The effect of ethynyl estradiol and desogestrel on proliferation and apoptosis hydatidiform mole trophoblast cell

Irawan Sastradinata, Andrijono, Wan Lelly Heffen, Bambang Sutrisna

Division of Oncology Gynaecology, Department of Obstetrics and Gynaecology, Faculty of Medicine, Universitas Sriwijaya Palembang, Indonesia.
Division of Oncology Gynaecology, Department of Obstetrics and Gynaecology, Faculty of Medicine, Universitas Indonesia Jakarta, Indonesia
Dharmais Cancer Centre Hospital, Jakarta, Indonesia
Faculty of Community Medicine, Universitas Indonesia, Jakarta, Indonesia

E-mail: wansastra@yahoo.com

Abstract. This study purpose to explore the effect of ethynyl estradiol and desogestrel on proliferation and apoptosis hydatidiform mole trophoblast cell. Hydatidiform mole tissue, trophoblast cells were isolated and culture at RPMI 20% FBS medium. Trophoblast cells divided into three groups observation. First group get ethynyl estradiol 10 nmol/mL, second group get desogestrel 100 nmol/mL, third group get DMSO 1%. Cells incubated and observe at 24, 48, 72, 96 hours. Cell cycle, apoptosis and βHCG was evaluated. The group of cells that get ethynyl estradiol in concentration 10 nmol/mL had cell proliferation index, amount cells and βHCG level higher than control after 72 hours observation. The group of cells that get desogestrel in concentration 100 nmol/mL have cell proliferation index, amount cells and βHCG level lower than control after 48 hours observation. There are no differences of apoptosis between the two group and control. Conclusion: Ethynyl estradiol will increase proliferation of hydatidiform mole trophoblast cell, while desogestrel will decrease proliferation of hydatidiform mole trophoblast cell. There is no effect of ethynyl estradiol and desogestrel on apoptosis of hydatidiform mole trophoblast cell.

1. Introduction

Hydatidiform Mole is an abnormal pregnancy that is characterized by proliferation of trophoblast cell, avascularity and hydropic degeneration of the chorionic villi [1]. The incidence rates of Molar pregnancies (hydatidiform mole) is still high in Asia. In Indonesia, it’s occurred about 1 out of every 47 (1/47) deliveries until 1 out of every 427 (1/427) deliveries. Molar pregnancies can become cancerous recognized as Malignant Trophoblastic Disease (MTD). The incidence rates of MTD between 15% and 28% [2,3,4]. Factors that contribute to the malignancy post hydatidiform mole are: DNA abnormalities, oncogenes, tumour suppressor genes, nutrition and hormonal status, including steroid hormones such as estrogen and progesterone [5].

The use of hormonal contraception in Indonesia is still high, no exception in post-molar pregnancy. The recommendation for use oral contraception for trophoblast patients is only for the patient who has normal β-HCG level on laboratory examination [6,7,8].

Oral contraception (OC) can be a risk factor but also be a protective factor for some Gynaecology cancer. OC are the risk factor for cervical cancer and breast cancer, but OC are the protective factor for ovarian cancer and endometrial cancer. The protective effect of course because of OC was contain progesterone hormone [9].
Estrogen hormone shown an important role for the carcinogenesis in breast cancer, endometrial cancer, liver cancer and some other cancers. This is because estrogen through estrogen receptor pathway may increase the activity of cell proliferation. In addition, some carcinogetic metabolites product of estrogen alone can cause DNA abnormalities. Estrogen can also decrease apoptosis and it will be additional risk for develop cancer. The role of progesterone in carcinogenesis has some different activity. Progesterone can induce proliferation and also inhibit proliferation depending on the type of organs and cell types.

![Figure 1. Factors in carcinogenesis GTN.](image)

It’s well known that apoptosis and proliferation are two things that play a role in carcinogenesis. The balancing of both effect on proliferation and apoptosis are important on carcinogenesis of gynaecologic patients [10-14].

One of the questions that until now still discussed are; how the risk of cancer for post molar patient who is using hormonal contraception. The absence of both in vitro and in vivo study on the effects of estrogen and progesterone on hydatidiform mole trophoblast cells is the background of this study. This study will try to know the role of ethiny estradiol and desogestrel in proliferation and apoptosis hydatidiform mole trophoblast cells.

**2. Methods**

This is an experimental study that examine the proliferation and apoptosis effect of ethiny estradiol and desogestrel on hydatidiform mole trophoblast cells. From April 2008 until March 2009 we collected 15 samples hydatidiform mole tissue from curettage and hysterectomy. Trophoblast cells was isolated from molar tissue and then cultured at medium RPMI supplemented with 20% foetal bovine serum (FBS), in 5% CO2 at 37oC. Only 7 samples (46%) shown good growth. The quality of trophoblast cells that indentified use Papanicolaou staining and cytology examination. Cytotrophoblast and sincitio trophoblast was able to be identified from good quality of trophoblast cells culture. The level β-HCG of medium culture supernatant also measured. Trophoblast cells culture produce β-HCG and was able to be measured. The Number of cells also measured. Cells amount more than 1x105/mL are good quality. Trophoblast cells culture good quality will include in experiment.

Ethiny estradiol and desogestrel was obtained from PT Sydna Pharma (Organon Indonesia), as raw material for Marvelon oral pill contraception.

The treatment given to the samples and the control as follow:

1. Preparation wells
   - Well 1 (A,B,C,D) ethynyl estradiol group
   - Well 2 (A,B,C,D) desogestrel group
   - Well 3 (A,B,C,D) control group
2. Treatment
- Well 1 (A,B,C,D) get treatment ethinyl estradiol 10 nmol/mL
- Well 2 (A,B,C,D) get treatment desogestrel 100 nmol/mL
- Well 3 (A,B,C,D) get treatment DMSO 1%

3. Each wells (A) incubate for 24 hours, each wells (B) incubate for 48 hours, each wells (C) incubate for 72 hours and each wells (D) incubate for 96 hours.

4. Number of cells was calculated. The pellet of cells was diluted with a solution tripane blue, only live cell can be seen and counted. cell then count using Haemocytometer. The cells concentration more than 1x10⁶/mL prepared for analysis.

5. Each culture cells were done examination as follow:
   - The number of cells
   - β HCG supernatant examination
   - Propidium iodide staining and cell cycle examination using flow cytometer
   - FITC-Annexin V staining and apoptosis examination using flow cytometer.

6. The β-HCG examination using Abbott AxSym total β-HCG reagent pack [15].

7. Cell cycle examination using BD Cycletest™ Plus DNA Reagent Kit. Cells were washed and staining according to the manufacturer’s protocol. Reading with FACSCalibur flow cytometer and verity soft ware ModfitLt 3.0, ModfitLt 3.2 [16].

8. Examine apoptosis by use the Annexin V-FITC apoptosis detection kit (BD Pharmigen, San Diego, CA, USA). Cells were washed and staining Annexin V-FITC and propidium iodide (PI) according to the manufacturer’s protocol. reading with FACSCalibur flowcytometer and software Cell Quest 5.1.

3. Results
The number of cells was counted as a routine procedure prior flowcytometer examination. using solution tripane blue, only live cell can be counted.

In the ethynyl estradiol group, the number of cells more than control group in 72 hours to 96 hours observation, while desogestrel group less than control in 24 hours to 96 hours examination. It’s shown that ethynyl estradiol will increased cell division and it’s significantly evident since 72 hours cells was incubated and also shown that desogestrel will inhibited cell division since 24 hours cells was incubated.

Cell cycle analysis at flow cytometry will give the information about percentage of cells on each phase of cell division. Specifically, group cells in G0/G1 phase, group cell in S phase, and group cell in G2/M phase.
Figure 3. The number of trophoblast cells.

Proliferation index calculated base on formula: Proliferation index (PI) = (G2/M + S) ÷ (G0/G1 + S + G2/M) x 100% [17].

The proliferation index of trophoblast cells as follow.

Figure 4. Proliferation Index of trophoblast cells.

The proliferation index of ethynyl estradiol group is higher than the control group in 48 hours to 96 hours observation, while in the group of desogestrel the proliferation index lower than the control group in 48 hours to 96 hours observation. It’s shown that ethynyl estradiol will increased cell proliferation and it’s significantly evident since 48 hours cells was incubated and also shown that desogestrel significantly will inhibited cell proliferation since 48 hours cells was incubated.

Figure 5. β-HCG Level of trophoblast cells.

The level of β-HCG examined with microparticle enzyme immunoassay using monoclonal/ polyclonal anti β-HCG reagent Abbott Axsym. The result of β-HCG level in each group as follow.

The ethynyl estradiol group shown β-HCG supernatant higher than control in 72 hours to 96 hours observation. In contrast with desogestrel group that β-HCG level less than control in 24 hours to 96 hours observation.
On examination apoptosis that stain use FITC-Annexin V, reading by using FACSCalibur flowcytometer, we get the percentage of cells that are in the early phase of apoptosis, percentage of cell in advance phase of apoptosis and early necrosis. The result of apoptosis as follow.

In the group ethynyl estradiol apoptosis lower than control at 72 hours and apoptosis higher than control in 24, 48, 96 hours of observation. In the group desogestrel the apoptosis lower than control in 72 hours and 96 hours, while apoptosis higher than control at 24 hours and 48 hours observation.

The result as mention above cannot give consistent result of the effect of ethynyl estradiol and desogestrel on apoptosis of trophoblast cells, so in this study we didn’t find any effect of ethynyl estradiol and desogestrel on apoptosis of trophoblast cells.

4. Discussions
The effect of estradiol on carcinogenesis as well as the influence of estradiol on cell proliferation has been demonstrated in some organ cells. The effect of estradiol effect will increase the activity of cell proliferation, but the effect of estradiol and progesterone on proliferation and apoptosis hydatidiform mole cells is not a simple mechanism and the only one factor, but also influenced by other factors such as growth factors and the presence or absence of estrogen or progesterone receptors on these cells. This study is limited to explore hormonal factors of ethynyl estradiol and desogestrel. This study didn’t prove the role of growth factors and receptors on trophoblast cells. This study is expected to give information about the effect of ethynyl estradiol and desogestrel on hydatidiform mole trophoblast cells. Preliminary data and these procedure experiences are expected to be used for future research. The result of this study proved that ethynyl estradiol will increase hydatidiform mole trophoblast cells proliferation. These results were consistent with previous studies done on breast cells, endometrial cells and uterine leiomyoma cells.

Yager JD and Zivadinovic D in different research have proven that estradiol will increase the proliferation of cell lines MCF-7 breast cancer. The mechanism of estradiol may increase cell proliferation as follows. Estradiol will affect cell proliferation through both estrogen receptor in the cell membrane, in the cytoplasm of cells as well as in the cell nucleus. Furthermore estradiol-receptor complex in the cytoplasm of cells will activate the gene in mitochondria and estradiol receptor complex in the cell nucleus to activate genes through the cell nucleus estrogen receptor element (ERE) found in the gene. Estradiol-receptor complexes in the cell membrane will pass the signal through two transduction signal pathways: (a) adenyl cyclase which increase cAMP through the protein kinase-A. (b) transduction signal pathways passed through Ras, Raf, MEK ½, ERK ½. Furthermore, estradiol through (mitogen –activated protein kinase) and (phosphatidylinositol-3 kinase) will increase the proliferation and apoptosis cells [17,18,19].

The result of this study found that desogestrel will inhibit proliferation of hydatidiform mole trophoblast cells. These result are consistent with research conducted by Tong W, which has proven that progesterone inhibits uterine epithelial cells proliferation [20].

The mechanism of progesterone that inhibit cell proliferation is as follows. Progesterone inhibits the estrogen activity in inducing Cyclin D1 and CDK4 in the nucleus of cells. Progesterone will also activate the Cyclin E-CDK2 kinase and Cycline A. As it known that Cyclin D1, CDK4, Cyclin E, Cyclin A-CDK2 play a role in the cell cycle when the cell cycle G1 phase and will enter phase S.
Progesterone in this case would inhibit the cell cycle at G1 phase in which will enter S phase. Research on endometrial cells proved that progesterone would inhibit endometrial cell proliferation via insulin growth factor (IGF). High progesterone will increase IGF-binding protein 1 (IGFBP1), furthermore will decrease concentration IGF1. As it known IGF1 through IGF-1R can activate the RAS/RAF which will induce cell proliferation through PI3K [21].

This research is still cannot conclude about effect of ethynyl estradiol and desogestrel on trophoblast cell apoptosis. Yager JD has proved that estradiol will decrease the apoptosis of breast cancer cell, and Liu J also proved that progesterone will decrease the apoptosis of uterine leiomyoma cells. Progesterone decrease the activity of cells apoptosis through progesterone receptors will interact with Bcl-2 gen promoter that will induce the expression of Bcl-2. As we known Bcl-2 is an anti-apoptotic gene that would inhibit the activity of apoptosis [22]. Receptor progesterone of trophoblast cells will inhibit apoptosis through down-regulation of the expression of Fas, Fas-L, caspase-8, caspase-3 and PARP [23,24].

This study cannot prove the effect of ethynyl estradiol and desogestrel on apoptosis hydatidiform mole trophoblast cells, this is a weakness of this study, of course it’s needed another next study with better design research. This study clearly proves about the influence of ethynyl estradiol on proliferation of hydatidiform mole trophoblast cells. That prove from both cell proliferation and also cell produce β-HCG. This study clearly proves about the anti-proliferative effect of desogestrel on hydatidiform mole trophoblast cells. That prove from both lower proliferation and also lower producing β-HCG.

There are two types oral pills are currently available, “Combined oral contraceptive” that contain hormones estrogen and progesterone and “minipill” that contain only progestin. By looking at the result of this research, the post molar pregnancy ladies can take minipill or get injection that contain only progesterone and should not be given combined oral pill or combined injection contraception to prevent malignant transformation become Gestational Trophoblastic Neoplasia.

Desogestrel is just one of many synthetic progesterone, we need new research on other types of progesterone such as levonorgestrel. We also need another design research that use combination both estrogen and progesterone in medium culture cells to know the balancing effect of both hormones in carcinogenesis. Hopefully, this research can provide benefits in the future.

5. Conclusions
Ethynyl estradiol at concentration 10 nmol/mL will increase proliferation of hydatidiform mole trophoblast cell that characterized by increase of: cell proliferation index, number of cells and cell producing β-HCG. Desogestrel at concentration 100 nmol/mL decrease proliferation of hydatidiform mole trophoblast cells. There is no effect of ethynyl estradiol and desogestrel on apoptosis of hydatidiform mole trophoblast cells.

6. References
[1] Andrijono 2007 Gestational Trophoblastic Neoplasia (Universitas Indonesia)
[2] Andrijono A and Muhilal M 2010 Prevention of post-mole malignant trophoblastic disease with vitamin A Asian Pac J Cancer Prev 11 567–70
[3] Steigrad S J 2003 Epidemiology of gestational trophoblastic diseases Best Pract. Res. Clin. Obstet. Gynaecol. 17 837–47
[4] Soper J T, Mutch D G, Schink J C and Gynecologists A C of O and 2004 Diagnosis and treatment of gestational trophoblastic disease: ACOG Practice Bulletin No. 53 Gynecol. Oncol. 93 575–85
[5] Ngan H Y S, Chan K K L and Tam K-F 2006 Gestational trophoblastic disease Curr. Obstet. Gynaecol. 16 93–9
[6] Parazzini F, Cipriani S, Mangili G, Garavaglia E, Guarnerio P, Ricci E, Benzi G, Salerio B, Polverino G and La Vecchia C 2002 Oral contraceptives and risk of gestational trophoblastic disease Contraception 65 425–7
[7] Palmer J R, Driscoll S G, Rosenberg L, Berkowitz R S, Lurain J R, Soper J, Twigg L B, Gershenson D M, Kohorn E I and Berman M 1999 Oral contraceptive use and risk of gestational trophoblastic tumors J. Natl. Cancer Inst. 91 635–40
[8] Costa H L F F and Doyle P 2006 Influence of oral contraceptives in the development of post-
molar trophoblastic neoplasia—a systematic review Gynecol. Oncol. 100 579–85

[9] Cibula D, Gompel A, Mueck A O, La Vecchia C, Hannaford P C, Skouby S O, Zikan M and Dusek L 2010 Hormonal contraception and risk of cancer Hum. Reprod. Update 16 631–50

[10] Yager J D 2000 Chapter 3: endogenous estrogens as carcinogens through metabolic activation JNCI Monogr. 2000 67–73

[11] Inoue A, Seino Y, Terasaka S, Hayashi S, Yamori T, Tanji M and Kiyama R 2007 Comparative profiling of the gene expression for estrogen responsiveness in cultured human cell lines Toxicol. Vitr. 21 741–52

[12] Suga S, Kato K, Ohgami T, Yamayoshi A, Adachi S, Asanoma K, Yamaguchi S, Arima T, Kinoshita K and Wake N 2007 An inhibitory effect on cell proliferation by blockage of the MAPK/estrogen receptor/MDM2 signal pathway in gynecologic cancer Gynecol. Oncol. 105 341–50

[13] Márquez-Garbán D C, Chen H-W, Fishbein M C, Goodglick L and Pietras R J 2007 Estrogen receptor signaling pathways in human non-small cell lung cancer Steroids 72 135–43

[14] Attalla H, Westberg J A, Andersson L C, Adlercreutz H and Mäkelä T P 1998 2-Methoxyestradiol-induced phosphorylation of Bel-2: uncoupling from JNK/SAPK activation Biochem. Biophys. Res. Commun. 247 616–9

[15] Birken S, Berger P, Bidart J-M, Weber M, Bristow A, Norman R, Sturgeon C and Stenman U-H 2003 Preparation and characterization of new WHO reference reagents for human chorionic gonadotropin and metabolites Clin. Chem. 49 144–54

[16] Tuschi H and Schwab C E 2005 The use of flow cytometric methods in acute and long-term in vitro testing Toxicol. Vitr. 19 845–52

[17] Yager J D and Davidson N E 2006 Estrogen carcinogenesis in breast cancer N. Engl. J. Med. 354 270–82

[18] Zivadinovic D, Gametchu B and Watson C S 2004 Membrane estrogen receptor-α levels in MCF-7 breast cancer cells predict cAMP and proliferation responses Breast Cancer Res. 7 R101

[19] Wang Q, Li X, Wang L, Feng Y-H, Zeng R and Gorodeski G 2004 Antiapoptotic effects of estrogen in normal and cancer human cervical epithelial cells Endocrinology 145 5568–79

[20] Tong W and Pollard J W 1999 Progesterone inhibits estrogen-induced cyclin D1 and cdk4 nuclear translocation, cyclin E- and cyclin A-cdk2 kinase activation, and cell proliferation in uterine epithelial cells in mice Mol. Cell. Biol. 19 2251–64

[21] Frost R A, Mazella J and Tseng L 1993 Insulin-like growth factor binding protein-1 inhibits the mitogenic effect of insulin-like growth factors and progestins in human endometrial stromal cells Biol. Reprod. 49 104–11

[22] Yin P, Lin Z, Cheng Y-H, Marsh E E, Utsunomiya H, Ishikawa H, Xue Q, Reierstad S, Innes J and Thung S 2007 Progesterone receptor regulates Bel-2 gene expression through direct binding to its promoter region in uterine leiomyoma cells J. Clin. Endocrinol. Metab. 92 4459–66

[23] Maruo T, Matsuo H, Shimomura Y, Kurachi O, Gao Z, Nakago S, Yamada T, Chen W and Wang J 2003 Effects of progesterone on growth factor expression in human uterine leiomyosarcoma Steroids 68 817–24

[24] Liu J, Matsuo H, Laoag-Fernandez J B, Xu Q and Maruo T 2007 The effects of progesterone on apoptosis in the human trophoblast-derived HTR-8/SV neo cells Mol. Hum. Reprod. 13 869–74

Acknowledgments
We thank you for all staff at Laboratory of cell biology, Dharmais Cancer Center Hospital Jakarta Indonesia.