chain reaction (PCR) technique, it has been detected p53 gene mutation in 30% hydatiform mole, 75% choriocarcinoma and without p53 gene mutation in normal chorionic villi.[7] Other studies have shown that p53 antibodies are found mainly in cancer patients with a specificity of 96%. Antibodies are primarily associated with p53 gene missense mutations and accumulation of p53 in the tumor. p53 gene mutations play a role in uncontrolled trophoblastic proliferation and neoplastic transformation.[8]

Natural estrogen hormones play a role in the process of carcinogenesis in several malignancies such as breast cancer, endometrial cancer, liver cancer, and several other cancer types. Estrogen receptor expression contribute to determine the process of carcinogenesis. This is supported by the fact about the occurrence of excessive expression of some of these cancers. Ethynil estradiol is a synthetic estrogen commonly used as a contraceptive pill that proven to play a role in the process of GTT carcinogenesis.[5] Until now there has been no research on the effects of natural estrogen 17β estradiol in HM cells.

In general, the fertility degree of post-hydatidiform mole has not changed. When not using contraception they will get pregnant soon after a normal menstrual return. Naturally, estrogen which is a female sex hormone, produced by the ovaries as part of the menstrual cycle. Estrogen synthesized in ovarian and extra ovarian tissues. In women of childbearing age, the secretions is from the ovaries cycle until menopause. Aromatase activity has also been detected in muscle, fat, and neural tissue. So, naturally, women is exposed by the endogenous estrogen for long periods of time.[9] Trophoblast cells of normal and pathological is histologically same which have estrogen receptor expression.[10]

Several theories have been advanced the occurrence of malignancy and one of them is a multiple estrogen receptor signal transduction pathway that will cause increased proliferation and inhibits apoptosis of endometrial stromal cell.[11] Shao proved the occurrence of carcinogenesis in MCF-7 Human Breast Cancer Cell-induced estrogen. Until now it is still unclear why and how the trophoblast cells could turn into a malignancy, so that suspected estrogen plays a role in the transformation of trophoblast cells.

Molar trophoblastic disease prevention efforts is needed, but until now the pathophysiology remains unclear and very little research has been done. To make a mole trophoblast cell research not possible in humans. Therefore, trophoblast cell culture medium is required in vitro that resembles the actual conditions and can be given different treatment desirely. Research on the role of estrogen in the transformation of trophoblast cells has not been done yet, so this study investigates the effects of estrogen (17β estradiol) on the expression of p53 in tissue culture of normal placenta and hydatidiform mole.

METHOD

This research was done experimentally between control and intervention group. Experiment was performed at the Laboratory of Physiology, Faculty of Medicine, Brawijaya University Malang. Research was conducted in July 2010. This study used tissue samples originating from hydatidiform mole after suction curretage and normal placental tissue samples from delivery by Caesarean section, a healthy mother does not suffer any pain with Hb level ≥ 10, with normal pregnancies.

The objectives of this study are to observe the expression of mutant p53 in trophoblast cells of the normal placenta tissue and HM cell culture which was not given any treatment or which was given 5 nm, 10 nm, 20 nm, 40 nm, and 80 nm 17ß estradiol exposure in which on each well performed up to 4 times repetition, then performed staining with primary mutant p53 antibodies. Then performed counting of 100 trophoblast cells, how many trophoblast cells that express mutant p53.

In this study, the independent variable is the dose of estrogen 17ß estradiol. While the dependent variable is the expression of p53 mutant N and HM cell culture.

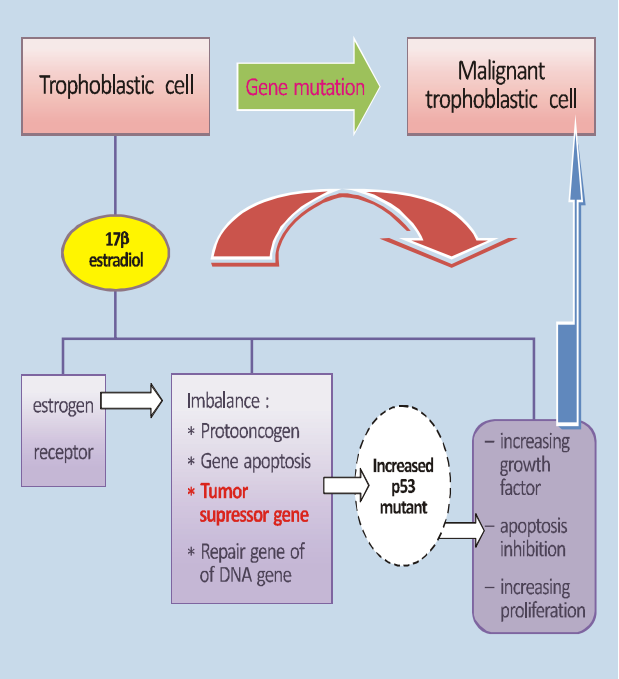

Figure 1. Conceptual framework.
17β estradiol E2 would bind to the receptor and cause the signal transduction that affects the cell activity. The theory of the malignancy through the estrogen receptor signal transduction pathways will lead to increased proliferation and inhibits apoptosis of cells. Tumor suppressor gene is a protein that easily activated when there is damage DNA. In gestational trophoblastic malignant or Choriocarcinoma, there are mutation of p53 protein that the cell proliferation was not inhibited, so that the cells continued to proliferate even though the cells produced a cell with mutated DNA and abnormal.

The results tabulated and presented in the form of tables and graphs and do statistical analysis using SPSS 13 for windows, calculated using one-way ANOVA, Tukey test, and correlation and regression test. Probability considered statistically significant if p value < 0.05 with 95% confidence interval.

Staining assessment and positive gradation were assessed with a light microscope big field 400x, then trophoblast cells that express mutant p53 is marked by a brown color visualization of nucleus trophoblast cells per 100 counted.

RESULTS

This study used normal placenta and HM cell culture without and with administration of estrogen (17β estradiol) in 5 doses (5 nm, 10 nm, 20 nm, 40 nm, 80 nm). This study objective is to see the expression of mutant p53 using mutant p53 monoclonal antibody after confluent cells.

The confluent cells is characterized by the cell has been attached to the attachment site and touch each other between cells. The distance between cells that regular and increasingly tight, flat surface marked by the appearance of the cell nucleus, plasma membrane, cytoplasm and extracellular matrix, with larger cell sizes. Staining was performed with mutant p53 monoclonal antibody which gives brown staining in nucleus cell.

The color brown is a visualization of crimogen dab (diaminobenzidine). Antibody-antigen complex mutant p53 will be recognized by biotin-labeled antibodies. Biotin on the secondary antibody binds with strep avidin harse rads perixidase (sahrp) which will visualize the brown color. Observations were carried out using a light microscope with 400x magnification, calculated from 100 trophoblast cells, how many are expressing p53 mutant cells.

The hypothesis H0 is accepted when determined by the significant value gained > alpha 0.05, while

Table 1. Mutant p53 expression in N cell culture.

Treatment	Sample						Mean±SD
	1	2	3	4	5	6	
Control	9	8	10	11	7	9	9.0 ± SD 1.58
17β estr 5 nm	10	11	9	11	8	10	9.8 ± SD 1.30
17β estr 10 nm	11	10	12	10	9	10	10.4 ± SD 1.14
17β estr 20 nm	10	12	11	10	11	11	10.8 ± SD 0.84
17β estr 40 nm	11	12	11	12	9	11	11.0 ± SD 1.22
17β estr 80 nm	12	10	13	10	11	11	11.2 ± SD 1.30

Table 2. Mutant p53 expression in HM cell culture.

Treatment	Sample						Mean ± SD
	1	2	3	4	5	6	
Control	55	49	60	59	57	56	56 ± SD 4.36
17β estr 5 nm	60	62	58	59	53	58	58.4 ± SD 3.36
17β estr 10 nm	68	66	63	74	71	68	68.4 ± SD 4.28
17β estr 20 nm	64	74	82	68	67	71	71 ± SD 7.14
17β estr 40 nm	88	86	94	91	87	89	89.2 ± SD 3.27
17β estr 80 nm	81	69	80	73	70	75	74.6 ± SD 5.59

Table 3. Mutant p53 expression in N cell culture.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig
Between Groups	17.368	5	3.473	2.217	0.086
Within Groups	37.600	24	1.567		
Total	54.967	29			

Table 4. Mutant p53 expression in HM cell culture.

ANOVA

	Sum of Squares	df	Mean Square	F	Sig
Between Groups	3614.800	5	722.960	30.634	0.000
Within Groups	566.400	24	23.600		
Total	4184.200	29			

H0 is rejected if the significance value obtained <alpha 0.05. H0 of this research is no different mutant p53 expression in N cell culture and HM cell culture with 17β estradiol exposure. While the H1 is the difference expression of mutant p53 in N cell culture and HM cell culture with 17β estradiol exposure.

Based on the analysis of variance showed significant value for HM cell culture is 0.000 ($p < 0.05$), so that H0 is rejected, and it can be concluded that there are differences in mutant p53 expression in N cell culture and HM cell culture with 17β estradiol exposure. While mutant p53 expression in N cell culture showed no significant differences in each treatment dose of 17β estradiol ($p = 0086 > 0.05$).

Multiple Comparisons

With this method, multiple comparisons will be made on the expression of mutant p53 in N cell culture with HM cell culture between each treatment of 17β estradiol in various doses. So to find out the differences of 17β estradiol in various doses on the expression of mutant p53 in N cell culture with HM cell culture, it can be seen from the results of Tukey test (Tukey's Test).

Differences mutant p53 expression in each treatment dose of 17β estradiol was seen in which the N cell culture tend to have a mutant p53 expression is lower than the expression of mutant p53 in hydatidiform mole tissue culture.

Table 5. Tukey Multiple Comparison Test Table for mutant p53 expression in N cell culture.

Comparison between treatment		Average of difference	Sig.	Conclusion
Control	17β estr 5 nm	–0.800	0.910	Not significantly different
	17β estr 10 nm	–1.400	0.503	Not significantly different
	17β estr 20 nm	–1.800	0.243	Not significantly different
	17β estr 40 nm	–2.000	0.156	Not significantly different
	17β estr 80 nm	–2.200	0.096	Not significantly different
17β estr 5 μg	17β estr 10 nm	–0.600	0.972	Not significantly different
	17β estr 20 nm	–1.000	0.801	Not significantly different
	17β estr 40 nm	–1.200	0.658	Not significantly different
	17β estr 80 nm	–1.400	0.503	Not significantly different
17β estr 10 μg	17β estr 20 nm	–0.400	0.995	Not significantly different
	17β estr 40 nm	–0.600	0.972	Not significantly different
	17β estr 80 nm	–0.800	0.910	Not significantly different
17β estr 20 μg	17β estr 40 nm	–0.200	1.000	Not significantly different
	17β estr 80 nm	–0.400	0.995	Not significantly different
17β estr 40 μg	17β estr 80 nm	–0.200	1.000	Not significantly different

Source: *Primary data are processed.*

Table 6. Tukey Multiple Comparison Test Table for mutant p53 expression in HM cell culture.

Comparison between treatment		Average of difference	Sig.	Conclusion
Control	17β estr 5 nm	– 2.400	0.968	Not significantly different
	17β estr 10 nm	–12.400	0.006	Significantly different
	17β estr 20 nm	–15.000	0.001	Significantly different
	17β estr 40 nm	–33.200	0.000	Significantly different
	17β estr 80 nm	–18.600	0.000	Significantly different
17β estr 5 μg	17β estr 10 nm	–10.000	0.035	Significantly different
	17β estr 20 nm	–12.600	0.005	Significantly different
	17β estr 40 nm	–30.800	0.000	Significantly different
	17β estr 80 nm	–16.200	0.000	Significantly different
17β estr 10 μg	17β estr 20 nm	– 2.600	0.955	Not significantly different
	17β estr 40 nm	–20.800	0.000	Significantly different
	17β estr 80 nm	– 6.200	0.362	Not significantly different
17β estr 20 μg	17β estr 40 nm	–18.200	0.000	Significantly different
	17β estr 80 nm	– 3.600	0.846	Not significantly different
17β estr 40 μg	17β estr 80 nm	14.600	0.001	Significantly different

Source: *Primary data are processed.*

DISCUSSION

Differences mutant p53 expression in N cell culture which is exposed to 17β estradiol

The statistical results indicate that mutant p53 expression in N cell culture showed no significant differences in each treatment dose of 17β estradiol (p = 0086 > 0.05). Because the average difference between each group treatments tested in this study do not differ too much, so that statistically the differences are not significant. From the results of this study showed that the dose at 80 nm 17β estradiol showed an average of highest mutant p53 expression on N cell culture rather than giving the dose of 17β estradiol on 40 nm, 20 nm, 10 nm and 5 nm. While the control group showed an lowest average of mutant p53 expression in N cell culture when compared to the treatment group which were exposed to 17β estradiol.

The absence of significant differences in the expression of mutant p53 in N cell culture which was exposed by 17β estradiol is because under normal circumstances, low p53 protein expression in the cytoplasm and inactive bind to the DNA. Active p53 protein accumulates in the nucleus in response to various cell stress.[12] In addition, the N cell culture that has no damage of DNA, will cause the concentration of p53 protein tended to be stable. So even if exposed to 17β estradiol, the mutant p53 expression in N cell culture will not show any significant increase or decrease, so that the results of data analysis mutant p53 expression in N cell culture which are exposed with some doses of 17β estradiol show no difference significant. Normal p53 (wild type) was able to suppress cell transformation caused by oncogene in culture and can inhibit cell tumorigenic potential in animals that were classified as suppressor gene p53. Although the mechanism of p53 is not known for sure, there are indications that p53 inhibit cell growth. There are several hypotheses regarding the mechanism of action: a) p53 recognizes and then binds itself to a "specific sequence" of DNA thought to be a certain part that functions as a regulator, b) p53 induces the activity of RNA polymerase, so it acts as a transcription factor.[13]

Differences mutant p53 expression in HM cell culture which is exposed to 17β estradiol

The statistical results indicate that mutant p53 expression in HM cell culture showed a significant difference at each treatment dose of 17β estradiol (p = 0.000 < 0.05). Therefore, the average difference between each group treatments tested in this study differs quite a lot, so that statistically there are significant differences. The existence of the effect of 17β estradiol begins when the expression of mutant p53 in HM cell culture become higher after being given treatment in the form of 17β estradiol on the dose of 5 nm compared with the expression of 17β estradiol in the control group. Then the expression of mutant p53 in HM cell culture is increasing when given doses of 17β estradiol at 20 nm and 40 nm. At a dose of 40 nm can show the highest expression of mutant p53. Expression of mutant p53 in HM cell culture decreased when given at doses 80 nm. Therefore, based on a descriptive according to average expression of mutant p53 in HM cell culture, it can be said that the treatment in the form of 17β estradiol showed a different effect or influence in increasing the expression of mutant p53 in HM cell culture when compared with control group.

The significant differences in the expression of mutant p53 in HM cell culture which was exposed to 17β estradiol is because it is influenced by the presence of 17β estradiol, which causes the concentration of mutant p53 protein can be increased in a dose of 5 nm, 10 nm, 20 nm and 40 nm, then decreased on dose of 80 nm. This is thought to be caused by the effect of 17β estradiol is experiencing saturation at doses higher than 40 nm, ie at doses of 80 nm, resulting in mutant p53 expression in HM cell culture to decline. Trophoblast cells of normal and pathological are the same histologically contained estrogen receptor expression.[10] In molar trophoblast cells, there are expressions of estrogen receptor alpha (ER-a) in villous cytotrophoblast (CT). With western blot analysis, there are estrogen receptors beta (ER-β) in the chorionic villi (CV) limited to the syncytiotrophoblast (ST).[14] 17β estradiol bind to estrogen receptors and cause signal transduction. Oxidative metabolism of estrogen through the cathecol, with cytochrome P-450 enzymes catalyze the oxidative metabolism of estradiol. Estrogen metabolites have carcinogenic properties, 3,4-quinone estrogen causes an unstable bond with adenine and guanine in the DNA causes depurination and mutation in vitro and in vivo. Reduction of estrogen quinon back into hydroquinon and catechol led to a chance of reactive oxygen species (ROS) formation and cause oxidative damage to lipids and DNA due to exposure to estrogen. Exposure to various doses of 17β estradiol on estrogen receptor saturation occurs at high doses, so that the average mutant p53 expression in HM cell culture at doses of 80 nm are lower than those exposed to 17β estradiol at doses of 40 nm.

Differences mutant p53 expression in N cell culture with HM cell culture which was exposed to 17β estradiol

The statistical results show that there are significant differences in mutant p53 expression in each treatment dose of 17β estradiol on N cell culture with tissue culture of hydatidiform mole (p = 0.000 > 0.05). Therefore, mutant p53 expression in each treatment dose of 17β estradiol was seen in which the N cell culture of mutant p53 expression tend to have lower expression than the mutant p53 in hydatidiform mole tissue culture. The difference between mutant p53 expression in normal placenta tissue culture with HM cell culture tested in this study differs quite a lot, so that statistically there are significant differences.

This is consistent with the statement that the p53 gene mutation is important in the pathogenesis of gestational trophoblastic and its progressivity in humans. With the polymerase chain reaction (PCR) technique, it has been detected p53 gene mutation in 30% hydatiform mole, 75% choriocarcinoma and without p53 gene mutation in normal chorionic villi.[7]

P53 gene mutations play a role in uncontrolled trophoblastic proliferation and neoplastic transformation.[8] During a stress on the cells that proliferate resulting in damage to DNA, the protein will be activated. The result of activation of this protein is the cessation of cell proliferation in G1 or G2 phase, and apoptosis. In Gestational trophoblastic malignant or choriocarcinoma, there are mutation of p53 protein so the cell proliferation was not inhibited, and the cells continued to proliferate even though the cells produced a cell with mutated DNA and abnormal.[15] Yaginuma and colleagues, detects the presence of abnormal p53 protein in all three choriocarcinoma cell types (SCH, JEG-3, Nuc-1). 20% protein p53 mutations occur in the early phase and 80% occurred in the final phase. The finding of p53 protein in choriocarcinoma cells staining indicating poor prognosis associated with this disease.[16,17]

While in normal circumstances, there is low p53 protein expression in the cytoplasm and inactive binding to DNA. Active p53 protein accumulates in the nucleus in response to various cell stress. In N cell culture which are no damage to DNA, will cause the concentration of p53 protein tended to be stable.[13]

CONCLUSION

Mutant p53 expression in N cell culture exposed to 17β estradiol showed no significant difference ($p > 0.05$). Expression of mutant p53 in HM cell culture which was exposed to 17β estradiol showed a significant difference ($p < 0.05$), and mutant p53 expression in N cell culture with HM cell culture which was exposed to 17β estradiol showed significantly different ($p < 0.05$), in which mutant p53 expression in N cell culture is lower than the expression of mutant p53 in HM cell culture.

SUGGESTION

Based on the conclusions and discussion of research results, we proposed the following suggestions. Through this research concerning the different mutant p53 expression in N cell culture with HM cell culture which was exposed to 17β estradiol, it is henceforth to be developed as a basis for further research. For subsequent studies, the results of this study can provide as preliminary studies to develop other similar research. In addition, further research needs to be done by extending the other variables that can increase the expression of mutant p53 in hydatidiform mole placenta tissue culture, apart from exposure to estrogen (17β estradiol). This research is still localized or in a single place that was in the General Hospital dr. Saiful Anwar Malang, meaning that the research results and conclusions apply only to populations, not to apply to the public, so that its scope should be expanded again. For example, by increasing the number of samples in the study of the wider population, in other words increasing the number of research areas, so that the conclusions of the research results can be more representative against the observed population.

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