Study of Apoptosis Induction of Hydatidiform Mole Trophoblastic Cell by the Administration of Retinoic Acid

ANDRIJONO
W.L. HEFFEN*

Department of Obstetrics and Gynecology
Faculty of Medicine University of Indonesia/
Dr. Cipto Mangunkusumo General Hospital
**Center for Research and Development of Dharmais Cancer Hospital*
Jakarta

Pendahuluan: Molahidatidosa merupakan kehamilan abnormal yang pada pemeriksaan histologi didapatkan proliferasi sel trofoblas. Sejumlah 80% penderita molahidatidosa akan mengalami regresi pascaevakuasi. Regresi spontan pascaevakuasi disebabkan karena sel trofoblas mempunyai aktivitas apoptosis. Sejumlah 20% penderita molahidatidosa menderita degenerasi keganasan yang secara klinis disebut PTG (Penyakit Trofoblas Ganas). Degenerasi keganasan ini mungkin disebabkan karena aktivitas proliferasi yang dominan sehingga proliferasi terjadi berkelanjutan pascaevakuasi. Mekanisme apoptosis pada molahidatidosa belum diketahui sepenuhnya. Asam retinoat yang merupakan zat aktif retinol atau vitamin A mempunyai aktivitas merangsang arest siklus sel dan merangsang apoptosis. Menarik untuk diteliti, apakah pemberian asam retinoat pada sel trofoblas molahidatidosa juga menginduksi apoptosis. Penelitian ini bertujuan membuktikan peningkatan aktivitas apoptosis pada sel trofoblas molahidatidosa yang diberikan asam retinoat. Penelitian ini memberi manfaat sebagai dasar penelitian kemoprevensi vitamin A pada molahidatidosa.

Bahan dan cara kerja: Penelitian menggunakan spesimen kultur sel trofoblas molahidatidosa. Kultur sel trofoblas diperoleh dengan mengkultur sel trofoblas yang diperoleh dari gelembung molahidatidosa. Kultur dengan media RPMI. Pada usia 24 jam, dilakukan perlakuan dengan pemberian ATRA (*all transretinoic acid*) dengan dosis 50 μg/ml, 100 μg/ml, 150 μg/ml dan 200 μg/ml. Pelarut yang digunakan adalah DMSO (*dimethyl sulfoxide*). Dilakukan analisis aktivitas apoptosis dengan *flowcytometry* pada 24 jam pascaperlakuan. Aktivitas apoptosis tergambar pada sitogram di kwadran kanan bawah, sedangkan jumlah sel hidup pada kwadran kiri bawah. Perhitungan sel dilakukan pada 1000 sel.

Hasil: Persentase apoptosis pada kontrol 60,64% sel hidup 7,09%. Persentase apoptosis pada 50 μg/ml 89,45%, 100 μg/ml sejumlah 87,23%, 150 μg/ml sejumlah 94.635 dan pada 200 μg/ml sejumlah 94,83%. Sedangkan sel hidup pada 50 μg/ml sejumlah 5,04%, pada 100 μg/ml 5,71%, pada 150 μg/ml sejumlah 3,14% dan pada 200 μg/ml sejumlah 2,66%.

Kesimpulan: Terdapat peningkatan persentase jumlah sel yang apoptosis dan penurunan sel yang hidup pada sel trofoblas yang diberikan ATRA.

[Maj Obstet Ginekol Indones 2008; 32-2: 99-104]

Kata kunci: apoptosis, molahidatidosa, sel trofoblas, asam retinoat

***Introduction**: Hydatidiform is an abnormal pregnancy which at histological examination shows the proliferation of trophoblastic cells. As high as 80% of hydatidiform mole patients would experience regression after the evacuation. Spontaneous regression after the evacuation occurred because trophoblastic cells had apoptosis activity. As high as 20% of hydatidiform mole patients suffered from malignancy degeneration which was clinically known as malignant trophoblastic disease (MTD). This malignancy degeneration may occur because of the dominant proliferation activity that took place continuously after the evacuation. Apoptosis mechanism in hydatidiform has not been completely understood. Retinoic acid which is an retinol active substance or vitamin A plays a role in stimulating the arrest of cell cycle and stimulating apoptosis. It was interesting to study on whether or not the administration of retinoic acid for hydatidiform mole trophoblastic cells would also induce apoptosis. The aim of this study was to demonstrate that the increased activity of apoptosis in hydatidiform mole trophoblastic cells receiving retinoic acid. This study would be beneficial in providing the basis for study of vitamin A chemoprevention in hydatidiform mole.*

***Material and methods**: The study made use of culture specimens of hydatidiform mole trophoblastic cells. The culture of trophoblastic cells was obtained by cultivating trophoblastic cells taken from hydatidiform mole bubbles. Culture with RPMI media. At the age of 24 hours, treatment was made with the administration ATRA (all transretinoic acid) at a dose of 50 μg/ml, 100 μg/ml, 150 μg/ml and 200 μg/ml. The solution used was DMSO (dimethyl sulfoxide). We performed analysis of apoptosis activity with flowcytometry in 24 hours after the treatment. The apoptosis activities were described in cytogram at the lower right quadrant, while the number of life cells at the lower left quadrant. The calculation of cell was done at 1000 cells.*

***Results**: The percentage of apoptosis in the control group was 60.64%, in the life cell 7.09%. The percentage of apoptosis at 50 μg/ml was 89.45%, at 100 μg/ml was 87.23%, at 150 μg/ml was 94.63%, at 200 μg/ml was 94.83%. On the other hand, the life cell at 50 μg/ml was 5.04%, at 100 μg/ml was 5.71%, at 150 μg/ml was 3.14%, and at 200 μg/ml was 2.66%.*

***Conclusion**: There was an increased percentage of cells with apoptosis and decreased percentage of life cells in trophoblastic cells receiving ATRA.*

[Indones J Obstet Gynecol 2008; 32-2: 99-104]

***Keywords**: apoptosis, hydatidiform mole, trophoblastic cells, retinoic acid*

INTRODUCTION

Hydatidiform mole is an abnormal pregnancy which at histological examination shows the proliferation of trophoblastic cells, avascular chorionic villi and hydrophic degeneration.[1] The standard management of hydatidiform mole was the evacuation of hydatidiform mole which was continued with clinical observation and the examination of hCG serum level.[2]

As high as 80% of patients underwent spontaneous regression, and another 20% experienced ma-

lignancy degeneration which was clinically known as malignant trophoblastic disease (MTD) or persistent hydatidiform mole. Regression after hydatidiform mole occurred because trophoblastic disease had the activity of apoptosis.

It was suspected that persistent hydatidiform mole or MTD occurs due to some affecting factors, such as gene factor which regulates cell proliferation, genes which regulate apoptosis and the factor of patient immunity. Cell proliferation is controlled by genes play a role as control in cell cycle.

The function of cell cycle occurs at the check point of G1 phase or S phase. The termination of cell cycle at the check point was known as G1 arrest and S phase arrest. Tumor suppressor genes (TSG) are the active genes which contribute to the arrest of cell cycle, such as p53, p21, p27, and other genes. If the function of TSG is disrupted, the control mechanism will also be disrupted. Cell cycle takes place without control. The failure of control function may be a factor responsible for the occurrence of persistent hydatidiform mole or MTD.[3-6]

The mechanism of apoptosis is a mechanism of body protection through cell suicide program. If the control function at the check point works, and the cell improvement does not occur, the cells that go through gene changes will be killed by apoptosis process, such that the mechanism of apoptosis takes place in conjunction with the control function, particularly by p53.[7,8,9]

Various genes play a role in the activity of apoptosis, the genes that stimulate apoptosis or the genes that inhibit apoptosis.

Vitamin A or retinol, with retinoic as its active metabolite play a role in the various cells. The roles of retinol or retinoic include, among other things, controlling of cell proliferation through its activity which stimulates G1 phase arrest and S phase arrest. Such mechanism occurs because retinol or retinoic has plays a part in enhancing the expression of p53, activity of p21, and suppressing cylin activity.[10]

In addition, retinoic plays a part in enhancing apoptosis in several cancerous cells. The stimulation of apoptosis by retinoic occurs through the enhancement of AP-1, stimulating caspase 7 and 9. Thus, retinol or retinoic has a suppressing effect on the growth of cancerous cells or has an impeding effect on the occurrence of cancer through two mechanisms, i.e. cell cycle and apoptosis.[10,11]

Retinol may enter the cell through the active mechanism or through retinol receptor. Inside the cell, the retinol is changed into retinoic active substance. In order to understand the role of retinoic in trophoblastic cells in the form of apoptosis activity, it is necessary to perform studies in trophoblastic cells.

The aim of this study was to understand the induction of apoptosis activity in hydatidiform mole trophoblastic cells receiving retinoic acid. This study was expected to provide benefits that vitamin A can be use as chemoprevention post hydatidiform mole malignancy.

METHODS

This study was a laboratory trial. The samples used were the culture of hydatidiform mole. The process of trophoblastic cell culture was performed independently in laboratories. The method of culture was cell culture with RPMI media, with the administration of growth factor, antibiotics and anti fungus. The selection of culture specimen and culture method was done by try out, because the culture of trophoblastic cells has not been reported yet. We conducted the treatment by administering ATRA (all transretinoic acid) in the growing cell culture. The doses of ATRA adminsitration were 50 μg/ml, 100 μg/ml, 150 μg/ml and 200 μg/ml. The evaluation of apoptosis activity was made by antibody Anexin V, and the examination of apoptosis made use of flowcytometry. The percentage of cell number undergoing apoptosis was at the lower right quadrant, while the percentage of life cell number was at the lower left quadrant. The study was conducted with the permission from Ethical Committee of the Faculty of Medicine University of Indonesia.

RESULTS

Isolation/culture of hydatidiform mole trophoblastic cells

The culture of trophoblastic cells was performed through various trials. After 7 trials, we obtained the method of sample collection and the appropriate processing of specimens. The samples were the fluid bubbles of hydatidiform mole. The fluid bubbles of hydatidiform mole were taken by injection needle.

The fluid obtained was injected into the media (RPMI). Later, we initiated the following staging: Mole cell was obtained through curettage evacuation. Specimens were taken from the mole bubbles with disposable syringe. The fluid bubbles were injected into the tubes containing RPMI media of 10% (FBS). Mole cells were washed two times with

PBS. Culture for 24 hours at 37˚C in 5% CO_2 incubator. After 24 hours, the cells appeared to proliferate, the medium was disposed from tissue culture flask and washed with PBS 2 times of 10 ml. ATRA was administered at doses of 50 μg/ml, 100 μg/ml, 150 μg/ml, 200 μg/ml within the well. Incubation for 24 hours in CO_2 incubator. Washed with cold PBS and centrifugation. Add 1 ml of the medium and calculate the cell number reaching 4-6 x 10^7/ml with hematocytometry. Cells were ready to be analyzed with flow cytometry

Evaluation

The activity of apoptosis was examined using antibody Annexin-V. Annexin-V would bind phosphaditilserin which was the residue of apoptosis cells on the surface of cell membrane.

The hCG examination was performed to demonstrate that the cells cultured were trophoblastic cells, and the results of beta hCG examination of culture media positively contained hCG. We performed repeat culture up to day 10. Analysis of the medium on day 10 showed that it still contained hCG.

Examination of apoptosis activity (flowcytometry)

Identification of apoptosis was performed in 24 hours after treatment. The examination of apoptosis was made with the administration of Anexin-V (*Annexin-V-fluorescein isothiocyanate/FITC*). The cells that underwent apoptosis were calculated with flowcytometry. The examination was performed in 1000 cells. The results of examination (cytogram) were presented at 4 quadrants. The examination of apoptosis activity with flowcytometry constituted the better examination than any other methods.[12,13]

The cells that underwent apoptosis were presented at the lower right quadrant, while the life cells at the lower left quadrant.[12,13]

Cytogram of control group and ATRA group

In the control (DMSO) of trophoblastic cell culture, we obtained 7.09% of life cells and 60.64% of the cells experienced apoptosis.

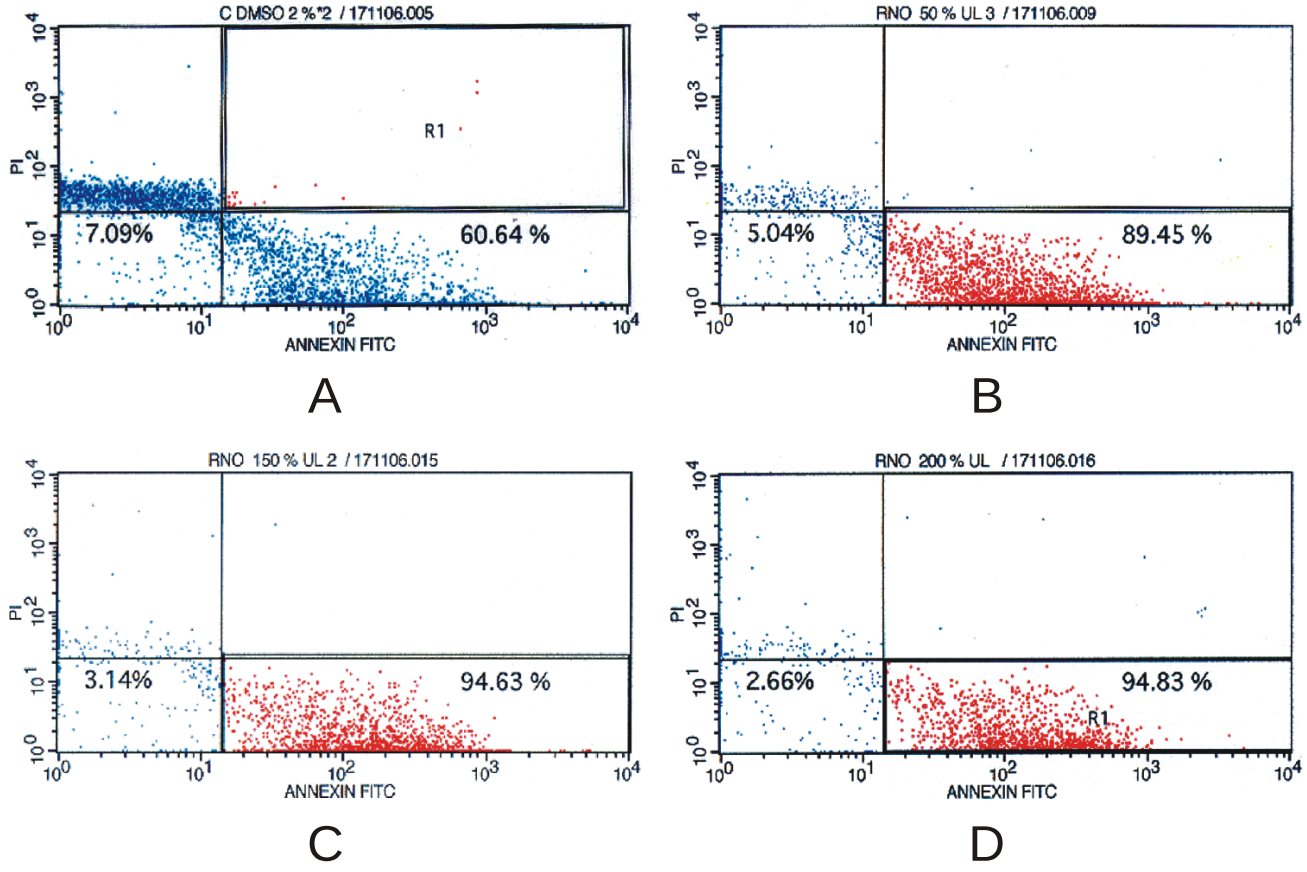

Figure 1. The results of flowcytometry cytogram examination (control)
Note: A: control group, B: ATRA 50 μg/ml, C: ATRA 150 μg/ml and D: ATRA 200 μg/ml

The life cells and the cells underwent apoptosis in the culture of trophoblastic cells receiving retinoic of 50 μg/ml, 100 50 μg/ml, 150 μg/ml, 200 μg/ml were show at table 1.

Table 1. Percentage of cell life and apoptosis

	CELL LIFE (%)	APOPTOSIS (%)
CONTROL	7.09	60.64
50 μg ATRA	5.04	89.45
100 μg ATRA	5.71	87.23
150 μg ATRA	3.14	94.63
200 μg ATRA	2.66	94.83

DISCUSSION

Signal of apoptosis resulting from the complex of retinoic-receptor acid

With the presence of RBP receptor in the trophoblastic cells (cytotrophoblast, syncytiotrophoblast, intermediate trophoblast) hydatidiform mole, it means that the retinol could enter the trophoblastic cells.

The entry of retinol into trophoblastic cells would be continued with the metabolism of retinol into retinoic acid. Retinoic acid would enter the cell nucleus, and bind to retinoic receptor at the cell nucleus. The complex of retinoic-receptor acid would produce signals (see Figure 2).

The binding of retinoic-receptor complex would result in various signals, such as the signal of cell cycle arrest at G_1 phase and S phase, and apoptosis signal.[10,11]

In our study, the administration of retinoic to the culture of trophoblastic cells caused an increase in the number of hydatidiform mole trophoblastic cells which underwent apoptosis.

In controls, in the observation after 24 hours, 7.09% of the cells were found to survive and 60.64% underwent apoptosis. This condition showed that the activity of apoptosis in the trophoblastic cells was reasonably high. This circumstance was consistent with the clinical condition, because the majority of hydatidiform mole cases experienced spontaneous regression. The spontaneous regression of hydatidiform mole was caused by the activity of apoptosis in trophoblastic cells.

The administration of retinoic to the culture of trophoblastic cells caused an increase in the number of cell undergoing apoptosis. The increase in percentage of apoptosis occurred with the increase in retinoic doses. With the administration of retinoic at a dose of 50 μg/ml, 5.04% of the life cells were found, and the number of cells undergoing apoptosis was 89.45%. On the other hand, with the administration of retinoic at a dose of 200 μg/ml, 3.18% of the life cells were found, and the number of trophoblastic cells undergoing apoptosis reached 93.81%. This finding showed that the administration of retinoic acid increased the activity of apoptosis in trophoblastic cells, and the percentage of apoptosis increased with the increase in the doses of retinoic administration.

The mechanism of apoptosis by retinoic acid occurred through the increase of TNF (tumor necrosing factor) expression, inhibition of Bcl-2, and the expression induction of p53. The complex of retinoic-receptor acid produced signals which directly stimulated *caspase* 9, and indirectly stimulated *caspase* 7.[14,15] Retinoic acid also induced apoptosis through *Apaf1* (*apoptotic protease activating factor 1*). Acid retinoic works as antagonist of transcription factor, activator protein-1 (*AP*-1) which played a role in the cell growth and cell differentiation.[15]

Retinoic acid causes the occurrence of G_1 phase arrest, the termination of that cycle occurred because of the decrease in G_1 cyclin protein, E cyclin, and also prevents the phosphorilation of *p*Rb. Thus, generally retinoic acid would cause G_1 arrest.[16]

Arrest at G_1 phase by retinoic acid could occur through several mechanisms, and one of the mechanisms taking place was the activation of *p*53 by retinoic acid. The increase of *p*53 activation caused the occurrence of arrest at G_1 phase. This mechanism apparently constituted a direct mechanism.[10,11]

Arrest at S phase occurred because there was an inhibition of E2F phosphorilation, expression of cyclin A could be suppressed and the complex of cyclin-cdk2 could not be formed. Therefore, the arrest at S phase could occur through two mechanisms, i.e. through *p*21 and through the inhibition of E2F phosphorilation.[10,11]

Retinoic acid also activated *p*53 and increased *p*53 level. The increase of non-mutant *p*53 would cause the activation of control mechanism of cell cycle. Gene *p*53 caused the occurrence of G_1 arrest, the termination of cell cycle at G_1 phase would make it possible for the cell to be repaired. In addition, *p*53 played a role in activating apoptosis mechanism (see Figure 2).[12,13]

The discovery of RPB (retinol binding protein) receptor at the outer cell membrane, the inner cell membrane and the cytoplasm of trophoblastic cells signified that retinol could go into the cytoplasm of trophoblastic cells.

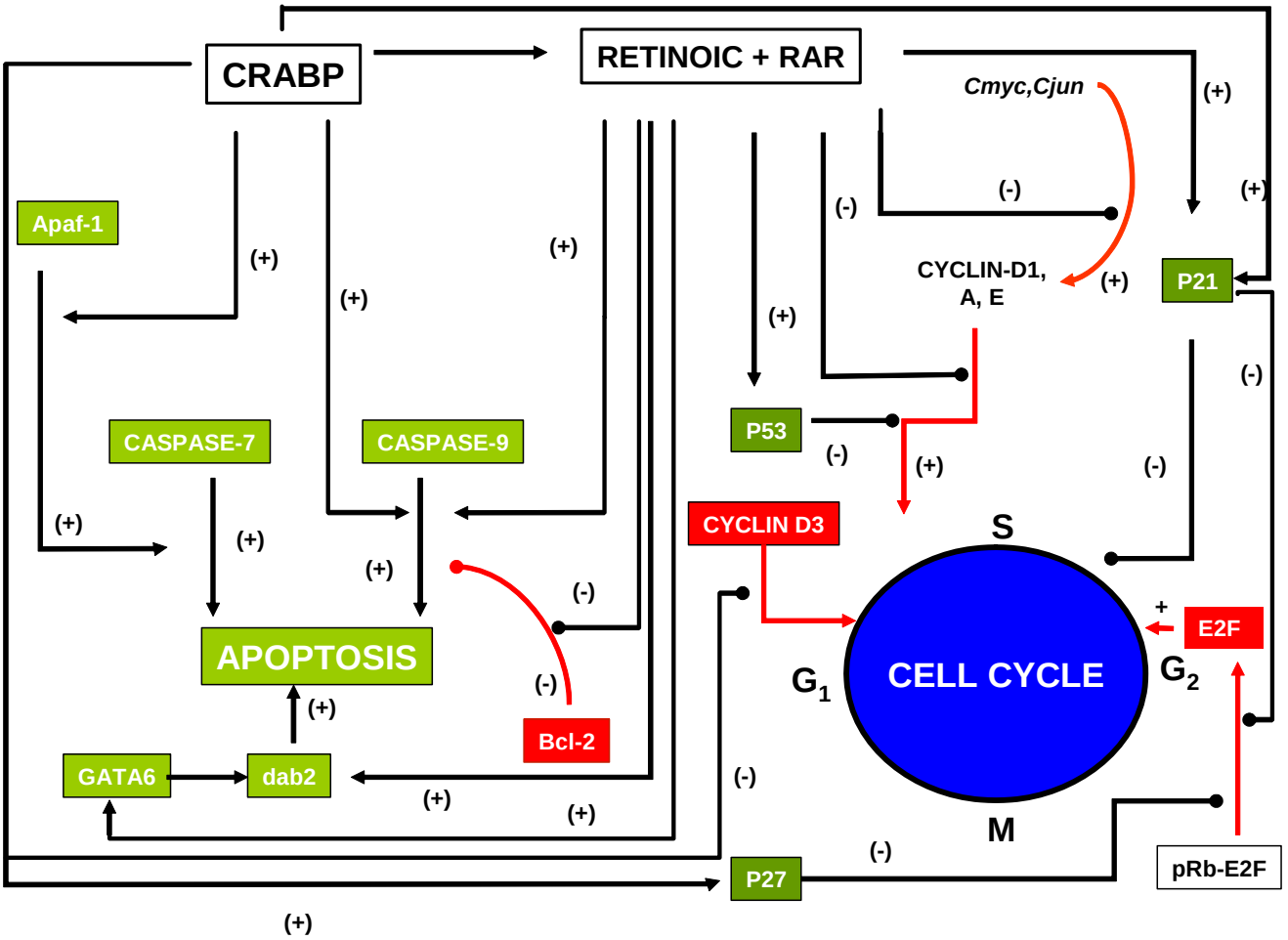

Figure 2. Signals of retinoic-receptor complex
Note: Retinoic inhibits cell cycle through stimulation of p53 and p21. Retinoic stimulates apoptosis through caspase stimulation.

Retinol inside the cytoplasm would be metabolized into retinoic acid, and the retinoic-receptor complex inside the cell nucleus would produce apoptosis signals and proliferation inhibition (see Figure 2).

In the study on the expression of retinoic-receptor in trophoblastic cells, it was demonstrated that trophoblastic cells had retinoic receptor. The presence of retinoic receptor in trophoblastic cells signified that retinoic could penetrate into hydatidiform mole trophoblastic cells.

The study on the activation of retinoic in trophoblastic cells showed that retinoic acid could induce the activity of apoptosis in trophoblastic cells.

In order to demonstrate the effects of vitamin A in hydatidiform mole trophoblastic cells as the effort to prevent malignancy degeneration following hydatidiform mole, it is necessary to conduct further study with clinical trials.

CONCLUSIONS

The administration of retinoic acid in trophoblastic cells increased the activity of apoptosis in trophoblastic cells.

REFERENCES

1. Tham KF, Ratnam SS. Current views on the management of trophoblastic tumors. Int J Gynecol Obstet 1995; 49: 77-89
2. Sasaki S. Clinical presentation and management of molar pregnancy. Best Practice and Research Clinical Obstetrics and Gynaecology 2003; 17(6): 885-92
3. MacDonald F, Ford CHJ, Casson AG. Molecular Biology of Cancer. Second edition, Garland Science/Bios Scientific Publishers, 2004: 2-4
4. MacDonald F, Ford CHJ, Casson AG. Molecular Biology of Cancer, second edition, Garland Science/BIOS Scientific Publisher, 2004: 61-72

5. Zornig M, Baum W, Hueber AO, Evan G. Programmed Cell Death and Senescence, in The Molecular Basis of Cancer, 2nd edition, Philadelphia, WB Saunders company, 2001: 19-40
6. MacDonald F, Ford CHJ, Casson AG. Molecular Biology of Cancer, 2nd edition, Garland Science/BIOS Scientific Publisher, 2004: 4-6
7. Tsao AS, Kim ES, Hong Wk. Chemoprevention of Cancer. CA Cancer J Clin 2004; 54: 150-80
8. Dragnev KH, Rigas JR, Dmitrovsky E. The Retinoids and Cancer Prevention Mechanisms. The Oncologist 2000; 5: 361-8
9. Greenwald P. Cancer chemoprevention. B Med J 2002; 324: 714-8
10. Budhu AS, Noy N. Direct Chanelling of Retinoic Acid between Cellular retinoic Acid-Binding Protein II and Retinoic Acid receptor Sensitizes Mammary Carcinoma Cells to Retinoic Acid-Induced Growth Arrest. Mol and Cell Biology 2002; 22: 2632-41
11. Zhang Y, Rishi AK, Dawson MI, Tschang R, Farhana L. S-phase Arrest and Apoptosis Induced in Normal Mammary Epithelial Cells by a Novel Retinoid. Cancer Res 2000; 60: 2025-32
12. Kravtsov VD, Daniel TO, Koury MJ. Comparative Analysis of Different Methodological Approaches to the in Vitro Study of Drug-Induced Apoptosis. Am J Pathol. 1999; 155: 1327-39
13. Yasuhara S, Zhu Y, Matsui T, Tipirneni N, Yasuhara Y, Kaneki M, Rosenzweig A, Martyn JAJ. Comparison of Comet Assay, Electron Microscopy, and Flow Cytometry for Detection of Apoptosis. J Histochem Cytochem 2003; 51: 873-85
14. Farias EF, Ong DE, Ghyselinck Nb, Nakajo S, Kuppumbatti Ys, Lopez RM. Cellular Retinol-Binding Protein I, a Regulator of Breast Epithelial Retinoic Acid Receptor Activity, Cell Differentiation, and Tumorigenicity. J J Nat Can Inst 2005; 97: 21-9
15. Donato LJ, Noy N. Suppression of mammary Carcinoma Growth by Retinoic Acid: Proapoptotic Genes Are Targets for Retinoic Acid Receptor and Cellular Retinoic Acid-Binding Protein II Signaling. Cancer Res 2005; 65: 8193-9