ORIGINAL ARTICLE

All-trans retinoic acid (atra) inhibits telomerase expression of BeWo choriocarcinoma cell (ATCC CCL-98)

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ABSTRACT

Introduction: Choriocarcinoma is malignant cancer originating from placental trophoblast. The incidence of this cancer is estimated at 0.57-1.1 per 1000 births in the United States of America, Australia, Europe, and New Zealand. The rate is much higher in South East Asia and Japan with two occurrences per a thousand births. Telomerase activity is an important part of the apoptotic process. Increased telomerase activity will result in cellular immortality and poor prognosis in cancer. Vitamin A possess an essential role in cell proliferation and differentiation. One of the active metabolites of vitamin A is All-Trans Retinoic Acid (ATRA).

Methods: In this study, we examined the role of ATRA against telomerase activity in choriocarcinoma cell. This cell was derived from BeWo cell line (ATCC CCL-98) and were given different doses of ATRA.

Results: From this study, Choriocarcinoma cell that was given ATRA in dosage of 50µg/ml inhibit telomerase activity by extending the cycle time of 39.51±0.09, compared to the control group with a cycle time of 37.62±0.43. Cycle length change consistently with higher dose of ATRA.

Conclusion: This study has proven that ATRA could inhibit telomerase activity by lengthening the cycle. Changes in the increase of ATRA doses in this experimental test need to be studied further on experimental animals, either administered as a single agent or as an addition to standard treatment of trophoblastic disease.

KEY WORDS:
ATRA, BeWo, Choriocarcinoma

INTRODUCTION

Choriocarcinoma is malignant cancer originating from placental trophoblast. Choriocarcinoma can be divided into two types, i.e., gestational and non-gestational. Gestational choriocarcinoma occurs mostly among woman of childbearing age, usually within a year of molar or non-molar pregnancy. Non-pregnancy choriocarcinoma may arise from germ cells or trophoblast differentiation in endometrial carcinoma. Twenty-five per cent of abortion cases were associated with tubal pregnancy, 25% with preterm pregnancy, and 50% from hydatidiform mole. Only 2-3% of hydatidiform mole became choriocarcinoma.[1] Choriocarcinoma main complain are intrauterine bleeding and rapid as well invasive proliferative response of tumour's mass to the blood vessels.[2]

Inhibitions in telomerase in cells causes telomere shortening and cell apoptosis. The above observation indicates that telomerase activity is a necessary condition for the perpetuation of selenium. Thus, telomerase will have important implications for human cancer research and management. Increased telomerase activity has also been found to be associated with poor prognosis in gastric carcinoma, meningioma, hepatocellular carcinoma, neuroblastoma, and leukemia. Few studies have been reported about telomerase activation in trophoblast disease.[3]

Vitamin A plays a substantial role in the control mechanisms against cell proliferation and differentiation. All-trams retinoic acid (ATRA) is the most biologically active metabolite of vitamin A that has been extensively studied as an anti-cancer agent. ATRA inhibits tumor cell growth by blocking cell cycle progression in G1 phase and regulating levels of CDK4 and 6, and cyclin D1 by regulating CDK G1-S (CKI) inhibitors, e.g., p16, p21, and p27. ATRA inhibits the E2F transcription factor and inhibit cell cycle in phase G1. However, ATRA's action mechanisms in this process is still unclear. Thus, a more extensive study is required to clarify the mechanisms by which ATRA regulates p16, p21, and p27.[4]

Retinoic acid, which is an active substance of retinol or vitamin A, has the capacity to stimulate cell cycle arrest and stimulating apoptosis. The role of vitamin A is vital in delaying or inhibiting the cancer process. The potential for prevention of cancer by beta-carotene and vitamin A presents promising results as chemoprevention in reducing the risk of cancer in humans.[5]

The likelihood of occurrence of malignant trophoblastic disease decreases in vitamin A administration, but the mechanism of vitamin A in malignant trophoblastic disease is not yet fully known.[6] A study by Chiu et al., showed that ATRA administration inhibited invasion and proliferation, and also increased the apoptosis of choriocarcinoma cells so that ATRA might be an alternative agent of choriocarcinoma treatment.[4]

Previous studies have shown the effect of ATRA on choriocarcinoma. However, to the best of our knowledge, there has not been no study conducted to examine the

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impact of ATRA dose on telomerase in choriocarcinoma cells.[7] This study is expected to contribute to the existing information about the role of vitamin A, particularly ATRA in choriocarcinoma. The complexity of mechanisms and effectiveness of doses administered by ATRA – that affect the telomere of choriocarcinoma cells needs to be studied, so that it can further extend our understanding regarding the importance this vitamin in preventing and treating choriocarcinoma.

MATERIALS AND METHODS

Cells

This study used a sample of choriocarcinoma cells derived from cell line choriocarcinoma BeWo from *American Type Culture Collection* CCL-98 which was performed by culture in Laboratorium Cell Culture/Tissue Laboratorium Faal, Medical Faculty of Universitas Brawijaya Malang, Central Diagnostic Lab, Saiful Anwar Public Hospital.

Choriocarcinoma Cell Culture BeWo (ATCC CCL-98)

The frozen cell line was taken from Liquid Nitrogen Vapor and subsequently thawed by immersing the cell line in a water bath at 37°C for two minutes and then decontaminated with ethanol spray 70%. Contents of the vial were inserted into a centrifuge tube containing 9ml complete culture medium (F-12K medium with 10% fetal bovine serum), centrifuged at 125x for 5-7 minutes and add 2-3ml trypsin-EDTA solution, the cell were observed using inverted microscope until the cells are separated. Adding completed culture, aliquots and culture were incubated at 37°C, then washed every two days until the cells are confluent.

ATRA Exposure Various Doses

After confluent, the cells were exposed to ATRA with six dose groups, i.e., 0µg/ml, 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml, 800µg/ml, then incubated for six hours. Each replication was done four times. Confluent cells were marked with cells attached to site attachments and touching or interconnecting between cells. The ATRA exposure by starting with 50µg, double the dose, and further multiply it according to the rule of thumb dosing multiplier to see which dose that ATRA are most effective by the resulting cell cycle graph in QTD real-time PCR.

HTERT Test

Cell or tissue granules, washed with PBS, repellet, and PBS were released slowly, the grain of tissue or cell can be stored at -80°C. Cell grains were suspended in 200µl of 1 x lysis Buffer/105-106 cells. The remaining material was placed in dry ice/ethanol and stored at -80°C.

Telomerase Activity Test Quantitative Telomerase Detection (QTD) real-time PCR

We then prepared the main mixture by mixing the reagents listed below except the mixture as the ingredients. The whole mixture together was blended and with the proper volume into the PCR tube or the thin wall of the PCR bowl. The PCR Quantification screen was displayed during PCR running and showed data that had been collected in real time with cycle threshold or CT value after the cycle was completed. The cell cycle threshold is a cycle that's statically increased significantly when Δ Rn detected the first time. Computed PCR was chosen instead of conventional one to eliminate human error. In PCR, it detects cell cycle after a particular time. The faster the cell identified the more rapid the cell cycle occurred. Furthermore, there were significant increases in time-to-detect-cell with the added ATRA, which increased in parallel with the added dose.

Data Analysis

Before the test of hypotheses was carried out, normality test was conducted by using the Shapiro-Wilk test. Assuming the sample data was not normally distributed, the data would then be tested using non-parametric analysis. The data normality test is a prerequisite in the analysis of statistical parametric approaches. ANOVA one-way test was used to test the hypotheses. The test was used to compare three or more groups of independent variables on a dependent variable with interval or ratio scale; the data must also be normally distributed. Otherwise, it must be verified by Kruskal-Wallis test; i.e., Group I: without added ATRA (all trans retinoic acid); Group II: ATRA 50µg/ml; Group III: ATRA 100µg/ml; Group IV: 200µg/ml; Group V: 400µg/ml; and Group VI: 800µg/ml.

RESULTS

RNA Quantity Measurement through Spectrometry

In this study, before the choriocarcinoma specimens were cultured in suspension form, the apecimens were divided into two large groups, i.e., samples that were exposed to ATRA, and those that were not.

Before the specimen was read with PCR, RNA quantity was measured by nanodrop technique using a device called spectrometry. Cultures – in the form of suspensions which were previously extracted into stocks – then taken from each stock of 5µl to aliquots for nanodrop. The results of nanodrop readings are shown in Table I.

Spectrometry was performed several times because there were samples in which the RNA quantity was too small, i.e., less than 1µg/µl; thus, need to find a better value. In ATRA specimens a dose of 400mL could not be performed by PCR due to very low RNA concentrations.

Measurement Result of Telomerase Activity of Choriocarcinoma with Digital PCR

Measurement of telomerase activity can be performed by quantitative PCR or qPCR method as shown in the Figure 1. The PCR curve shows that the increase of cyclical (time) was directly proportional to the administration of various doses that increased gradually.

Observation Results Cycle Time

The measurement of choriocarcinoma telomerase activity which was treated with various ATRA dosages is shown in Table III. From the table shows the data from computer that showing capture of the gradual cycle increase starting from the first non-ATRA-controlled control specimen and followed by specimens given various of ATRA dosage.

Table I: RNA Quantity Measurement using Spectrometry

Sample	RNA Concentration (µg/µL)
Control 1	0.85
Control 2	0.96
Control 3	174.05
Dose of ATRA 50 1	1.44
Dose of ATRA 50 2	0.57
Dose of ATRA 100	6.80
Dose of ATRA 200 1	1.40
Dose of ATRA 200 2	2.19
Dose of ATRA 400 1	-0.02
Dose of ATRA 400 2	-0.10
Dose of ATRA 800 1	3.71
Dose of ATRA 800 2	5.24

Table II: Cycle time of Choriocarcinoma Telomerase activity treated with ATRA

Sample	Average Threshold cycle	Experimental Cycle Threshold Changes	Control Cycle Threshold Changes	Cycle Threshold Changes	Expression of Multiple Changes Telomerase Activity
0	37.62				
50	39.51	1.89	1.08	0.81	1.75
100	41.22	1.71	1.08	0.63	1.54
200	42.65	1.43	1.08	0.35	1.27

Table III: Comparison of the Influence of ATRA on Choriocarcinoma Cell Culture BeWo (ATCC CCL-98) toward Cycle Time

Observation Group	N	Mean ± SD	p-value
Control	4	37.62±0.43[a]	<0.000
ATRA 50 µg/mL	4	39.51±0.09[b]	
ATRA 100 µg/mL	4	41.22±0.58[c]	
ATRA 200 µg/mL	4	42.65±0.55[d]	
ATRA 800 µg/mL	4	43.54±0.51[d]	

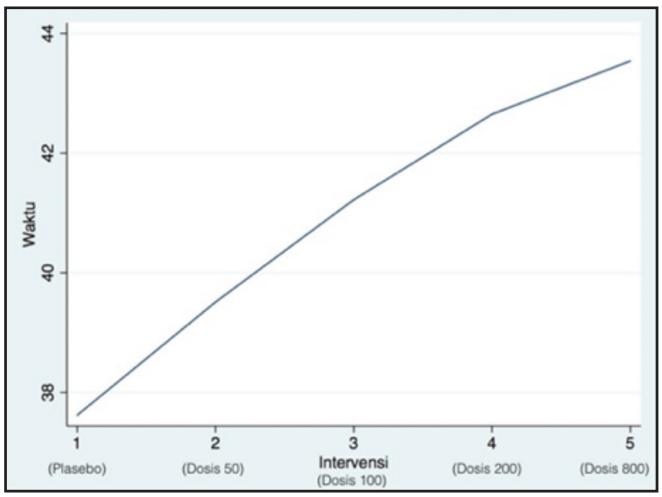

Fig. 1: Telomerase Activity of Choriocarcinoma using PCR.

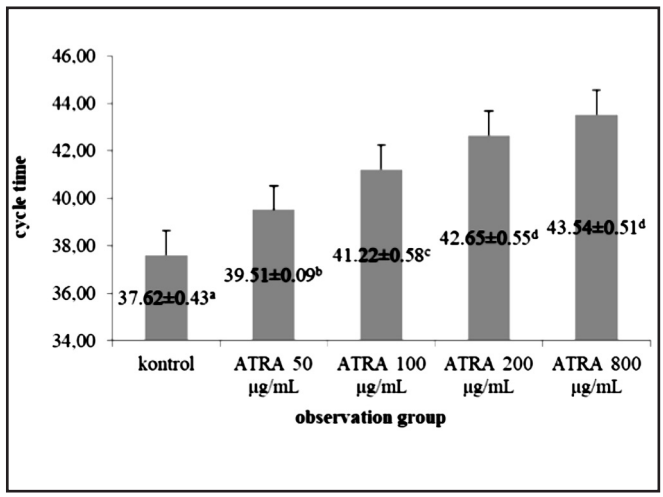

Fig. 2: Histogram of Cycle Time over Various Dosage of ATRA treatment.

Comparative Test Results between Groups
Based on one-way ANOVA test result in cycle time data obtained, there was a significant difference mean time cycle of the five groups of sample observations. This was indicated by the p-value <0.001 as shown in Table III. Furthermore, multiple comparisons with Tukey test are shown in Table II. Furthermore, the average cycle time in the five sample groups is presented in full view on the histogram (bar chart) in Figure 2.

The data from the computer showed the capture of the increasing gradual cycle, starting from the first non-ATRA-controlled control specimen which is followed by specimens that were given various variations of ATRA dosage. This suggests that an ATRA-treated choriocarcinoma cell with very high telomerase activity was captured more quickly in the machine's readout cycle. Meanwhile, specimens with given ATRA doses showed decreasing telomerase activity as indicated by larger cycle arrest along with increasing ATRA doses.

The average cycle time histogram in the control group showed ATRA dosages of 50µg/ml, 100µg/ml, 200µg/ml, and 800µg/ml. There appeared to be an increase in the mean cycle time as an ATRA dose was added to the BeWo choriocarcinoma cell culture. The highest average cycle time was found on the highest stem in the treatment group with ATRA dosage of 800µg/ml. In other words, the 200µg/ml dose could be considered the most effective dose to increase cycle time or prolong the cycle time, compared to different doses. The trend of change between the observation groups is presented in Figure 2.

Trend average cycle time change
There was an increase of trend in PCR detection time with consecutive ATRA added to the cell line. With initial dose of ATRA of 50µg/ml have significant increase of time compared with no ATRA, which further increased at doses of 100µg/ml, 200µg/ml, and 800µg/ml at its highest time an increase in the mean cycle time as the ATRA dose was added to the *BeWo Choriocarcinoma Cell* culture. Hence, the hypothesis test demonstrated that there was an effect of giving various doses of ATRA to the time of telomerase expression cycle in *BeWo Choriocarcinoma Cell* culture.

Induction of apoptosis and differentiation is critical in the administration of chemotherapy for the eradication of cancer cells, including in trophoblast disease in which the primary treatment is chemotherapy. The retinoic acid and its receptors are essential in the growth and development of the foetus which also possesses a substantial role in trophoblast invasion, implantation, and differentiation. Therefore, one type of retinoic acid All-Trans Retinoic Acid (ATRA) is expected to inhibit proliferation by adding ATRA to the effectiveness of methotrexate as a primary treatment.

Using the Shapiro-Wilk test, the data of this research followed the normal distribution (p>0.05), meaning that all samples were normally distributed.

This study successfully showed that by giving the ATRA dose of 50µg/ml, ATRA could inhibit telomerase activity by prolonging the cycle time to 39.51±0.09 cycle compared to the control group (without the active ingredient) with the value of the cycle with a value 37.62±0.43 with value p<α.Changes in time extension cycles occur consistently shown in histogram images showing higher doses (800mg/dl) indicating the longest cycle time. Changes in dosing trends are also correlated straight with the cycle time extension.

According to Andrijono et al., prolonged vitamin A deficiency would become a proliferation factor of hydatidiform mole and indubitably the form of its choriocarcinoma.[7] Andrijono also found that 73.21% of people with moles had a low retinol deposit in the liver. Andrijono et al., continued to assert that 200,000IU could be used as prevention of malignant trophoblastic disease (PTG) in which one of them was mola.[7] At a dose of 200,000, it would increase the levels of retinol in the blood and also induced apoptosis and cell regression that began to show the nature of the malignancy; hence, the mole did not turn into choriocarcinoma. This high level of ATRA dose at 200,000 did not cause side effects such as SGOT SGPT increase. This appears very interesting because ATRA had been able to reduce the incidence of malignant forms of choriocarcinoma.

According to Qin et al., and Guo et al., retinoid substances including ATRA may decrease telomerase activity by increasing cells in the G0/G1 phase than in S.[9,10] The authors also cited that the increased proportion of cells in the G0/G1 phase experienced or induced apoptosis in T cells in Leukemia.[8]

The mechanism of why apoptosis is well described in a study by Chiu et al., where CDK G1-S (CKI) which members are p16, p21 and p27 regulated by CDK 4, 6 DAN Cyclin can inhibit E2F transcription so that the cancer cells are unable to divide and over time degradation to apoptosis occur.[4]

In other words, this study has proven that ATRA could inhibit telomerase activity by lengthening the cycle. Changes in the increase of ATRA doses in this experimental test need to be studied further on experimental animals, either administered as a single agent or as an addition to standard treatment of trophoblastic disease.

CONCLUSION
By giving the ATRA dose of 50ug/ml, ATRA could inhibit telomerase activity of BeWo Choriocarcinoma Cell by prolonging the cycle time to 39.51±0.09 cycle compared to the control group (without the active ingredient) with the cycle value of 37.62±0.43

CONFLICT OF INTEREST
None.

ETHICS APPROVAL
This study has been approved by Ethical Committee of Medical Faculty Brawijaya University.

AUTHOR'S CONTRIBUTIONS

All authors contributed equally in this study.

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