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EXPRESSION OF CASPASE-1 AND CASPASE-3 mRNA IN THE DEVELOPING AND REGRESSING OVARY OF THE CHICKEN EMBRYO

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In the chicken female embryo the gonads develop asymmetrically. Only the left ovary becomes a functional gonad and the right one regresses. The aim of this study was to evaluate the participation of apoptosis in the processes of development and regression by detection of mRNA expression of initiator caspase-1 and executive caspase-3 in the left and right ovaries at days 12 (E12) and 15 (E15) of embryogenesis. Total RNA from the ovaries was isolated and the mRNA expression of the analyzed genes was detected by using the RT-PCR method. The expression of caspase-1 and caspase-3 was observed in the left and right ovaries of the chicken embryo at days 12 and 15 of embryogenesis. In the left ovary the expression of the two analyzed genes did not differ between the two analyzed stages. In the right ovary the expression of caspase-1 mRNA significantly decreased on the 15th day of incubation in comparison with the 12th day, whereas the expression of caspase-3 significantly increased on the 15th day. The observed changes in mRNA expression of caspases suggest that initiator caspase-1 is expressed in the early phase of ovary regression, while caspase-3 is expressed in the later stage, which affects the execution of apoptosis.

Key words: caspases, chicken embryo, ovary

INTRODUCTION

In chicken embryos, up to day 6 of incubation (E6) the gonadal sex is morphologically identical and bipotential (ROMANOFF, 1960). From E8 to E10, the gonads differentiate and develop into asymmetric ovaries in ZW heterozygotes and into symmetric testes in ZZ homozygotes. Particular growth and development of the left ovary during embryogenesis makes it 2.4-fold longer than the right ovary in newly-hatched chicks. Starting at E7, the ovarian cords in the left ovary develop to form a cortex, whereas the medulla is composed of epithelium derived from medullary cords (primary cords). In contrast, the right ovary stops its further development from day 7 onward and finally the right ovary has only a medulla (UKESHIMA and FUJIMOTO, 1991, SMITH and SINCLAIR, 2004, GONZALEZ-MORAN, 2005).

In the female the hormonally regulated failure of germ cells at different stages of pre- and postnatal development is considered a normal physiological course of action ensuring the greatest ability to ovulate viable, fertilizable oocytes. The

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total amount of primary oocytes within the ovary of a chicken has been estimated to increase from 28,000 at E9 to 680,000 at E17, and then to decrease to 480,000 by the time of hatching. Only a small fraction of these primary oocytes (200-500) develops to the preovulatory stage within the lifespan (Johnson and Woods, 2007).

Susceptibility to apoptosis correlates with the expression of factors which act as a stimulator or suppressor of this process. The key regulators of apoptosis are caspases, a family of intracellular cysteine proteases linked both to the initial and final stages of apoptosis in almost every type of vertebrate cells (for review, see Johnson, 1996, 2002). Six caspase orthologues have been characterized in Aves (chicken caspase-1,-2,-3,-6,-8 and -9) and each of these is expressed within the ovary (Johnson et al., 1997, 1998; Johnson and Bridgham, 2000; Hrabia and Jankoś, 2011). The caspase family includes initiator caspases (-1, -2, -8, -9, -10) which can auto-catalyze to form active subunits and executioner caspases (-3, -6, -7) which require other proteases, such as the initiator caspases, for processing.

Apoptosis is regulated temporally and spatially, changing the patterns reflecting the phases of differentiation, morphogenesis and growth of tissues and organs. Several studies on cell death in embryonic chicks have been carried out. Most of them focused on individual organ systems: heart (Pexieder, 1975), sclerotome (Sanders, 1997), tail bud (Schoenwolf, 1981) or neural crest (Lumsden et al., 1991). The onset of cell death was detected before gastrulation and TUNEL-positive cells were localized around 3 hours after the onset of incubation (Hirata and Hall, 2000). Apoptotic cells were detected also in the gastrulating embryo (Sanders and Wride, 1997).

The mechanism of apoptosis in the developing and regressing ovaries of the chicken embryo is not known, therefore the aim of the present study was to analyse the mRNA expression of initiator caspase-1 and effector caspase-3 in the left and right female gonads on days 12 and 15 of embryogenesis.

MATERIALS AND METHODS

The experiment was carried out in accordance with the principles and procedures of the Local Animal Ethics Committee in Krakow. Fertilized eggs (n=180) of Hy-Line Brown strain were set in an incubator (Masalles 65 DIGIT) and were incubated under standard conditions [1-18 days of incubation: t = 37.8°C, relative humidity (RH) = 55%, 19-21 days of incubation; t = 37.2°C, RH = 70%]. They were candled on day 5 of the incubation to eliminate unfertilized eggs and dead embryos. On embryonic days 12 (E12) and 15 (E15) the embryos were decapitated. They were sexed and subsequently the left and right ovaries were isolated for RT-PCR analysis.

RT-PCR

The collected tissues were placed in RNAlater and stored at -20^{0}C until total RNA isolation. The total RNA of each tissue was isolated by using TRI-Reagent (Molecular Research Center, Cincinnati, USA), according to the method described by Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). The tissues were homogenized by using UltraTurrax T25 (IKA-Labortechnik, Staufen, Germany), and then phase separation was made with bromochloropropane. RNA was precipitated from the aqueous phase by mixing with isopropanol, washed with 75 % ethanol and subsequently dissolved in pure RNAase free water (Promega, Madison, USA). The concentration of RNA was measured spectrophotometrically using Biophotometer (Eppendorf, Hamburg, Germany). Each sample was tested for RNA degradation by separation on agarose gel and spectrophotometrical analysis of the absorbance 260/280 nm ratio. The 260/280 nm ratio of RNA was 1.65-1.85. To exclude genomic DNA contamination, RNA samples were treated with DNAse (RQ1 RNase-Free DNase, Promega). Reverse transcription (RT) reactions were performed in 20 µl volume. The reaction mixture contained 5 µg of total RNA, 200 U M-MuLV reverse transcriptase (Fermentas, Vilnius, Lithuania), 0,5 µg oligo(dT)$_{18}$ as a primer, 1 mM of each dNTP, 20 U Ribonuclease Inhibitor (Fermentas) and 4 µl of 5 x reaction buffer containing 250 mM Tris-HCl, 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT (Fermentas). The resulting first strand cDNA (1 µl) was used for PCR reactions with primers specific for caspase-1 and caspase-3. As a reference gene, GAPDH was used. The sequences of primers, their genomic localization and product

TABLE 1. Characterization of primers and PCR conditions used in this study.

Gene	GenBank Accession Number	Primer sequence	PCR product	Annealing temperature
Caspase-1	AF031351.1	F: 5'-GATACGTGACTCCATCGACCC-3' R: 5'-CTTCTTCAGCATTGTAGTCC-3'	313 bp	55°C
Caspase-3	AF083029	F: 5'-AGCAAGCGAAGCAGTTTTGT-3' R: 5'-TGCGTTCCTCCAGGAGTAGT-3'	300 bp	62°C
GAPDH	K01458	F:5'-GTGGAGAGATGACAGAGGTG-3' R:5'-AACAAGCTTGACGAAATGGT-3'	349 bp	60 °C

size are shown in Table 1. The 12.5-µl PCR reaction mixture contained 1µl of cDNA, 0.2 mM of each dNTP, 0.2 µM of each primer, 1.5 mM $MgCl_2$ and 0.312 U of TaqDNA Polimerase (Fermentas). GAPDH gene expression was acquired in a separate sample. All the PCR reactions were performed using the Thermocycler Gradient (Eppendorf). The cycle profiles were programmed as follows: initial template denaturation for 5 min at 95°C, denaturation at 95°C for 30 s, annealing at gene specific temperature (Table 1) for 30 s, followed by extension at 72°C for 30 s. Thirty (GAPDH and caspase-3) or 27 (caspase-1) cycles of the appropriate profile were run, and the final extension step for each gene amplification was increased to 7 min at 72°C. Amplification products were separated by electrophoresis on 1.5 % agarose gel containing ethidium bromide. The PCR products were semiquantitatively estimated from the density of the gel band using the Scion Image for Windows Software (Scion Corporation, Maryland, USA). The relative density of each gene product was compared with GAPDH products, and expressed as the mean of 6 samples.

Statistical analysis

For statistical evaluation of the results two-way analysis of variance (ANOVA) followed by Duncan's multiple range test was applied. Values are expressed as the mean ± SEM from 6 determinations and considered significantly different at $P<0.05$.

RESULTS

The expression of caspase-1 and caspase-3 was detected in the regressing right and developing left ovaries in two analysed stages of chicken embryo development (Fig.2, 3). In the left ovary the expression of the two analyzed genes did not differ between the two analyzed stages. In the right ovary the expression of caspase-1 mRNA (Fig. 3A) significantly decreased on the 15th day of incubation (0.19±0.06) in comparison with day 12 (0.46±0.11) whereas the expression of caspase-3 (Fig. 3B) significantly increased on the 15th day (1.49±0.22) in comparison with day 12 (0.82±0.18). The expression of caspase-1 mRNA at E15 was significantly lower in the right ovary than in the left one and it was also significantly lower in comparison with the mRNA expression of caspase-3 in the same tissue.

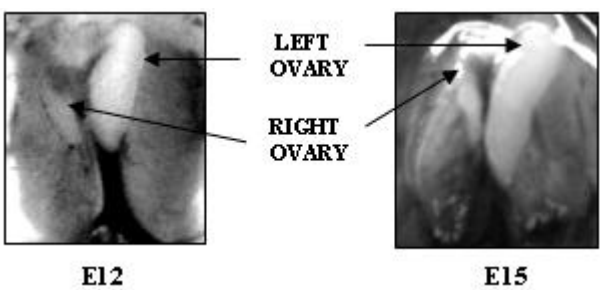

Fig. 1. Regressing (right) and developing (left) ovaries of the chicken embryo.

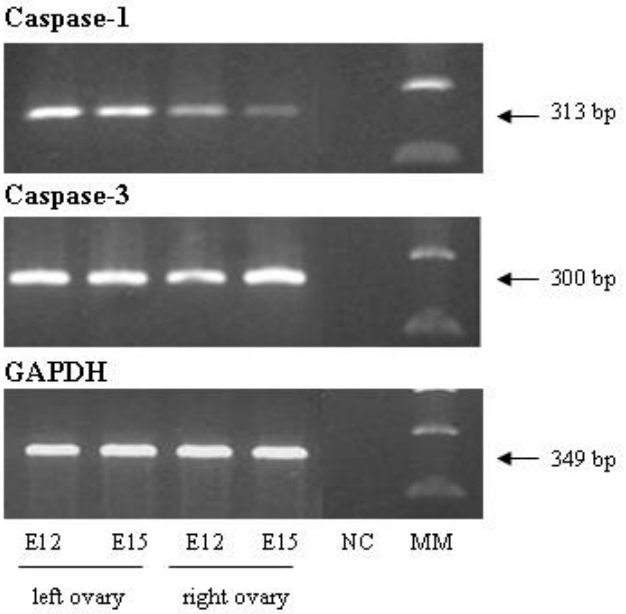

Fig. 2. The expression of mRNA encoding: caspase-1, caspase-3 and GAPDH in the female gonads of the chicken embryo using the RT-PCR technique. E12, E15 – embryonic days 12 and 15, respectively; NC-negative control without cDNA; MM – molecular marker (100-1000bp).

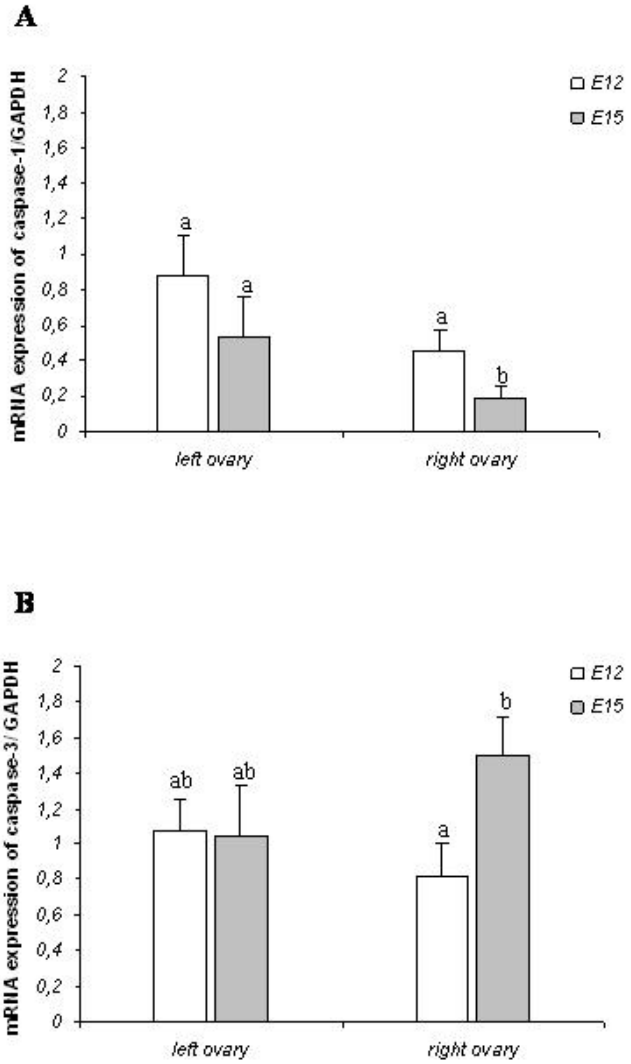

Fig. 3. Semiquantitative analysis of the mRNA expression of caspase-1 (**A**) and caspase-3 (**B**) in the ovary of the chicken embryo. The values are expressed as means ± SEM; n=6. Bars without common superscripts are significantly different ($p<0.05$).

DISCUSSION

In the present study the mRNA expression of caspase-1 and caspase-3 was detected in the developing left and regressing right ovaries of the chicken embryo at days 12 and 15 of incubation. There are some data indicating that caspase activation can support the processes which are relevant for life (Abraham and Shaham 2004; Launay et al, 2005), not only for apoptotic death. Apoptosis may serve multiple functions during development, including: removal of damaged, excess or abnormal cells; sculpting of structures during morphogenesis; removal of structures, for example during metamorphosis. Many authors show that apoptosis may be a feature of rapid growth in specific regions at early stages of development. Zakeri et al. (2005) showed for the first time that nonlethal caspase activity occurs before implantation. Apoptotic cells have been previously detected in the early phase of the chicken embryogenesis at sites of intensive growth, morphogenesis and cell migration (Hirata and Hall, 2000), as well as in the ovary of embryonic and post-hatched Japanese quail (Yoshimura and Nishikori, 2004). Caspase-1 (Johnson et al., 1998, Hrabia and Jankoś 2011), caspase-2 (Johnson et al. 1997, Hrabia and Jankoś 2011), caspase-3 (Johnson and Bridgham 2000; Hrabia and Jankoś 2011) and -6 (Johnson and Bridgham 2000) have been previously detected in the hen ovary. It has also been found that caspases-1, -2 and -3 are involved in regression of the chicken ovary during moulting induced by long-term feed withdrawal (Anish et al., 2008).

Increased concentrations of activated caspase-3 was observed in granulosa cells from atretic follicles whereas in healthy follicles almost exclusively

the inactive (unprocessed) form of caspase-3 was detected (see review JOHNSON and BRIDGHAM 2002). In the current investigation significant increase in caspase-3 mRNA expression in the regressing right ovary on the 15th day of embryogenesis in comparison with day 12 was observed. It may suggest that caspase-3 is a key apoptosis effector during gonadal tissue regression in the female chicken during embryogenesis, like in adults.

In the present study the expression of caspase-1 mRNA was detected in the developing and regressing ovaries and it significantly decreased in the regressing ovary on E15, whereas caspase-3 increased at this stage. It may suggest that in the earlier phase of tissue regression initiator caspase participates in the regulation of this process, whereas in the later phase effector caspases play a key role in this course of action.

Gonadal development in the chicken depends on estrogens. They play a major role in the regulation of growth, development, homeostasis and programmed cell death (AMSTERDAM et al., 1997). Estradiol has been detected in the chicken gonads as early as day 3.5 of embryogenesis, while synthesis of estradiol is started in the ovarian medulla between days 6.5 and 12.5 and in the ovarian cortex on day 13.5 of embryogenesis (WOODS and EARTON, 1978). Additionally, estrogen receptor was expressed in the left but not the right ovarian cortex (GASC 1980; SCHEIB 1983; ANDREWS et al., 1997; NAKABAYASHI et al., 1998). Estrogens prevent apoptosis through ER-dependent mechanisms and promote cell survival, at least in part, by diminishing the expression of genes encoding apoptosis promoting proteins. Caspase expression may be also regulated by steroid hormones. This suggestion may be supported by observations of MONROE et al. (2002) who demonstrated that in the chicken oviduct caspase-1 and -2 are transcriptionally stimulated by estrogen withdrawal, whereas caspase-3 and -6 gene expression levels are not influenced by estrogens, suggesting that initiator caspases are regulated differently than executioner caspases. In the present study caspase-1 mRNA expression decreased in the regressing ovary, where estrogen receptors were not expressed and protective estrogen activity against programmed death was not present. MONROE et al. (2002) showed that caspase-2, 3-like and -6 enzyme activity levels became elevated when the chicken oviduct was undergoing apoptosis. It may suggest that the expression of caspases in chicken reproductive tissue regression is regulated posttranscriptionally. The fact that estrogen regulation of caspase-1 gene expression is directly correlated with apoptosis in the oviduct, as well as in mammary gland and ovary in mammals, suggests that caspase-1 plays a critical role in apoptosis in reproductive tissues (MONROE et al., 2002; MARTI et al., 1999; RUEDA et al., 1999). In the current investigation caspase-1 mRNA expression was lower than caspase-3 mRNA expression in the regressing ovary. The amounts of mRNA transcript encoding caspase-1 compared with mRNA transcript encoding caspase-3 was extremely low also in hen granulosa cells (JOHNSON et al., 1998). This observation is in agreement with previous study of MONROE et al. (2002), who shown generally lower activity level of initiator caspases than executioner caspases in the chicken oviduct.

To summarize, results of the present study suggest, that caspases not only regulate apoptotic death but they also regulate developmental processes in the chicken reproductive tissues during embryogenesis. Nevertheless, the specific mechanism regulating caspases expression and activity during development and regression of the ovary requires further studies.

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