ROLE OF LEPTIN IN OXIDATIVE STRESS AND SURVIVAL OF HUMAN GRANULOSA LUTEINIZED CELLS IN VITRO

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Summary. The aim of this study was to investigate the effect of leptin on oxidative status and survival of human granulosa luteinized cells (GLCs) in vitro. The human GLCs were isolated from follicular fluids of women undergoing IVF procedure and cultured in DMEM /HAM F12 with 10% FCS for 24 h. To study the oxidative status and survival of GLCs, after a 24-hour culture period, the cells were supplemented with 0, 1, 10, 100 ng/ml leptin in DMEM/ HAM F12 plus 1% FCS and cultured for another 24 h. The level of malondialdehyde (MDA), formed during the decomposition of lipid peroxidation products in cells, was measured by TBARS Assay Kit. Using the specific substrate (AcDEVD-PNA), the caspase-3 activity was measured as an indicative marker for apoptosis. Treatment with a physiological dose of leptin (10 ng/ml) decreased MDA level, while the higher dose of leptin (100 ng/ml) resulted in increased MDA level, compared to control cells (p < 0.05). Moreover, the treatment of GLCs with 100 ng/ml of leptin led to a significant increase in caspase-3 activity compared to control cells or cells treated with 10 ng/ml leptin (p < 0.05). The higher concentration of MDA was related to higher caspase-3 activity (R = 0.5789, p = 0.049). In conclusion, leptin plays an important role in oxidative metabolism and survival of human granulosa luteinized cells.

Key words: apoptosis, human granulosa luteinized cells, leptin, oxidative stress

INTRODUCTION

Leptin is a 16-kDa adipocyte-secreted protein, which plays an important role in reproductive physiology. The expression of leptin and its receptors have been demonstrated in human granulosa and theca cells, oocytes, endometrial cells and pre-implantation embryos [1, 2, 3]. Leptin is involved in ovarian steroidogenesis, follicular development and differentiation [4], oocyte matu-
ration [5] and early embryo development [6]. Reactive oxygen species (ROS) play both physiologic and pathologic role in the female reproductive tract. The low levels of ROS affect processes such as oocyte maturation, fertilization, embryo development and pregnancy. Last evidences suggested that oxidative stress is related to ovarian pathology and infertility [7]. Apoptosis is a form of cell death in which the programmed sequence of events leads to the elimination of old, unnecessary and unhealthy cells without releasing of harmful substances into the surrounding area. The apoptosis is an essential component of human ovarian function and development and is involved in oogenesis, folliculogenesis, oocyte selection and luteal regression. The rate of granulosa cell apoptosis is related to the number of preovulatory follicles, the number of retrieved and fertilized oocytes in stimulated cycles [8] and play a role in the etiology of unexplained infertility [9].

The aim of this study was to investigate the effect of different leptin concentrations on oxidative status and survival of human granulosa luteinized cells in vitro.

**MATERIAL AND METHODS**

*Isolation of human granulosa luteinized cells*

Human granulosa luteinized cells (GLCs) were isolated from follicular aspirates (obvious blood contamination were excluded) obtained from women undergoing in vitro fertilization-embryo transfer (IVF-ET) program. After gradient centrifugation on Histopaque-1077 (Sigma), GLCs were aspirated from interphase and washed with culture medium (DMEM/HAM’s F 12/10% FCS, Sigma). Cell viability was determined by trypan blue exclusion method.

*In vitro culture of GLCs*

Isolated GLCs from at least three women were pooled and suspended in DMEM/HAM’s F-12 / 10% FCS, supplemented with penicillin (100 IU/ml) and streptomycin (100 mg/ml) at 60 x 10^4 cells/mL and cultured in 96-well plates (Orange Scientific). The plates were incubated at 37°C/5% CO_2_ and after a 24-hour culture period and washing, the cells were supplemented with 1, 10, 100 ng/ml [2, 10] leptin (Santa Cruz Biotechnology) in DMEM/HAM’s F-12/1% FCS and cultured for another 24 h. Control cells were cultured in DMEM/HAM’s F-12/1% FCS, only.

*Oxidative stress assay*

Intracellular oxidative stress was evaluated by the TBARS kit (BioAssay Systems) according the manufacture protocol. The intensity of the color reaction at 535 nm was measured by Multiplate Reader (LKB) and the concentration of the end products, formed during the decomposition of lipid peroxidation products, malondialdehyde (MDA), was calculated by standard curve and a formula set.

*Caspase-3 activity assay*

Intercellular caspase-3 activity was determined using the Colorimetric kit (R&D) according the manufacture protocol. Briefly, the cells were lysed with lysis
buffer, the soluble fraction was obtained and assayed for caspase-3 activity using AcDEVD-PNA as specific substrate. The intensity of color reaction at 405 nm was measured by Multiplate Reader (LKB) after incubation for 2 h at 37°C in a humidified 5% CO₂ incubator.

**Statistical analysis**

The statistical analysis of results from three independent experiments was done by one-way ANOVA, followed by the Newman-Keuls test and by the correlation matrices. A p-value less than 0.05 was considered significant.

**RESULTS**

Effect of different leptin concentrations on oxidative status of GLCs

After supplementation of GLCs in vitro with 1, 10, 100 ng/ml, the lowest level of MDA was measured in cells treated with 10 ng/ml of leptin compared to control. The highest dose of leptin (100 ng/ml) resulted in increased MDA level, compared to the cells supplemented with 10 ng/ml of leptin and to the control cells (Fig. 1, p < 0.05).

Effect of different leptin concentrations on survival of GLCs

The highest level of GLCs apoptosis was established in cells treated with highest (100 ng/ml) concentration of leptin compared to controls and to cells treated with 10 ng/ml of leptin. The lowest caspase-3 activity was measured in GLCs supplemented with 10 ng/ml of leptin (Fig. 2, p < 0.05).

Relationship between MDA level and Caspase-3 activity in GLCs

The correlation analysis showed that the level of MDA in GLCs was positively related to the activity of caspase-3. The highest concentration of MDA was related to the highest level of GLCs apoptosis (Table1, R = 0.5789, p = 0.049).

![Fig. 1. Relationship between leptin concentrations and oxidative status of GLCs (MDA levels)](image-url)
DISCUSSION AND CONCLUSION

The ovarian steroidogenesis, oocyte maturation, ovulation, implantation, the formation of blastocyst, luteolysis and luteal maintenance in pregnancy are physiological processes modulated by ROS. ROS and antioxidants remain in balance under normal physiological conditions. When the balance is disrupted towards an elevated level of ROS, the oxidative stress occurs [7]. In the presence of higher leptin concentration, ROS production was stimulated by inflammatory cells, endothelial cells, and other cell types [11, 12]. The process of lipid peroxidation is one of the oxidative conversion of polyunsaturated fatty acids to products known as malondialdehyde [13]. The concentration 10 ng/ml of leptin has been frequently reported as “physiological concentration” in the blood circulation of humans, as well as in the blood plasma of different animal species [14]. In this study the effect of different leptin concentrations (1, 10, 100 ng/ml) on oxidative status (levels of MDA) of GLCs, in vitro, was investigated. The level of MDA was lowest in GLCs treated with 10 ng/ml, compared to control cells and to other treated cells. Oxidative stress was significantly higher (highest MDA level) in the cells supplemented with the highest dose of leptin.
Our results are in agreement with the findings of other authors [15, 16], who have found, that the elevated leptin level was associated with increased oxidative stress.

Leptin has a dual effect on the cell survival and can suppress or promote ovarian apoptosis. Our results showed that leptin concentration of 10 ng/ml suppressed apoptosis of GLCs in vitro, while the activity of caspase-3 was highest in GLCs treated with 1 and 100 ng/ml. The similar effect of the various concentrations of leptin was reported by Sirotkin, where the chicken ovarian cells treated with leptin in concentrations of 1 ng/ml and 100 ng/ml showed the higher expression of p53, compared to cells treated with 10 ng/ml leptin, and that the concentration of 100 ng/ml of leptin induced highest expression of Bax in human granulosa cells [17, 18].

Leptin may exert anti-apoptotic effects through the maintenance of Bcl-2 expression [19]. In our preliminary study we found that the in vitro luteinized porcine granulosa cells treated with 10 ng/ml of leptin showed higher Bcl-2 and lower p53 expression [20]. The increased expression, both of pro-apoptotic (Bax) and of antiapoptotic (Bcl) peptides and the Bcl/Bax ratio, resulted in suppression of ovarian cell apoptosis [21].

The ROS and resulting oxidative stress play pivotal role in apoptosis. The lipid peroxides are markers of oxidative stress. In this study we found, that the high concentration of MDA was related to the higher caspase-3 activity in GLCs and confirmed that the oxidative stress and apoptosis are closely linked physiological events [22].

In conclusion, leptin plays an important role in oxidative metabolism and survival of human granulosa luteinized cells.

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**REFERENCES**


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