EXPRESSION OF ATRIAL NATRIURETIC PEPTIDE, PROGESTERONE, APOPTOSIS-RELATED PROTEINS AND CASPASE-3 IN *IN VITRO* LUTEINIZED AND LEPTIN-TREATED PORCINE GRANULOSA CELLS

JULIETA DINEVA[1], ANNA K. WOJTOWICZ[2], KATARZYNA AUGUSTOWSKA[2], IVAILO VANGELOV[1], EWA L. GREGORASZCZUK[2], MARIA D. IVANOVA[1]

[1]*Institute of Biology and Immunology of Reproduction, "Acad. K. Bratanov", Bulgarian Academy of Science, Sofia, Bulgaria*
[2] *Laboratory of Physiology and Toxicology of Reproduction, Department of Animal Physiology, Institute of Zoology, Jagiellonian University, Krakow, Poland*
E-mail: maria_d_ivanova@yahoo.co.uk

Objective. Our aim was to examine the expression patterns of ANP, the rate of apoptosis bcl-2 and p53 expression and caspase-3 activity and progesteron (P) production in porcine granulosa cells (pGCs) stimulated in vitro for luteinization and after treatment with leptin.

Methods. Freshly isolated prepubertal pGCs were cultured as monolayers for 24 h, subsequently FSH was supplemented for 24 h, and finally LH was added to a part of the cells for 24 h to induce luteinization. The effect of leptin on in vitro luteinized pGCs was tested by the addition of 10 ng/ml human recombinant leptin (hrL) 24 h after LH administration. Indirect immunofluorescence for ANP, bcl-2 and p53 expression was used, P production was assayed by direct enzyme immunoassay (EIA) and colorimetric AcDEVD-Pna assay for caspase-3 activity was applied.

Results. Stimulation of prepubertal pGCs with FSH resulted in a moderate expression of ANP and elevation in P production. When FSH treatment was followed by LH, the pronounced expression of ANP was observed in all cells. Suppressive effect of FSH and LH on p53 expression and caspase-3 activity with parallel increase in bcl-2 expression and increased P production was observed. The treatment of *in vitro* luteinized (FSH/LH-stimulated) pGCs with leptin did not influence the expression of ANP. However, in FSH/LH plus leptin treated cells the concomitant increase in bcl-2 expression and parallel inhibitory effect on p53 expression and caspase-3 activity was noted, compared to control cells without any significant increase in P production.

Conclusion. The present data demonstrated the positive relationship between ANP expression and P production in pGCs stimulated for luteinization *in vitro* by FSH and LH, as well as their antiapoptotic role mediated presumably by cGMP accumulation in the luteinized pGCs. A direct anti-apoptotic effect of leptin on in vitro luteinized pGCs, without any significant modulation of P production, was documented.

Key Words: Granulosa cells luteinization – Atrial natriuretic peptide (ANP) – Progesteron – Leptin – Apoptosis

Luteinization is the final stage of follicular differentiation, which is triggered *in vivo* by preovulatory gonadotrophin surge and involves a large-scale remodeling of the ovulatory follicle (MCGEE et al. 1997). Gonadotrophins, steroids and a number of autocrine and paracrine factors stimulate the proliferation, differentiation and survival of granulosa cells (CHUN and HSUEH 1998; RUIZ-CORTES et al.2003; SWENSSON 2003). All of them further affect the process of granulosa cells luteinization through the control of apoptosis (MATSUBARA et

al. 2000). Ovarian natriuretic peptide system is under the regulatory effect of gonadotrophins, and ANP content showed cyclic changes with the highest rate of its production in proestrus (KIM et al. 1992; GUTKOWSKA et al. 1999). In granulosa cells, ANP showed a stimulatory effect on a cGMP transduction sequence, resulting in the increased progesterone secretion (ACOSTA et al. 1999). Our study of ANP expression in porcine granulosa cells demonstrated the strongest immunostaining for ANP in such cells isolated from large preovulatory follicles (IVANOVA et al. 2003). The relationship between ANP and progesterone, as survival factors in human luteinized granulosa cells (GLCs), was shown too (DINEVA et al. 2004).

Leptin, a product of the obese gene, is a cytokine with multiple activities mediated through leptin receptor isoforms expressed in ovarian cells (CIOFFI VAN BLERKOM et al. 1997). The expression of leptin receptors in granulosa and luteal cells is varying during pig ovarian cell differentiation (SONG et al. 1999). It has been shown that leptin modulates steroidogenesis in a dose-dependent manner and may play a role in the process of luteinization (STOKLOSOWA et al. 1982).

The model for *in vitro* luteinization of porcine granulosa cells was used to follow up the expression of ANP, bcl-2, p53 as well as P production and caspase-3 activity in relation to apoptosis. To gain further insight into the direct leptin action on the ovary, we investigated the effect of leptin on in vitro luteinized porcine granulosa cells.

Material and Methods

Isolation and culture of ovarian follicles. Parker medium M199, trypsin, and calf serum (CS) were purchased from the Laboratory of Sera and Vaccines (Lublin, Poland). Antibiotic/antimycotic solution (100x) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Porcine ovaries from prepubertal animals were obtained from a local abattoir and collected into a bottle filled with sterilized ice-cold saline and transported to the laboratory within approximately 1.5 h elapsed from the slaughter to the arrival at laboratory.

Granulosa cells (GCs) were subsequently prepared according to the technique described by STOKŁOSOWA et al. (1992). After isolation, GCs were washed three times in M199, collected and resuspended in M199 supplemented with 5 % calf serum (M199/CS).

Experimental procedure. To examine the expression patterns of ANP, bcl-2 and p53, the rate of apoptosis and P production in pGCs stimulated in vitro for luteinization, we cultured freshly isolated prepubertal pGCs as monolayers for 24 h, subsequently FSH was supplemented for 24 h , then LH was added to a portion of cells for 24 h to induce luteinization and, finally, a part of FSH/LH-stimulated cells was treated with leptin for additional 24 h. Control cells were cultured with addition of only 10 % fetal calf serum. The supernatants were collected after 96 h of culture and stored at -20 °C for determination of P production, while the adherent cells were washed and fixed for immunoflurescent analysis or frozen for measurement of caspase-3 activity.

Analysis of progesterone concentration. Progesterone in the culture medium was assayed by using a direct enzyme immunoassay (EIA) as described by SZAFRANSKA et al. (2002). The standard curve range was from 0.39 to 100 ng/ml and the effective dose for 50 % inhibition (ID50) of the assay was 4.5 ng/ml. The intra- and inter- assay coefficients of variation were 6.6 % and 9.2 %, respectively.

Caspase-3 activity. Cultured cells were lysed with a lysis buffer containing 50 nm Hepes (pH 7.4), 100 nM NaCl, 0.1 % CHAPS, 1nM EDTA, 10 % glycerol and 10 nM DTT. The soluble fraction of the cell lysate was then assayed for caspase-3 activity using AcDEVD-Pna (Sigma) substrate for caspase-3 as described in the manufacturer's protocol.

Detection of ANP, Bcl-2, p53 with IIF. The GCs were permeabilized with 0.1% Triton X-100 for 4 min and after washing the cells were incubated with primary antibodies: Mab anti-ANP supernatant, obtained and characterized at the Institute of Biology and Immunology of Reproduction (Sofia, Bulgaria), 1:1 dilution in PBS/BSA; 0.1 %; Mab anti-p53, Clone BP53-12, mouse ascites fluid (Sigma, Saint Louis, MO. USA) 1:800 dilution in PBS/BSA; 0.1%; Mab anti-Bcl-2,Clone Bcl-2-100, mouse ascites fluid (Sigma, Saint Louis, MO, USA) 1:2000 dilution in PBS/BSA, overnight, followed by washing with PBS-Tween and then incubated with secondary antibody (Sw/am IgG labeled with FITC), diluted 1:20, 1 h, Sevac, Czech Republic). After intensive washing in PBS-Tw, slides were mounted with Mowiol. The expression of ANP, Bcl-2 and p-53 was examined using fluorescence microscope (Leitz Labor-Lux) and scored: from + to +++ and documented with Kodak color film.

Results

Expression of ANP in pGCS stimulated with FSH or FSH/LH for luteinization and after leptin treatment of the luteinized cells. Stimulation of prepubertal pGCs with FSH resulted in a moderate ANP immunostaining with membrane, submembrane and cytoplasmic localization in the great number of cells, compared to control cells where only single cells showed faint ANP immunostaining (Fig.1a,1d). When FSH treatment was followed by LH, the pronounced expression of ANP was observed in all cells (Fig.1b). The leptin treatment of in vitro luteinized (FSH/LH stimulated) pGCs did not influence the expression of immunoreactive ANP (Fig.1c).

Progesterone production in pGCs stimulated with FSH or FSH/LH for luteinization and after leptin treatment of luteinized cells. Data of the P production are shown in Fig.2. The effect of gonadotrophins on in vitro luteinization was manifested by the elevated P production. The highest level of P production was observed in FSH/LH-stimulated cells. Box-plot analysis showed the increased P production after FSH, FSH/LH and FSH/LH plus leptin treatments, compared to control cells ($P < 0.05$). The leptin dose of 10 ng/ml, used 24 h after LH, did not influence significantly the P production of in vitro luteinized pGCs ($P^* < 0.38$).

Expression of bcl-2 and p53 in pGCs stimulated with FSH or FSH/LH for luteinization and after leptin treatment of the luteinized cells. Immunostaining for bcl-2 after FSH treatment showed a moderately positive reaction in stimulated cells compared to controls (Fig. 3a, 3d). The number of positive cells increased and immunostaining was intense as a consequence of LH stimulation (Fig. 3b). After leptin treatment of in vitro luteinized pGCs, the strong immunostaining for bcl-2 was observed in a great number of cells (Fig. 3c). The opposite picture was demonstrated for proapoptotic p53 protein (Fig. 4a, 4b, 4c, 4d). The number of p53-positive cells was greater in control cells and incidences of positive cells were lowered by gonadotrophin treatment, as well as by leptin.

Caspase-3 activity in pGCs stimulated with FSH or FSH/LH for luteinization and after leptin treatment of the luteinized cells. The data of caspase–3 activities are presented in Fig. 5. The proteolytic assay used in this study is specific for cleavage of the DEVD-sequence and the increased activity is still related to the apoptotic process. The obtained results showed the significant inhibitory effect of FSH, FSH/LH and FSH/LH+leptin treatment on caspase-3 activity compared to control cells ($p<0.05$). The higher inhibitory effect of leptin on caspase-3 activity was documented, but it was not statistically significant when compared to FSH/LH-treated cells (Fig. 5).

Discussion

One purpose of this study was to determine how the model for in vitro luteinization of prepubertal pGCs after FSH or FSH/LH stimulation is related to ANP expression and P production. As mentioned, luteinization is a large–scale remodeling process that occurs in response to stimulation with gonadotrophins and induced paracrine and autocrine growth factors (Matsubara et al. 2000). Our study showed that the treatment of prepubertal pGCs with FSH induced the rise of immunoreactive ANP expression in the cultured cells, as well as stimulation of P production. The observed increase in ANP expression in cultured cells, as well as higher P level after LH treatment, were suggestive of the role of ANP in the pGC luteinization, as was previously shown for bovine GCs (Acosta et al. 1999) and for follicular development in rats (Gutkowska et al. 1999). The same positive relationship between ANP and P production was shown in rat and human GCs (Johnson et al. 1997; Pascador et al. 1999) and in our earlier study with human luteinized granulosa cells (Dineva et al. 2004), despite discrepant data on the effect of ANP on P production, cited by Jankowsky et al. (1997). When in vitro luteinized pGCs were treated with the so-called physiological leptin dose of 10 ng/ml (Shikone et al. 1996), we did not observe any influence on the expression of immunoreactive ANP . The lack of a significant increase in P production after leptin treatment in our experimental model may be due to the time of leptin administration we used, i.e. 24 h after LH supplementation, when luteinization was fully expressed. The patterns of ANP immunostaining and P production in FSH/LH-stimulated cells support this suggestion.

The ultimate vulnerability of cells to diverse apoptotic stimuli is determined by the relative expression of various proapoptotic and antiapoptotic proteins, which provide checkpoint control over cell cycle progression and induction of apoptosis (Meikranntz et al. 1995; Ruiz-Cortes et al. 2000). FSH is the most important factor for follicle growth in the preovulatory phase and a lack of FSH could lead to follicular atresia through apoptosis of GCs. It is known that LH is indispensable for luteinization and luteal cell survival (Murphy et al.

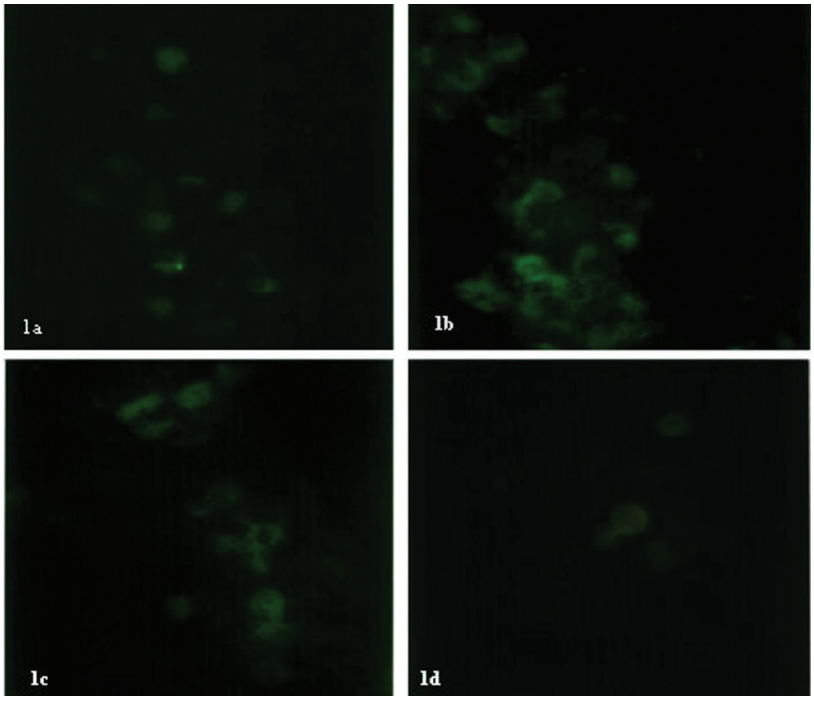

Fig. 1 ANP immunostaining of in vitro culturedpGCs: 1a) moderate ANP immunostaining in FSH treated cells; 1b) strong immunostaining in cells after FSH and LH treatment; 1c) ANP immunostaining in FSH/LH plus leptin treated pGCs, where no changes in ANP immunostaining was documented compared to FSH/LH treated cells; 1d) control cells: weak immunostaining is visible only in few cells.

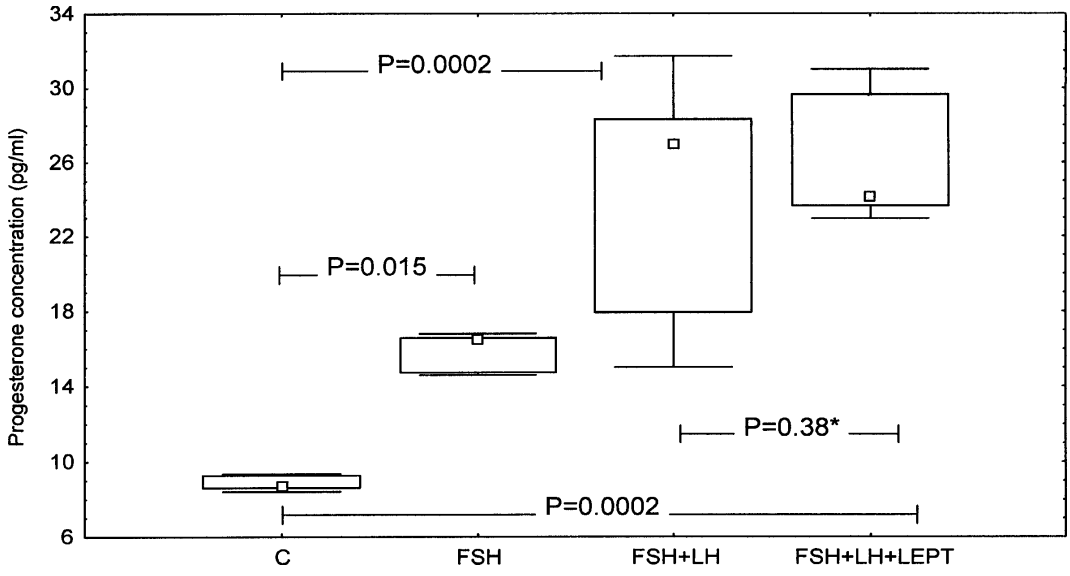

Fig. 2 Comparison of P production after different treatment of prepubertal pGCs in vitro. Data are displayed as a box-plot with median value, range of values (min-max) and interquartile range (box), where increased P production after gonadotropin treatment is evident ($p<0.05$). The dose of 10 mg/ml leptin treatment did not influence significantly P production of in vitro luteinized pGCs ($p=0.38$)

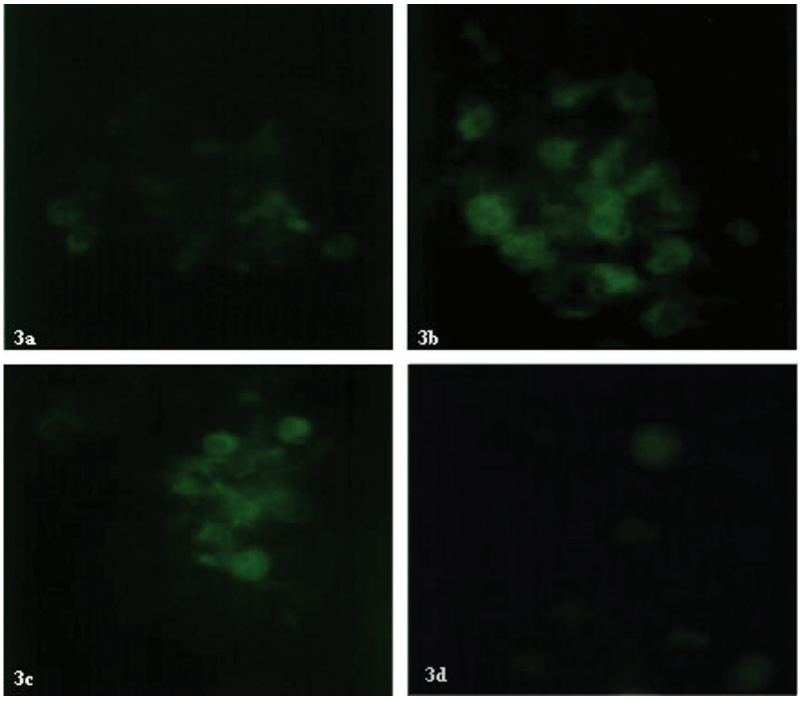

Fig. 3 Expression of antiapoptotic bcl-2 protein in pGCs: 3a) moderate immunostaining of cells treated with FSH; 3b) moderate immunostaining of cells treated with FSH/LH; 3c) intense immunostaining for bcl-2 in FSH/LH plus leptin treated cells; 3d) control culture

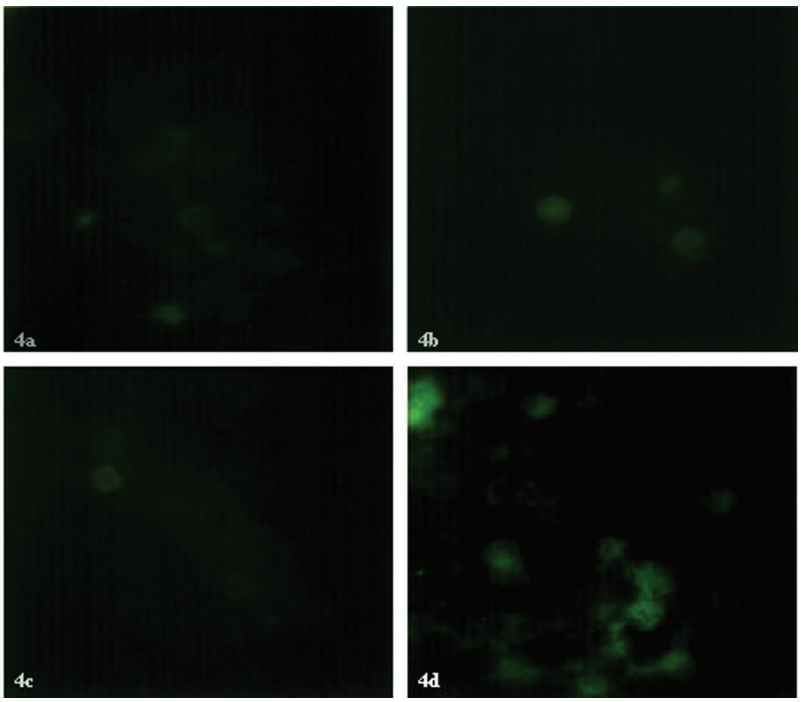

Fig. 4 Expression of proapoptotic p53 oncoprotein in pGCs; 4a) a few positive cells after FSH treatment are visible; 4b) weak immunostaining in great number of cells treated with FSH/LH; 4c) weak immunostaining in some cells after FSH/LH plus leptin treatment; 4d) the great number of positive cells for p53 seen in control culture

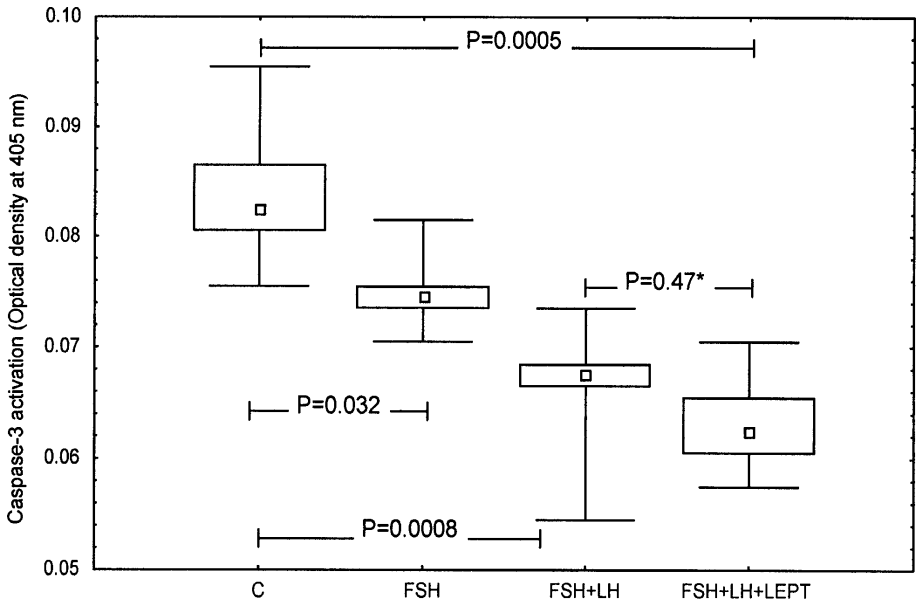

Fig. 5 Box-plot analysis of caspase-3 acitvity among different treatments of GCs in vitro. The inhibitory effect of FSH, FSH/LH and FSH/LH plus leptin treatment is shown as compared to control cells (p<0.05). The lower caspase-3 activity in FSH/LH treated cells was without any significant difference as compared to FHS/LH treated cells (p<0.48)

2000; Ruiz-Cortes et al. 2000). The observed suppressive effect of FSH and LH treatment on p53 expression and simultaneously stimulatory effect on the expression of bcl-2, the main survival promoter in the ovary (Song et al. 1999) appear to justify the conclusion that luteinization of GCs decreased the susceptibility to apoptosis (Swenson et al. 2003). This event seems to be at least partly mediated by P and ANP in the luteinized GCs. Pronounced ANP binding to its specific guanylyl-cyclase receptors stimulates cGMP formation and P secretion (Noubani et al. 2000). On the other hand, cGMP, that is a second messenger of NO, seems to participate in the survival pathways at all stages of follicle growth (Chun and Hsueh 1998; McGee et al. 1997).

Among executor caspases (caspase-3, -6, and –7), caspase-3 appears to be upstream of caspase-6 and -7 and, therefore, its activation represents a critical point in transmission of the apoptotic signal. The activation of caspase-3 is a central event in the apoptotic process in which numerous signaling pathways converge and through which multiple downstream substances are cleaved (Ishizaki et al. 1998). There is a strong linkage between the activation of caspase-3 and apoptotic degradation of genomic DNA. A specific caspase-3 activated DNase (DFF40 or CAD) has been identified and characterized as an enzyme which is involved in the internucleosomal fragmentation of DNA and, finally, in apoptotic cell death (Widlak et al. 2000). The results presented showed the inhibition of caspase-3 activity by FSH, FSH/LH treatment and the parallel elevation of ANP expression and P production. This supports the putative role of ANP, P and cGMP in GC luteinization and survival. Moreover, the presented data clearly showed anti apoptotic action of leptin. This observation is in agreement with data reported by Almog et al. (2001) who showed that leptin dramatically reduced incidence of follicular apoptosis measured by TUNEL in leptin-treated rats. These authors demonstrated a pronounced elevation (up to 16.3-fold) in Bcl-2 expression during 7-14 days of leptin injections compared to a Bcl-2 expression in controls. This is also in agreement with our recent data indicating that leptin acts as a antiapoptotic factor during corpus luteum formation (Gregoraszczuk et al. 2005).

In summary, the present investigation demonstrated the positive relationship between ANP expression and P production in pGCs stimulated for luteinization in vitro by FSH and LH, as well as the antiapoptotic role of FSH/LH, mediated presumably by cGMP accumulation (indirectly shown by ANP immunostaining) after luteinization of pGCs. The dose of 10 ng/ml leptin given 24 h after LH did not significantly influence P production in the in vitro luteinized prepubertal

pGCs. Direct anti-apoptotic effect of leptin on in vitro luteinized pGCs was documented.

Acknowledgements

We would like to thank prof. I. Kehayov and prof. St .Kyurkchiev (Institute of Biology and Immunology of Reproduction, Sofia,Bulgaria) for the gifz of Mab.anti-ANP; Dr. B. Szafranska (University of Warmia and Mazury, Olsztyn, Poland) for the gift of P antiserum (code: SO/914). This work was supported in part by the Interacademic (Bulgarian Academy of Science , Sofia, Bulgaria and Jagiellonian University, Kraków, Poland) Joint Project; NF "Sci.Researches"- Project N#B1507/05

References

Acosta TJ, Berisha B, Ozawa T, Kunidata S, Schams D, Miyamoto A: Evidence for a local endothelin- angiotensin natriuretic peptide system in bovine mature follicles in vitro: Effects on steroid hormones and prostaglandin secretion. Biology of Reproduction **61,** 1419-1425,1999

Almog B, Gold R, Tajima K, Dantes A, Salim K, Rubinstein M, Barkan D H,Homburg R, Lssing JB, Nevo N, Gertler A, Amsterdam A: Leptin attenuates follicular apoptosis and accelerates the onset of puberty in immature rats. Mol Cell Endocrionol **183,** 179-91, 2001

Barkan D, Jia H, Dantes A, Vardimon L, Amsterdam A, Rubinstein M: Leptin modulates the glucocorticoid-induced ovarian steroidogenesis. Endocrinology **140,** 1731-1738, 1999

Chun SY, Hsueh AJW: Paracrine mechanisms of ovarian follicle apoptosis. J Reprod Immunol **39,** 63-75, 1998

Cioffi van Blerkom Y, Antczak M, Shater A, Wittmer S, Snodgrass HR: The expression and its receptors in preovulatory human follicles. Molec Human Reprod **3,** 467-473, 1997

Dineva J, Nikolov G, Vangelov, I Konakchieva R, Ivanova M: Apoptosis of human granulosa luteinized (GLCs) and cumulus cells (CCs) in relation to age of women. CR Acad Bulgare des Sciences **57,** 113-116, 2004

Duggal PS, Van der Hooek KH, Milner CR, Ryan NK, Armstrong DI, Magoffin DA, Norman RJ. The in vitro and in vivo effect of exogenous leptin on ovulation in the rat. Endocrinology **141,** 1971-1976, 2000

Gregorasczuk EL, Ptak A. In vitro effect of leptin on Growth Hormone(GH) and Insuline-like Growth Factor–1 (IGF-1) stimulated progesterone secretion and apoptosis in developing and mature corpora lutea of pig ovaries. J Reprod Dev **51,** 727-733, 2005

Gutkowska J, Jankowski M, Sairam M, Fujio N, Reis AM, Mukaddam-Daher S, Tremblay J: Hormonal Regulation of Natriuretic Peptide System during Induced Ovarian Follicular Development in the Rat. Biol Reprod **61,** 162-170, 1999

Ishizaki Y, Jacobson MD, Raff MC: A role for caspase in lens fiber differentiation. J Cell Biol.**140,** 153-158, 1998

Ivanova M, Gregoraszczuk EL, Augustowska K, Kolodziejczyk J, Mollova MV, Kehayov IR: Localization of natriuretic peptide in pig granulosa cells isolated from ovarian follicles of various size. Reprod Biology **3,** 173-189, 2003

Jankowski M, Reis AM, Mukaddam-Daher S, Dam T-V, Farooki R, Gutkowska J: C-type natriuretic peptide and guanylyl cyclase receptors in the rat ovary are modulated by the estrus cycle. Biol Reprod **56,** 59-66, 1997

Johnson KM, Hughes FMJ, Fong YY, Mathur RS, Williamson HO, Gorospe WC: Effect of atrial natriuretic peptide on rat ovarian granulose cell steroidogenesis in vitro. Am J Reprod Immunol **31:** 163-168, 1994

Kim SH, Cho KW, Lim SH, Hwang YH, Ryn H, Oh SH, Seul KH, Yeong GB, Yoon S: Presence and release of immunoreactive atrial natriuretic peptide in granulosa cells of the pig ovarian follicle. Regulatory Peptides **42,** 153-162, 1992

Kitawaki J, Kusuki I, Koshiba H, Tsukamoto K, Honjo H: Leptin directly stimulates aromatase activity in human luteinized granulosa cells. Molec Hum Reproduction **5,** 708-713, 1990

Matsubara H, Ikuta K, Ozaki J, Suzuki Y, Suzuki N, Sato T, Suzomori K: Gonadotropins and cytokines affect luteal function through control of apoptosis in human luteinized granulosa cells. JClin Endocrinol Metab **85,** 1620-1626, 2000

McGee E, Spears N, Minami S, Hu SY, Chun SY, Biling H, Hsueh AJW: Preantral ovarian follicles in serum-free culture: Supression of apoptosis after activation of the cyclic guanosine 3',5'-ponophosphate pathway and stimulation of growth and differentiation by follicle-stimulating hormone. Endocrinology **138,** 2417-2424, 1997

MEIKRANTZ W, SCHLEGEL R: Apoptosis and the cell cycle. J Cell Biochem **58,** 160-174, 1995

MURPHY DM: Models of luteinization. BiolReprod **63,** 2-11, 2000

NOUBANI A, FAROOKHI R, GUTKOWSKA J: B-type natriuretic peptide receptor expression and activity are hormonally regulated in rat ovarian cells. Endocrinology **141,** 551-558, 2000

PASCADOR N, STOCCO D, MURPHY B: Growth factor modulation of steroidogenic acute regulatory protein and luteinization in pig ovary. Biol Reprod **60,** 1453-1461, 1999

RICHARDS JS: Hormonal control of gene expression in the ovary. Endocrine Reviews **15,** 725-751, 1994

RUIZ-CORTES ZT, MEN T, PALIN MF, DOWNEY BR, LACROIX DA, MURPHY BD: Porcine leptin receptor: molecular structure and expression in the ovary. Molecular Reproduction Dev **56,** 465-474, 2000

RUIZ-CORTES ZT, MARTEL-KENNES Y, GEVRY NY, DOWNEY BR, PALIN MF, MURPHY BD: Biphasic effects on leptin in porcine granulosa cells. Biol Reproduction **68,** 789-796, 2003

SHIKONE T, YAMOTO M, KOKAWA K, YOMASHITA K, NISHIMORI K, NAKANO R: Apoptosis of human corpora lutea during cyclic luteal regression and early pregnancy. J Clin Endocrinol Metab **81,** 2376-2380,1996

SONG A, KUANG Y, DIXIT VM, VINCENZ C: Boo, a novel negative regulator of cell death, interacts with Apaf-1. EMBO **18,** 167-170 1999

STOKLOSOWA S, GREGORASZCZUK EL, CHANNING CP: Estrogen and progesterone secretion by isolated cultured porcine thecal and granulose cells. Biol Reprod **26,** 943-952, 1982

STREEGERS EAP, HOLLANDERS JMG, JONGSMA HW, HEIN PR: Atrial natriuretic peptide and progesterone in ovarian follicular fluid. Gynecol Obstetr Invest **28,** 185-187, 1990

SWENSON E: Regulation of apoptosis and steroidogenesis in ovarian preovulatory granulose cells PhD Thesis, Goteborg University, Goteborg 2003

SZAFRANSKA B, ZIECIK A, OKRASA S: Primary antisera against selected steroids or proteins and secondary antisera against gamma-globulins - an available tool for studies of reproductive processes. Reprod Biol **2,** 187-204, 2002

WIDLAK P: The DFF40/CAD endonuclease and its role in apoptosis. Acta Biochim Pol. **47,** 1037- 1044, 2000

Corresponding author: Assoc. prof. Maria D. Ivanova
Institute of Biology and Immunology of Reproduction,
Bul. Tzarigradsko shosse 73,
Sofia 1113, Bulgaria
e-mail: maria_d_ivanova@yahoo.co.uk
phone: 0359 2 971 01 54;
fax: 0359 2 720 022