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Apoptosis (Programmed Cell Death) of Human Ovarian Granulosa Cells after Inhibin Treatment *in vitro* – Immunohistochemical and Histochemical Study

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The present study is designed to explore in vitro effects of inhibin on programmed cell death (apoptosis) of ovarian granulosa cells, obtained from young cyclic women, women in IVF (in vitro fertilization) and from premenopausal women. The immunoexpression patterns of late apoptosis marker caspase-3, and proliferation-marker Ki-67, as well as the histochemical localization of NADPH-diaphorase were studied in cells cultered either with or without inhibin. Our semiquantitative findings clearly show an anti-apoptotic effect of inhibin on ovarian granulosa cells from all three patients groups investigated, in full concert with its stimulatory influence on ovarian estrogen production.

Key words: apoptosis, ovary, inhibin.

Introduction

Apoptosis is a form of programmed cell death process, important for tissue development and homeostasis and is triggered by external and internal stimuli. The apoptosis is also important in tumorogenesis and a key mechanism by which chimioterapeutic agents kill the cells [1, 6].On the other hand, in the ovary the follicular atresia and the regression of corpus luteum are accompanied by apoptosis [1, 9, 12, 14]. Some studies have pointed out the relationship between apoptosis of ovarian granulosa cells and the prognosis for pregnancy of women taking part in an in vitro fertilization program [2]. Concerning the regulation of the apoptosis in different tissues several molecules as cytokinies, growth factors, peptide hormones, steroids and some other have been reported [13].The same factors participate in the performance of the balance between cell proliferation and the programmed cell death during the apoptotic process. The apoptosis pathway followed by human ovarian granulosa cells was not well defined.

To obtain more detailed information on proliferation and apoptosis patterns in human ovarian granulosa cells isolated from women of different hormonal status and age and to test probable in vitro effects of inhibin on these patterns, we applied immunohistochemically the marker of cell proliferation (Ki-67) and the marker of effector phase of apoptosis (caspase-3). Having in mind the stimulatory effect of inhibin A on estrogen biosynthesis in isolated human ovarian granulosa cells [5] we applied the histochemical reaction for NADPH-diaphorase (marker of NO synthase) in our study.

Material and Methods

The ovarian granulosa cells were obtained from women (aged 27-31 years as a normo-ovulatory controls); women undergoing in vitro fertilization (IVF) and from premenopausal women (aged 45-51 years). The patients have given their informed consent to the study.

Ovarian granulosa cells were isolated from antral follicles by the non-enzymatic needle puncture method and from the follicular fluid.

Granulosa cells were cultured in Dulbecco's minimal essential medium /DMEM/ in the presence of 10% fetal calf serum (FCS) with or without inhibin A (at dose 10ng/ml, Sigma).

The immunohistochemical reactions for Ki-67 and caspase-3 were performed by avidin-biotin peroxidase methods. The histochemical reaction for NADPH-diaphorase was visualized after the method of Scherer – Singler et al., modified by B r u n i n g et al.[4] in phosphate buffered saline (PBS) - pH 7,2, containing 1mg/ml NADPH and 0,1 mg/ml nitroblue tetrazolium (Sigma Chemicals, St.Louis,Mo).

The percentages of granulosa cells positive for Ki 67, caspase 3 and NADPH-diaphorase have been calculated. The percentage of positive cell was calculated out of 450 randomly selected cells counted in the light microscope.

Results

The reaction for Ki 67 was positive in cell nucleus (Fig.1).The obtained values of the percentages of Ki 67 positive granulosa cells pointed out the highest number in the cultures from women after IVF and the lowest value in cultures from premenopausal patients (Table 1, 2 ,3). After treatment with inhibin A increased the number of Ki 67 positive ovarian granulosa cells from women of the three investigated groups (tables 1, 2, 3).

The reaction for caspase-3 was positive in the cell cytoplasm (Fig. 2).

The percentages of the caspase-3 positive granulosa cells were the highest in the premenopausal women and the lowest in the cell cultures from women after IVF (Fig. 4, 5, 6). The applied inhibin provoked depletion of the values of caspase 3 positive granulosa cells (Fig. 4, 5, 6).

The histochemical reaction for NADPH diaphorase in the cell cytoplasm (Fig.3) of cultured cells demonstrates the diminution of the percentages of the NADPH – diaphorase positive cells in the three investigated groups (Fig. 7).

Fig.1. Immunohistochemical reaction for Ki-67 in the granulosa cells nuclei (× 400)

Fig. 2. Immunohistochemical reaction for caspase-3 in the granulosa cell cytoplasm (× 400)

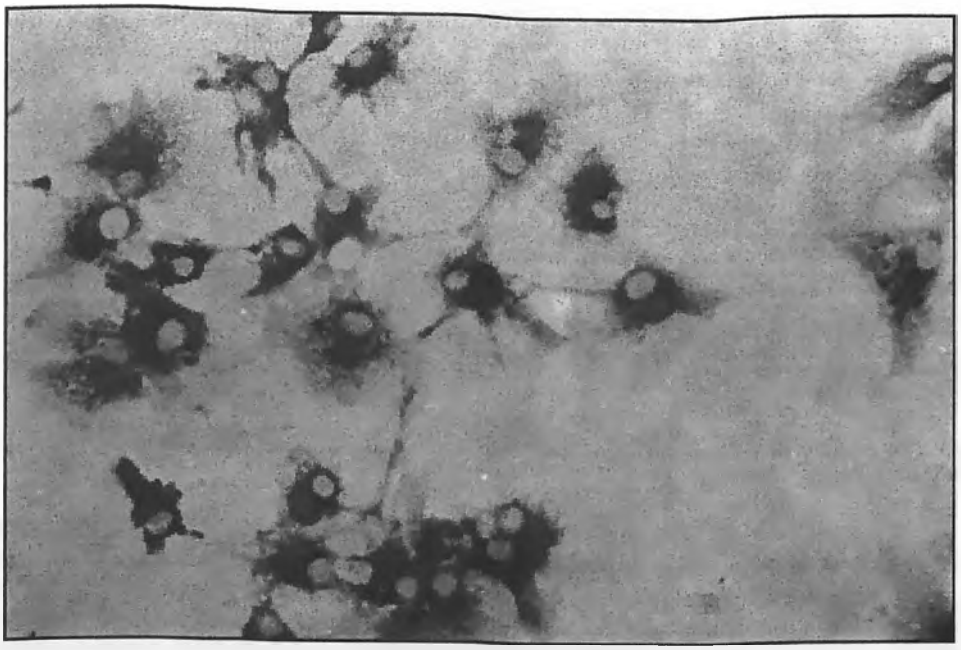

Fig. 3. Histochemical reaction for NADPH-diaphorase in the cytoplasm of the granulosa cells (× 100)

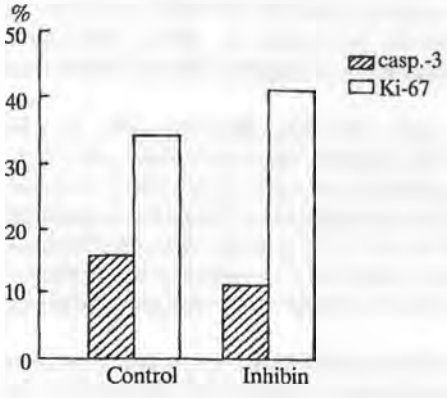

Fig. 4. Caspase-3 and Ki-67 immunopositive GCs from normally cycling women

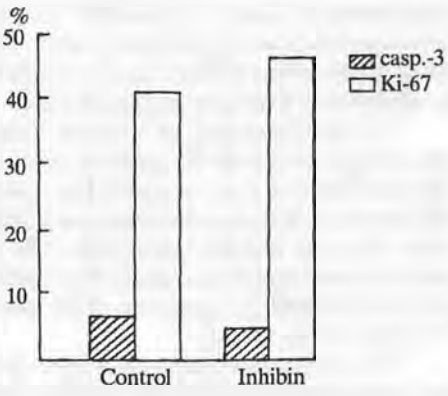

Fig. 5. Caspase-3 and Ki-67 immunopositive GCs from women after IVF

Discussion

The obtained data about the in vitro apoptosis of ovarian granulosa cells from women with different hormonal status using the immunohistochemical reactions for Ki 67 and caspase 3 and the histochemical expression of NADPH diaphorase could be discussed in connection to the role of estrogen in apoptotic process; the specific association between cell proliferation and apoptosis and, on the other hand, in connection to the participation of inhibin in the paracrine regulation of these processes.

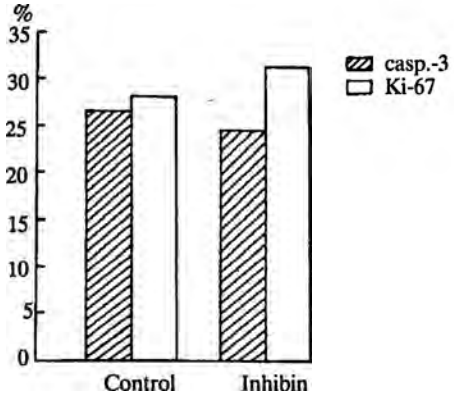

Fig. 6. Caspase-3 and Ki-67 immunopositive GCs from premenopausal women

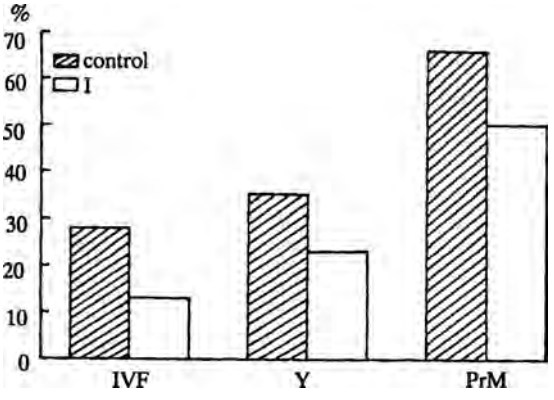

Fig. 7. NADPH-d positive GCs from the three groups of patients

Benifla et al. [2] have pointed out the potential role of apoptosis in human reproduction and Billig et al. [3] considered estrogen as an inhibitor of ovarian granulosa cell apoptosis. Our study confirmed this supposition because the number of the granulosa cells positive for caspase 3 /one of the specific cell human death enzymes after Gallaher et al.[8]/ revealed the highest number in cultures from premenopausal women, contrary to the small number of caspase 3 positive granulosa cells in women after IVF, when the estrogen in the follicular fluid is the highest [7].The results from histochemical study of NADPH diaphorase activity (marker of NOS activity) of investigated ovarian granulosa cells confirmed the presumed by other authors[13] proapoptotic effect of NO, one of compounds produced by granulosa cells and considered as inhibitor of estrogen production [5].

The proliferation of ovarian granulosa cells after the obtained data for the percentages of the Ki 67 positive cells showed the highest value in women after IVF. The proliferation was considered as a phase of apoptotic process [1] but there are some differences in the balance between cell proliferation and apoptosis of hormone-dependent tissue like the ovarian granulosa cells [10]. Baisch [1] implied that proliferation pathways and apoptotic signal of transduction are connected (for instance high value of proliferation and low apoptosis of the granulosa cells in cultures from women undergoing IVF seen in our study).

The *in vitro* application of inhibin A which inhibits the FSH secretion and stimulates the estrogenesis [4] provoked changes in the percentages of granulosa cell positive for Ki 67, caspase 3 and NADPH d in all investigated women confirming its stimulatory effect on the estrogen production and inhibitory effect on the apoptotic process. Inhibin could be suggest as a responsive marker of granulosa cell differentiation with autocrine and paracrine action.

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