

## Ultrastructural Changes of Corpus Luteum after Ovarian Stimulation at Implantation Period

Mandana Beigi Boroujeni<sup>\*1</sup>, Nasim Beigi Boroujeni<sup>2</sup>, Mojdeh Salehnia<sup>3</sup>, Elahe Marandi<sup>1</sup>  
and Masoud Beigi Boroujeni<sup>4</sup>

<sup>1</sup>Dept. of Anatomical Sciences, School of Medicine, Lorestan University of Medical Sciences, Khorramabad; <sup>2</sup>Dept. of Clinical Sciences, School of Veterinary Medicine, Shahid Bahonar University of Kerman, Kerman; <sup>3</sup>Dept. of Anatomical Sciences, School of Medicine, Tarbiat Modares University, Tehran; <sup>4</sup>Dept. of Biology, School of Basic Sciences, Payame Noor University of Tehran, Tehran, Iran

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### ABSTRACT

**Background:** To achieve multiple oocytes for *in vitro* fertilization, ovulation induction is induced by gonadotropins; however, it has several effects on oocytes and embryo quality and endometrium receptivity. The aim of this study was to assess ultrastructural changes of corpus luteum after ovarian induction using human menopausal gonadotropin (HMG) and human chorionic gonadotropin (HCG) during luteal phase at implantation period. **Methods:** Female NMRI mice (6-8 weeks) were divided into control and stimulated groups. In the control group, the mice were rendered pseudopregnant and in the ovarian induction group, the mice were rendered pseudopregnant after the ovarian induction. The samples were obtained from the ovary in each group at the same time during luteal phase at implantation period. Ultrastructural changes were assessed using electron microscopy study. **Results:** Our results displayed some identifiable changes in ultrastructure of corpus luteum in ovarian induction group. These changes included enhancement of the apoptosis and intercellular space, whereas the angiogenesis was decreased. The findings indicated a decline in organelle density in the cytoplasm of ovarian induction, such as mitochondria, endoplasmic reticulum and polyribosome. Furthermore, chromatin condensation of nuclei was observed in some cells. **Conclusion:** The ovarian induction using HMG and HCG resulted in some ultrastructural changes on the corpus luteum at implantation period, which could affect on the pregnancy rate. *Iran. Biomed. J. 16 (1): 33-37, 2012*

**Keywords:** Corpus luteum, Luteal phase, Ultrastructure, Ovarian induction

### INTRODUCTION

Corpus luteum is a transient endocrine unit that originates from an ovulated follicle. It is found two classes of steroidogenic cell types in corpus luteum named theca and granulosa cells. If the pregnancy occurred, corpus luteum activity would be kept through secretion of human chorionic gonadotropin (HCG) and anti-luteolysis factors, which associated with neuroendocrine reflexes and transmission of these signals to the corpus luteum [1, 2].

The corpus luteum has a principal role in production of steroidal hormones, including estradiol, progesterone and androgen [1]. Secretions of corpus luteum result in some changes in glands and vessels of the uterus, finally these changes cause to embryo reception. Therefore, the appropriate function of corpus luteum has a significant role in the pregnancy

maintenance and differentiation between fertile and infertile cycles [1, 2].

In assisted reproductive techniques, ovarian stimulation is used to approach multiple oocytes, whereas this method leads to reduction in pregnancy rate by a disturbance in the hormonal balance [2-4]. Development of embryos after oviductal transfer was lesser in the ovarian induction group compared to the normal group [5].

Recently, ultrastructural changes of granulosa cells were studied in corpus luteum of rhesus monkeys during implantation [6]. It was reported the human ovarian hyperstimulation might result in dysplasia occurrence in ovarian epithelial cells [7]. Anderson *et al.* [8] demonstrated that hyperstimulation with pregnant mare serum gonadotropin and HCG caused an increase in DNA mitochondria proliferation of corpus luteum. Although corpus luteum has an essential role in

\*Corresponding Author; Tel.: (+98-661) 6200 133; Fax: (+98-661) 6200 133; E-mail: mandbe2000@yahoo.com

pregnancy maintenance [1, 2], there are a few studies relevant to the effect of ovarian stimulation on ultrastructure of corpus luteum cells [7, 8]; thus it needs more investigation.

## MATERIALS AND METHODS

**Animals.** NMRI female mice, aged 6-8 weeks, were kept under 12 h light/12 h dark condition. The mice were randomly divided into two groups (5 in each group): 1) control group, which was rendered pseudopregnant by vaginal stimulation using a plastic soup and 2) ovarian induction group, which was stimulated using an intraperitoneal injection of human menopausal gonadotropin (HMG, 10 IU), followed by another injection of HCG (10 IU) after 48 h. In the evening of the second injection, the mice were rendered pseudopregnant the same as the method of control group.

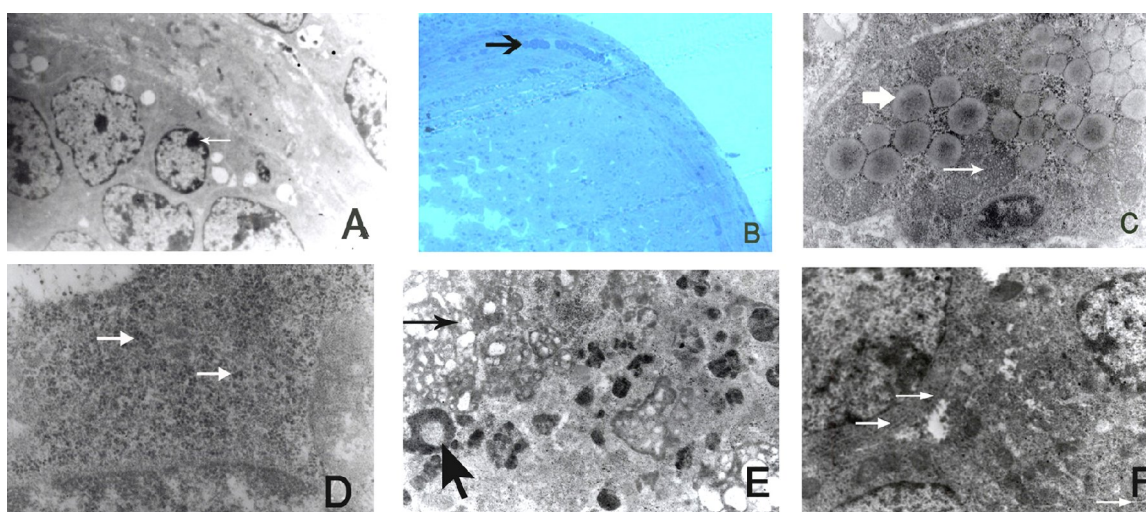
**Tissue preparation.** The mice from each group were sacrificed by cervical dislocation at the same time during luteal phase at implantation period, 4.5 days after HCG injection or pseudopregnancy. The samples were obtained from the ovary and prepared for electron microscopy study.

**Electron microscopy.** The samples were fixed using 2/5% glutaraldehyde in phosphate-buffered saline (pH 7.4) at 4°C for 2 h and then washed two times in cacodylate buffer (0.1 M cacodylate buffer working

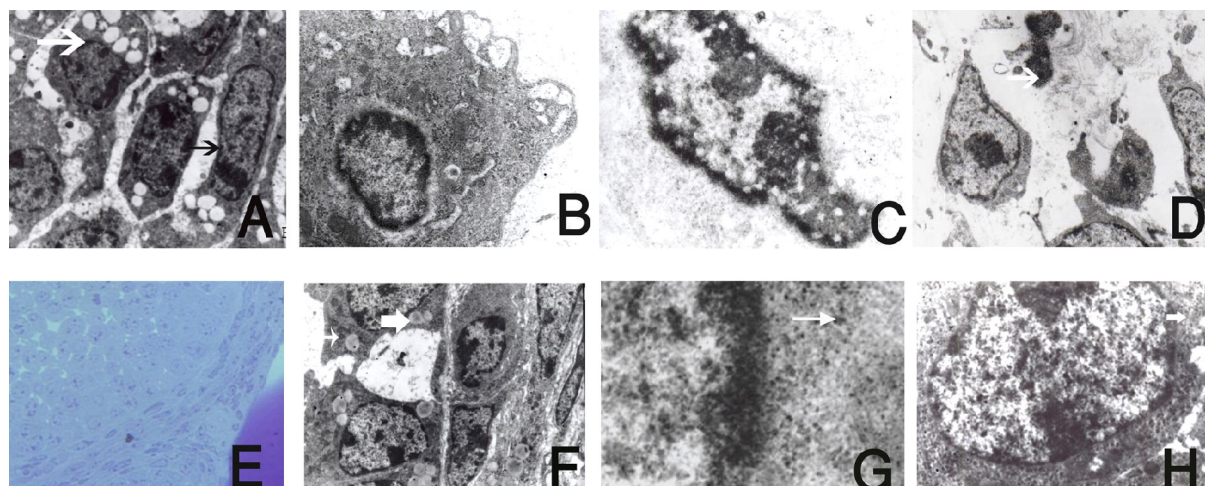
solution, pH 7.4) for 15 min. Post-fixation of samples were carried out with 1% osmium tetroxide in the same buffer for 2 h. Then, the samples were washed two times in deionized water. After dehydration in a graded ethanol series, specimens were placed two times in acetone dilution for 2 h. For impregnation, the samples were transferred into 1:1 resin/acetone and then placed in propylene oxide and embedded in Epon 812. Sectioning was performed with ultramicrotome and semithin sections (0.5  $\mu$ m) were stained with toluidine blue for light microscopy. After observation of corpus luteum in sample study, ultrathin sections were contrasted with uranyl acetate and lead citrate and examined by electron microscopy (Zeiss EM 900, Germany).

## RESULTS

Granulosa cells in the control group were morphologically identifiable with distinct cytoplasm borders and extensive adhesion areas. Nuclei of these cells were almost round, regular membrane with euchromatin and contained 1-2 nucleoli (Fig. 1A), whereas granulosa cells in the ovarian induction group were irregular in shape and showed various shapes like round and long. Also, the nuclei of these cells in this group were irregular in shape with 1-2 nucleoli. Furthermore, the granulosa cells in this group had irregular cellular margin, increased intercellular space and darker nuclei (Fig. 2A).



**Fig. 1.** Micrograph of corpus luteum in the control group 4.5 days after pseudopregnancy. (A) shows the corpus luteum (magnification  $\times 3,000$ ) and the arrow shows the nucleoli of granulosa cell; (B) shows clearly the vascular semithin section of corpus luteum (magnification  $\times 400$ ) and the arrow shows the vascular section; (C) shows high density of lipid droplet and mitochondria in corpus luteum cell (magnification  $\times 7,000$ ) and the thick and thin arrows show the lipid droplet and mitochondria, respectively; (D) shows high density of polyribosomes in the granulosa cell (magnification  $\times 12,000$ ) and the arrows show the polyribosomes; (E) shows extensive golgi apparatus in the granulosa cell (magnification  $\times 20,000$ ) and the thick and thin arrows show the golgi apparatus section and corpus luteum body, respectively and (F) shows extensive endoplasmic reticulum in the granulosa cell (magnification  $\times 7,000$ ). And the arrows show the endoplasmic reticulum.



**Fig. 2.** Micrograph of corpus luteum in the ovarian induction group 4.5 days after pseudopregnancy. (A) shows corpus luteum (magnification  $\times 3,000$ ) and the white and black arrows show the nucleoli and the intercellular space, respectively; (B) some of the corpus luteum cells showed apoptotic criteria. Cell membrane ruffling is visible in the apoptotic cell (magnification  $\times 4,400$ ); (C) shows marginated, condensed heterochromatin of nuclei in the apoptotic cell (magnification  $\times 12,000$ ); (D) and the arrow show the apoptotic body (magnification  $\times 3,000$ ); (E) shows semithin section of corpus luteum. There is not observed vascular section (magnification  $\times 400$ ); (F) shows low density of lipid droplet and mitochondria in corpus luteum cell (magnification 4,400) and the thick and thin arrows show the lipid droplet and mitochondria, respectively; (G) shows low density of polyribosomes in the granulosa cell (magnification  $\times 12,000$ ) and the arrow shows the polyribosomes and (H) shows endoplasmic reticulum in the granulosa cell (magnification  $\times 7,000$ ) and the arrow shows the endoplasmic reticulum.

Some of the corpus luteum cells in ovarian induction group showed apoptotic criteria including cell membrane ruffling, high chromatin density in the nuclei perimeter, and apoptotic body formation (Fig. 2B, 2C and 2D).

In addition, the assessment of semithin sections in control group indicated obvious vascular sections in techa compare to ovarian induction group (Fig. 1B, 2E).

Lipid droplets in the control group had high density and were associated with a large number of mitochondria (Fig. 1C), but lipid droplets in the ovarian induction group showed low density and were accompanied by the presence of a few mitochondria (Fig. 2F).

High density of polyribosomes was clearly observed in the granulosa cells in control group compared to ovarian induction group (Fig. 1D, 2G).

Extensive golgi apparatus associated with abundant aggregation corpus luteum body was observed in the granulosa cells in control group (Fig. 1E).

In addition, endoplasmic reticulum in the granulosa cells in control group was more extensive than ovarian induction group (Fig. 1F, 2H).

## DISCUSSION

In the present study, ultrastructural analysis on the corpus luteum cells in ovarian induction group

indicated heterochromatin nuclei and darker schema in these cells than control group. Centurine *et al.* [9] findings on morphometric and ultrastructural analyses of the granulosa cells after GnRH agonist administration lead to increase in clear cell numbers than dark cells. This issue indicated high activity of clear cells in steroidogenesis. The result of the present study indicated darker cells schema in ovarian induction group than control group, thus these findings may be associated with low activity for steroidogenesis. Ultrastructural evaluation in ovarian induction group declared that some corpus luteum cells have nuclei with peripheral heterochromatin and ruffling cell membrane. Moreover, apoptotic body was observed in some areas that this schema rarely observed in control group. Based on ultrastructural findings, apoptotic cells had symmetry with the nucleus ultrastructural changes in apoptosis process and cell-programmed death [10-13].

Atresia appearance may be happened in follicular cells in each step of follicular phase. In addition, apoptosis in the granulosa cells leads to cellular death motivation in adjacent cells [12]. Additionally, it is declared that levels of apoptosis in human granulosa cells increase after ovarian stimulation [14]. The present data is compatible with the recent study, as our present findings approved increased intercellular space in various areas of the corpus luteum in ovarian induction group. It seems that ovarian stimulation acts through reduction of intercellular adhesions and results

in intercellular space formation. Finally, it will lead to increase in apoptosis induction rate in corpus luteum cells. Cell-cell adhesions and interactions in the granulosa cells are effective in cell signaling, intercellular substrates transfer and hemostasis maintenance. Considered together, increased intercellular space in the granulosa cells leads to deficiency in proliferation and maturation [15, 16]. Moreover, electron microscopy findings in various studies have indicated that potentiality of follicular stimulating hormone on ultrastructure of granulosa cells results in separation and distance formation between granulosa cells [16, 17]. Therefore, it is concluded that increased intercellular space will be accompanied by reduction in material exchange and intercellular signaling. Finally, these happenings cause reduction in quality of the corpus luteum. As reported previously, there are a large number of mitochondria in natural granulosa cells in corpus luteum [18, 19]. Also, our findings clearly indicated a high density of mitochondria and polyribosomes in various sections of granulosa cells in control group. Evaluation of sections in corpus luteum cells in the ovarian induction group clearly showed low number of mitochondria and polyribosomes than in the control group. Nuclear changes such as irregular nuclear membrane and heterochromatin nucleus, associated with reduction in the mitochondria number, were observed in most sections in the ovarian induction group. Other researchers found similar changes during comparative cellular stress [3]; our present data confirmed this idea.

According to the recent studies, encircled areas by endoplasmic reticulum and golgi apparatus enhanced in the corpus luteum cells during implantation period. It seems that these changes are necessary for steroidogenesis [6]. Our report represents low extension of these organelles in ovarian-induced cells than control group.

Concurrent with steroidogenesis, the mitochondria number will be enhanced in follicular cells. Existence of potent relation among the endoplasmic reticulum, mitochondria and vesicles is indicative on substrate transition among these organelles [17]. Some researchers implied that mitochondria aggregation is effective in prevention of chromatin pyknosis [18]. Also, other researchers demonstrated that ovarian induction using HCG and pregnant mare serum gonadotropin leads to disturbance in scattered mitochondria in the follicular cells [3]. It seems that all of these changes will be ended in decreased efficiency and inappropriate function of the corpus luteum cells. This point has an important role in pregnancy maintenance and differentiation between fertile and infertile cycles [1, 2]. Therefore, ovarian stimulation acts through possible unfavorable changes in the corpus luteum and causes reduction in pregnancy rate.

In the present study, observed capillary sections in the ovarian induction group were lesser than control group. Blood flow supporting throughout an enriched-capillary web play a substantial role in development of corpus luteum, as the corpus luteum transforms to the most enriched vascular organ in the body at 7<sup>th</sup> day after implantation [20, 21]. On the other hand, the first phenomenon in the corpus luteum destruction is occurrence of apoptosis in the vascular endothelial and decline in vascular endothelial cells in the corpus luteum [22]. A recent study indicated that natural level of HCG during pregnancy acts as an angiogenic factor through vascular endothelial growth factor synthesis [23]. Berndt *et al.* [24] declared that physiologic level of HCG is responsible for enhanced angiogenesis and the number of pericytes. Assessment of various vascular sections in the cases with HMG and HCG treatment resulted in comparative reduction in corpus luteum angiogenesis. This difference between our results compared with previous reports may be due to some diversity, including higher dose of HCG in the ovarian induction than to physiological HCG levels, concurrent HMG administration and other interface reasons that are needed to be more investigated. Additionally, researchers have also shown that ovarian induction decreased endometrial levels of natural killer cells and vascularization index but increased vascular endothelial growth factor levels [25].

Regarding ultrastructural changes on corpus luteum cells due to ovarian induction using HCG and HMG, note that ovarian induction results in unfavorable changes in corpus luteum; as a consequence, it could affect on success level and pregnancy rate.

## REFERENCES

1. Buffet NC, Bouchard P. The neuroendocrine regulation of the human ovarian cycle. *Chronobiol Int.* 2001 Nov; 18(6):893-919.
2. Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev.* 2000 Jan; 80(1):1-29.
3. Miyamoto K, Sato EF, Kasahara E, Jikumaru M, Hiramoto K, Tabata H, Katsuragi M, Odo S, Utsumi K, Inoue M. Effect of oxidative stress during repeated ovulation on the structure and functions of the ovary, oocytes, and their mitochondria. *Free Radic Biol Med.* 2010 Aug; 49(4):674-81.
4. Beigi Boroujeni M, Salehnia M, Khalatbary AR, Pourbeiranvand S, Beigi Boroujeni N, Ebrahimi S. Effect of ovarian stimulation on the endometrial apoptosis at implantation period. *Iran Biomed J.* 2010 Oct; 14(4):171-7.
5. Kito S, Yano H, Ohta Y, Tsukamoto S. Superovulatory response, oocyte spontaneous activation, and embryo development in WMN/Nrs inbred rats. *Exp Anim.* 2010;



- 59(1):35-45.
6. Booher C, Enders AC, Hendrickx AG, Hess DL. Structural characteristics of the corpus luteum during implantation in the rhesus monkey (*Macaca mulatta*). *Am J Anat.* 1981 Jan;160(1):17-36.
7. Ghen G, Penault-Llorca F, Le Bouedec G, Mishellany F, Dauplat MM, Jaffeux P, et al. Ovarian epithelial dysplasia after ovulation induction: time and dose effects. *Hum Reprod.* 2009 Jan;24(1):132-8.
8. Anderson WA, Matsumoto LH, Melo A, Wyche JH, Perotti L, Ryan RJ. Replication of mitochondrial DNA in the gonadotropin-primed corpus luteum of the rat. *J Submicrosc Cytol Pathol.* 1988 Jul;20(3):477-89.
9. Centurine L, Giampietro F, Sancilio S, Piccirilli M, Artese L, Tiboni GM, et al. Morphometric and ultrastructural analysis of human granulosa cells after gonadotrophin-releasing hormone agonist or antagonist. *Rep Biomed. Online.* 2010 May;20(5):625-33.
10. Al-Zi'abi MO, Fraser HM, Watson ED. Cell death during natural and induced luteal regression in mares. *Reproduction.* 2002 Jan;123(1):67-77.
11. Bhardwaj JK, Sharma RK. Scanning electron microscopic changes in granulosa cells during follicular atresia in caprine ovary. *Scanning.* 2011 Jan;33(1):21-4.
12. Inoue N, Matsudo F, Goto Y, Manabe N. Role of cell death ligand receptor system of granulosa cells in selective follicular atresia in porcine ovary. *J Reprod Dev.* 2011 Apr;75(2):169-75.
13. Wong DH, Villanueva JA, Cress AB, Duleba AJ. Effects of resveratrol on proliferation and apoptosis in rat ovarian theca-interstitial cells. *Mol Hum Reprod.* 2011 Apr;16(4):251-9.
14. Giampietro F, Sancilio S, Tiboni GM, Rana RA, Di Pietro R. Levels of apoptosis in human granulosa cells seem to be comparable after therapy with a gonadotropin-releasing hormone agonist or antagonist. *Fertil Steril.* 2006 Feb;85(2):412-9.
15. Khan-Dawood FS, Yang J, Dawood MY. Expression of gap junction protein connexin-43 in the human and baboon (*Papio anubis*) corpus luteum. *J Clin Endocrinol Metab.* 1996 Feb;81(2):835-42.
16. Andrade ER, Maddox-Hyttel P, Landim-Alvarenga Fda C, Viana Silva JR, Alfieri AA, Seneda MM, et al. Ultrastructure of sheep primordial follicles cultured in the presence of indol acetic acid, EGF and FSH. *Vet Med Int.* 2010 Dec;2011:670987.
17. Nottola SA, Cecconi S, Bianchi S, Motta C, Rossi G, Continenza MA, Macchiarelli G. Ultrastructure of isolated mouse ovarian follicles cultured *in vitro*. *Reprod Biol Endocrinol.* 2011 Jan;13:9:3.
18. Katska-Ksiazkiewicz L, Alm H, Torner H, Heleil B, Tuchscherer A, Rynska B. Mitochondrial aggregation patterns and activity in *in vitro* cultured bovine oocytes recovered from early antral ovarian follicles. *Theriogenology.* 2011 Mar;75(4):662-70.
19. Davoodian N, Mesbah F, Kafi M. Oocyte ultrastructural characteristics in camel (*Camelus dromedarius*) primordial to large antral follicles. *Anat Histol Embryol.* 2011 Apr;40(2):120-7.
20. Irving-Rodgers HF, Friden BE, Morris SE, Mason HD, Brannstrom M, Sekiguchi K, et al. Extracellular matrix of the human cyclic corpus luteum. *Mol Hum Reprod.* 2006 Sep;12(9):525-34.
21. Reynolds LP, Grazul-Bilska AT, Redmer DA. Angiogenesis in the corpus luteum. *Endocrine.* 2000 Feb;12(1):1-9.
22. Davis JS, Rueda BR, Spaniel-Borowski K. Microvascular endothelial cells of the corpus luteum. *Reprod Biol Endocrinol.* 2003 Nov;1:89.
23. Michel RM, Aguilar JL, Arrieta O. Human chorionic gonadotropin as an angiogenic factor in breast cancer during pregnancy. *Med Hypotheses.* 2007;68(5):1035-40.
24. Berndt S, Blacher S, Perrier d'Hauterive S, Thiry M, Tsampalas M, Cruz A, et al. Chorionic gonadotropin stimulation of angiogenesis and pericyte recruitment. *J Clin Endocrinol Metab.* 2009 Nov;94(11):4567-74.
25. Junovich G, Mayer Y, Azpiroz A, Daher S, Iglesias A, Zylverstein C, et al. Ovarian stimulation affects the levels of regulatory endometrial NK cells and angiogenic cytokine VEGF. *Am J Reprod Immunol.* 2011 Feb;65(2):146-53.