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Immunolocalization of Leptin Receptor and mRNA Expression of Leptin and Estrogen Receptors as well as Caspases in the Chorioallantoic Membrane (CAM) of the Chicken Embryo*

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The chicken chorioallantoic membrane (CAM) is used as a model in tests of angiogenesis, the biocompatibility of materials as well as tumor invasive potential. To assess the properties of CAM tissue, the localization of leptin receptor in the CAM, and the mRNA expression of two leptin receptor isoforms, estrogen receptors (ERα and ERβ) and caspases (-1 and -3) in the CAM on embryonic days 12 (E12), 15 (E15) and 18 (E18) were investigated. The leptin receptor was immunolocalized in each structure of the CAM (chorionic epithelium, allantoic epithelium, mesodermal layer and the walls of blood vessels) and did not change among analyzed stages of embryonic development (E12, E15 and E18) and between sexes. Expression of mRNA of genes encoding leptin and estrogen receptors as well as caspases was detected in the CAM of female and male chicken embryos at all three analysed stages of development. The relative mRNA expression of the long form of leptin receptor exceeded that of its short isoform. The mRNA expression of ERα was significantly higher than ERβ as well as caspase-3 in comparison with caspase-1. There were no differences in mRNA expression of these genes between sexes and among analyzed developmental days. The results indicate that the CAM is a target tissue for leptin as well as for estrogens and that CAM development is partially regulated by caspase-1 and caspase-3 dependent cell death. These results should be taken into consideration in studies in which the CAM is used as an experimental model.

Key words: leptin receptors, estrogen receptors, caspases, chorioallantoic membrane, chicken embryo.

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The chicken chorioallantoic membrane (CAM) is an extra-embryonic structure formed by fusion of the chorion and allantois during early avian development. The CAM is responsible for carrying out three significant processes: (1) respiration, (2) storage of urea, ammonia and uric acid produced by the embryo, (3) resorption of Ca^{2+} from the shell through the chorionic epithelium to be transported into the vasculature of the embryo.

CAM development starts at day 4 of incubation. Undifferentiated blood vessels grow very rapidly until day 8, originating a network of capillaries that migrate to reside in the area at the base of the chorion and mediate gas exchange with the external environment. Rapid capillary proliferation continues until day 11 and the vascular system attains its final arrangement three days before hatching (AUSPRUNK *et al.* 1974). Angiogenesis is the formation of new blood vessels from the existing vasculature, a complex multistep process involving the proliferation, migration, and remodeling of endothelial cells in response to growth factors and cytokines produced by endothelial and non-endothelial cells. One of these factors is leptin, a member of the polypeptide hormone family (SIERRA-HONIGMANN *et al.* 1998; PARK *et al.* 2001;

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SUGANAMI *et al*. 2004; SCHIEKOFER *et al*. 2005). Leptin has been found in many vertebrates; however, the existence of leptin in birds has been controversial (SHARP *et al*. 2008). The interaction between leptin and leptin receptor (LEPR) is well established; it is conserved across diverse taxa (RØNNESTAD *et al*. 2010; HAMMOND *et al.* 2012; PROKOP *et al.* 2012, 2014), but surprisingly in the *in-silico* evaluation of 35 taxa of PROKOP *et al.* (2012), only bird leptins did not form stable complexes with their receptors. Recently it has been indicated that avian blood contains a leptin-like molecule that specifically binds to LEPR (OHKUBO *et al*. 2014). The putative leptin gene of zebra finch has only 26% and 29% amino acid sequence identity with human and mouse leptin, respectively (HUANG *et al.* 2014). In turn, FRIEDMAN-EINAT *et al*. (2014) discovered the same gene in the rock dove with 30% identity to the human ortholog. These discoveries indicate that previous reports (TAOUIS *et al*. 1998) and database depositions of leptin genes in birds with more than 90% identity with mammalian leptin, the credibility of which was immediately undermined (FRIEDMAN-EINAT *et al.* 1999), are probably due to contamination of samples with mammalian leptin.

Previously it was considered that the principal site of leptin synthesis in birds is the liver (TAOUIS *et al*. 1998; ASHWELL *et al.* 1999). Recently it has been discovered that leptin expression occurs almost exclusively in the pituitary of the zebra finch (HUANG *et al.* 2014), whereas in the rock dove predominant expression was recorded in the liver and gonads, and poor expression in the pituitary (FRIEDMAN-EINAT *et al.* 2014). Leptin binds to leptin receptors on the plasma membrane, activating several intracellular processes via the Janus kinase (Jak) and STAT pathway (DENVER *et al.* 2011). The genes encoding the long form (HOREV *et al.* 2000; OHKUBO *et al.* 2000; PACZOSKA-ELIASIEWICZ *et al.* 2003; RICHARDS & POCH 2003) as well as the short isoform of leptin receptor (LIU *et al*. 2006) have been cloned and detected in birds.

The process of differentiation of cells and development of embryonic tissue depends on a variety of mechanisms regulating the elimination of cells, including programmed cell death, i.e. apoptosis. Caspases are key regulators of apoptosis and have been characterized in birds (chicken caspase-1,-2,-3,-6,-8 and -9) (JOHNSON *et al*. 1997, 1998; JOHNSON & BRIDGHAM 2000). Two general pathways involving initiator caspases have been identified: an upstream pathway promoted by the activation of tumor necrosis factor receptor (STRASSER & NEWTON 1999), and an alternative pathway involving an initiator caspase and release of mitochondrial cytochrome C (EKERT *et al.* 2001). Autoactivation of either caspase-8 or caspase-9 eventually initiates the processing of an effector caspase (-3, -6 or -7), and the full potentiation of the caspase cascade. Pyroptosis, another type of programmed cell death, is triggered by caspase-1 after its activation by various pathogens and other activating signals. Formation of the inflammasome results in processing of inactive procaspase-1 into an active caspase-1, which subsequently activates the proinflammatory cytokines (ARAI *et al*. 1990) and induces pyroptosis (VANAJA *et al.* 2015). Similar to oncosis and unlike apoptosis, pyroptosis results in cellular lysis and release of cytosolic contents to the extracellular space (MIAO *et al*. 2011).

Over the past few decades efforts have been made to reduce, replace, and refine experiments for ethical use of experimental animals. The chicken CAM is used as an experimental model in tests of the biocompatibility/toxicology of organic and inorganic materials (SPANEL-BOROWSKI 1989; VALDES *et al*. 2002), assessments of the ability of bacterial strains to invade epithelial barriers (ADAM *et al*. 2002), analysis of metastatic (LOKMAN *et al*. 2012) and developmental growth potencies of grafted tumor and embryonic tissue samples (NAVARRO *et al.* 2003), and the angiogenic response to a defined stimulus (RIBATTI *et al*. 2001; RICHARDSON & SINGH 2003). However, the functional and physiological properties of the CAM are not fully understood. Experimental treatment of the CAM with human leptin stimulated neovascularization (BOULOUMIE *et al.* 1998; RIBATTI *et al.* 2001), suggest the presence of leptin receptors in this tissue. In order to partially characterize the endocrine properties of the CAM, mechanism of CAM development and sensitivity to hormones, we immunolocalized leptin receptor (the leptin binding domain of the receptor) in this membrane and analyzed the mRNA expression of the long and short forms of the leptin receptor as well as estrogen receptors (α and β). Additionally, in order to assess the participation of programmed cell death in CAM growth and development, the mRNA expression of caspases (-1 and -3) was also analyzed.

Material and Methods

Fertilized eggs (n=50) of the Ross Broiler Breeder 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%). They were candled on day 5 of the incubation to eliminate unfertilized eggs and dead embryos and subsequently, 36 eggs were selected on embryonic days 12 (E12), 15 (E15) and 18 (E18) (six embryos of each group). They were decapitated and sexed after

analysis of morphology of gonads. Fragments of the chorioallantoic membrane of female and male embryos were isolated for histological analysis or kept frozen in liquid nitrogen until RNA isolation.

Immunohistochemical localization of leptin receptor in the chicken chorioallantoic membrane

Fragments of the CAM were fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.4) and embedded in paraffin by routine procedure. The 6 μm thick sections of the CAM were stained by: (1) routine haematoxylin-eosin technique or (2) immunohistochemical technique with specific antibody for chicken leptin receptor, for which sections were dewaxed in xylene and hydrated through a series of graded ethanol and microwaved in 10mM citric acid (pH 6.0) for antigen retrieval. Next, they were incubated in 1% H_2O_2 to inhibit endogenous peroxidase activity. Afterwards, sections were incubated with normal goat serum and then with rabbit antiserum raised against recombinant leptin-binding domain (anti-LBD; aminoacids 419-624) of chicken leptin receptor purified by affinity chromatography. The sections were then incubated with the secondary antibody (biotinylated goat anti-rabbit, 1:350 in PBS; Vector Laboratories) and with the avidin-biotin-peroxidase complex StreptABComplex/HPR (DakoCytomation, Denmark). The colour reaction was developed by incubation with a DAB-H_2O_2 mixture. The negative control was incubated without primary antibody or the primary antibody was preincubated with recombinant chicken LBD before incubation with tissue sections. The resulting immunocytochemical staining was the same as in the negative control, confirming the specificity of the primary antibody against leptin receptor.

RNA isolation

The total RNA of each tissue was isolated by using TRI-Reagent (Molecular Research Center, Cincinnati, USA), according to the manufacturer's protocol (modified method described by CHOMCZYNSKI and SACCHI (1987)). The tissues were homogenized by using UltraTurrax T25 (IKA-Labortechnik, Staufen, Germany) and subsequently phase separation was done with bromochloropropane. RNA was precipitated from the aqueous phase by mixing with isopropanol, washing with 75 % ethanol and dissolving in pure RNAse free water (Promega, Madison, USA). The concentration of RNA was measured spectrophotometrically using a Biophotometer (Eppendorf, Hamburg, Germany). Each sample was tested for RNA degradation by separation on agarose gels and spectrophotometrical analysis of absorbance at 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 and PCR reactions for leptin receptors, estrogen receptors and caspases

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 $MgCl_2$, and 50 mM DTT (Fermentas). The resulting first strand cDNA (1 μl) was used for PCR reactions. The expression of mRNA encoding leptin receptor (long and short isoforms), estrogen receptors (α and β) and caspases (-1 and -3) in the CAM was determined. 18S rRNA was used as a reference gene. The primer sequences, their localization in the genome, and product size are shown in Table 1. The 25 ml PCR reaction mixture contained 1 μl of cDNA, 0.2 mM of each dNTP, 0.2 μM of each primer, 1.5 mM $MgCl_2$, and 0.625 U of TaqDNA polymerase (Fermentas). 18S rRNA gene expression was acquired in a separate sample. All PCR reactions were performed in a 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. Table 1 lists the number of cycles and PCR profiles; the final extension step for amplification was increased to 7 min at 72°C. Amplification products were separated by electrophoresis on 1.5 % agarose gels containing ethidium bromide. The PCR products were semi-quantitatively estimated from the density of the gel band using Scion Image for Windows Software (Scion Corporation, Maryland, USA). The relative density of each gene product was compared with 18S rRNA products, and expressed as the mean of 6 samples.

Statistical analysis

Two-way analysis of variance (ANOVA), with two main effects: sex and stage of embryo development, followed by Duncan's multiple range test was applied to analyze the data. Values are expressed as the mean ± SEM from six determinations and considered significantly different at $P<0.05$.

Results

Immunohistochemical analysis of leptin receptor in the CAM tissue

Immunohistochemical analysis of the CAM showed that leptin receptor is localized both in the female and male embryos at embryonic days 12 (E12), 15 (E15) and 18 (E18). The intensity of the immunopositive reaction for leptin binding domain of leptin receptor did not significantly differ between the chorionic epithelium and allantoic layer with extended blood vessels. There were no differences in leptin receptor immunoreactivity among the analyzed stages of development or between sexes. Preincubation of 1^{o} antibody against leptin binding domain with chicken LBD or incubation of tissue slides with TBST instead of 1^{o} antibody against leptin receptor abolished staining (Fig. 1).

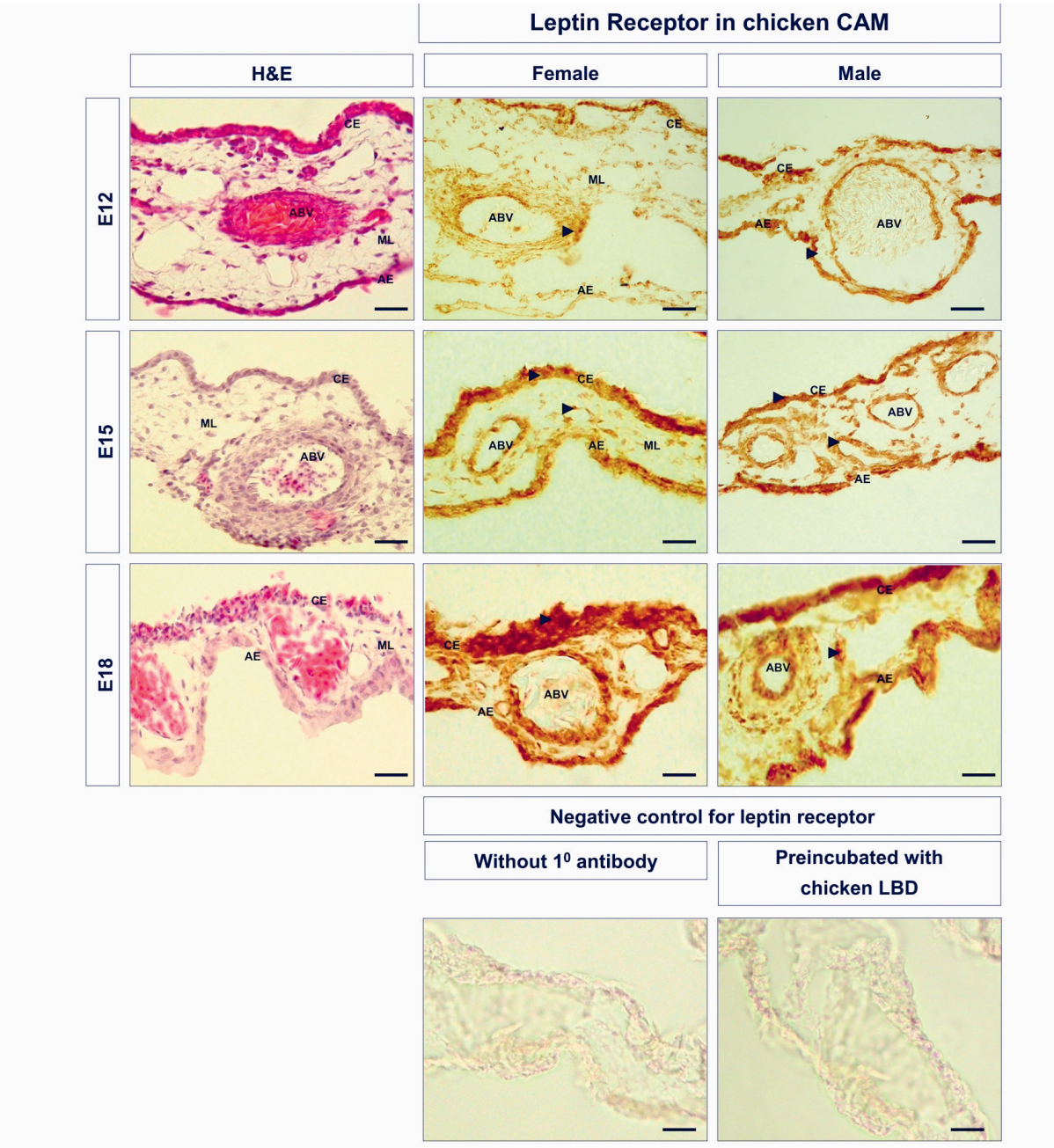

Fig. 1 Haematoxylin and eosin staining of chorioallantoic membrane (CAM) of chicken embryos and immunolocalization of leptin receptors in the female and male embryonic CAM on day 12, 15 and 18 of embryogenesis (E12, E15 and E18). Negative control tissue was incubated without 1^{0} antibody or 1^{0} antibody was preincubated with recombinant protein – chicken LBD (leptin binding domain of chicken leptin receptor). ABV – allantoic blood vessels, CE – chorionic epithelium, AE – allantoic epithelium, ML – mesodermal layer, ▶ immunoreactive cells (leptin receptor). Scale bar = 20 μm.

Table 1

Positions of oligonucleotide primers in mRNA sequence, GeneBank Accession Numbers, sequences of amplified gene primers, annealing temperature of PCR reaction, number of cycles and product size

Gene	Position	Gene Bank Accession Number	Primers sequences: (5'-3')	Annealing temperature (°C)	Number of cycles in PCR	Product size (bp)
LepR (Long isoform)	2641-2660 3125-3144	AF169827	F: CCAAAGATGGGTATGCCAGT R: GCCCACCTGATCTGGAGTTA	60	30	504
LepR (Short isoform)	1-18 197-216	AY34719	F: GAATGAAGAAACTGCTCTGG R: TACAACTCATGTGCTGCATT	56	30	216
ERα	1522-1542 1801-1821	X03805.1	F: GTGCCTTAAGTCCATCATCCT R: GCGTCCAGCATCTCCAGTAAG	58	30	300
ERβ	1374-1394 1657-1677	AB036415	F: TGATATGCTCCTGGCCATGAC R: CTTCATGCTCAGCAGATGCTC	55	30	304
Caspase-1	1183-1203 1476-1495	AF031351.1	F: GATACGTGACTCCATCGACCC R: CTTCTTCAGCATTGTAGTCC	55	27	313
Caspase-3	696-715 934-956	AF083029.1	F: TTAGATTCTGGTATTGAAGC R: GAAATCCTGTCGAGTGGAGCAGG	62	30	261
18S rRNA	160-179 308-326	AF173612	F: CGCGTGCATTTATCAGACCA R: ACCCGTGGTCACCATGGTA	60	30	167

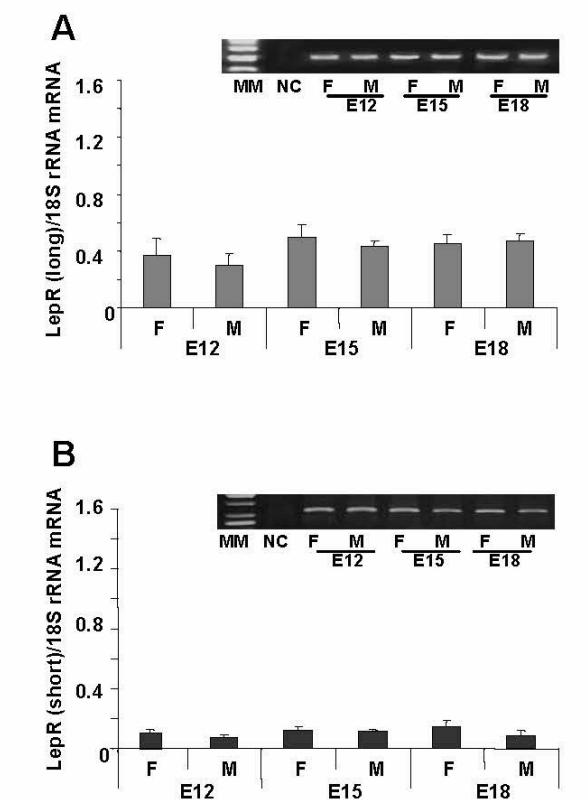

Fig. 2. Semiquantitative analysis of mRNA expression of long (A) and short (B) isoforms of leptin receptor in the chicken CAM (means ± SEM, n=6). F – female group, M – male group. E12, E15, E18 – days 12, 15, and 18 of chicken embryogenesis.

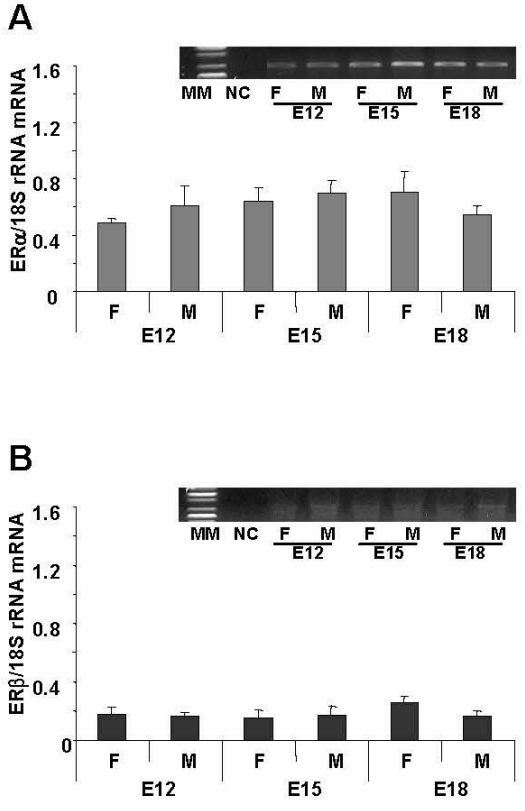

Fig. 3. Semiquantitative analysis of mRNA expression of estrogen receptor α (A) and β (B) in the chicken CAM (means ± SEM, n=6). F – female group, M – male group. E12, E15, E18 – day 12, 15, and 18 of chicken embryogenesis.

mRNA expression of leptin receptors, estrogen receptors and caspases in the CAM

The analysis of mRNA expression revealed that both long and short isoforms of leptin receptor mRNA are expressed in the chicken CAM on the 12th, 15th, and 18th day of embryogenesis. The mean relative expression of the long form of leptin receptor was not significantly different among analyzed stages of embryogenesis or between sexes. The values were 0.437 ± 0.041 in female and 0.401 ± 0.066 in male CAM. The expression of mRNA encoding the short form of leptin receptor was significantly lower than expression of the long form of receptor (0.120 ± 0.020 and 0.420 ± 0.061, respectively). There were no differences in the short isoform of leptin receptor mRNA expression between female and male CAM (0.138 ± 0.018 and 0.101 ± 0.018, respectively) (Fig. 2).

The mRNA expression of estrogen receptors α and β was detected in the chicken CAM in each analyzed stage of embryogenesis. The expression of ERα (0.613 ± 0.064) was significantly higher than the expression of ERβ (0.179 ± 0.021). Neither mRNA expression of ERα nor ERβ was significantly different in male and female CAM (Fig. 3).

As shown in Fig. 4, mRNA expression of caspase-1 and caspase-3 was detected in all examined experimental groups. The relative expression of caspase-1 was significantly lower than caspase-3 (0.597 ± 0.041 and 1.193 ± 0.031, respectively). The mRNA expression of caspase-1 as well as caspase-3 did not differ between female and male CAM (Fig. 4).

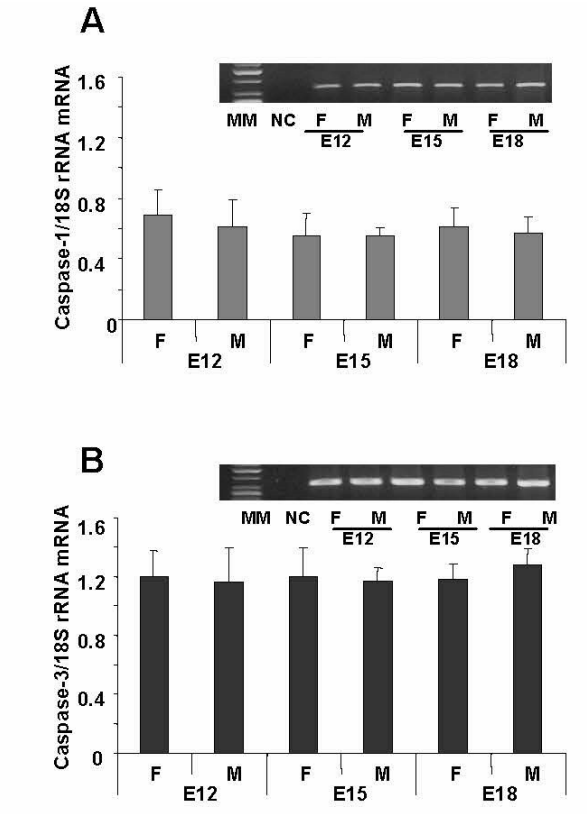

Fig. 4. Semiquantitative analysis of mRNA expression of caspase-1 (A) and caspase-3 (B) in the chicken CAM (means ± SEM, n=6). F – female group, M – male group. E12, E15, E18 – day 12, 15, and 18 of chicken embryogenesis.

Discussion

In the present study, for the first time the leptin binding domain of leptin receptors was immunolocalized in the male and female CAM. There were no differences in the intensity of reactions between sexes and among analyzed stages of embryogenesis, confirmed by mRNA expression analysis. It was also shown that both long and short isoforms of leptin receptor mRNA are expressed in the CAM membrane. It cannot be excluded that the presence of receptors for leptin in the CAM may be related to the process of angiogenesis. This assumption is supported by an investigation in which quail embryos treated with leptin had a significant increase in vascular complexity and density (TALAVERA-ADAME *et al.* 2008). On the other hand, SU *et al.* (2012) observed the opposite effect, i.e. significant antiangiogenic activity of leptin, but, interestingly, only in females. This suggests that the mechanism of leptin action on angiogenesis is complicated and requires further study. Nevertheless, detection of leptin receptor mRNAs and protein in the CAM tissue suggest that the influence of leptin on CAM development may depend on the ability of leptin receptors localized in the CAM tissue to interact with the circulating leptin hormone.

Beyond affecting angiogenesis, leptin may also be involved in the regulation of calcium absorption from the egg shell, the major source of calcium for the development of high-calcium consuming organs, e.g. the skeleton, muscles and brain (TUAN *et al.* 1991; BLOM & LILJA 2004). The domestic chicken begins to mobilize calcium between days 7 and 8 of incubation, corresponding to the initiation of mineralization of skeletal tissue. However, until day 10, the egg yolk is the main source of calcium (TUAN *et al.* 1991; KARLSON & LILJA 2008), between days 10 and 12, calcium is also mobilized from the shell. At the end of embryonic development, more than 80% of the calcium is derived from the shell (SIMKISS 1975; TUAN 1987). It cannot be excluded that leptin also participates in the process of calcium resorption from the egg shell and incorporation into the embryonic body. The mRNA expression of leptin receptors did not change among analyzed stages of development, whereas the rate of incorporation of calcium into the chorioallantois is not stable during embryogenesis. It is greater for 15- than 12-day-old embryos (CROOKS & SIMKISS 1975). The key step for ion transport through the membrane is the 14th

day of incubation (MORIARTY & HOGBEN 1970) and leptin may be one of many regulatory proteins involved in the process of mineralization in chicken embryos.

This assumption can be supported by a previous study (GRZEGORZEWSKA *et al*. 2008) that detected the expression of leptin receptor mRNA and protein in the oviduct of the laying hen. Taking into consideration this observation, it cannot be excluded that leptin is involved in a variety of calcium transport processes in birds. It is well known that leptin may act as a regulator of the bone formation process (PARHAMI *et al.* 2001; GALUS & WŁODARSKI 2004; HANDSCHIN *et al*. 2007; DIMITRI *et al.* 2015), corroborated by the expression of leptin receptor in chicken bones (MAURO *et al.* 2010).

The mRNA expression of estrogen receptors α and β has been detected in the CAM tissue of both male and female chicken embryos at E12, E15 and E18. So far, in the available literature, the simultaneous expression of both isoforms of estrogen receptors in the chicken CAM has not been reported, however ALBERGOTTI *et al.* (2009) demonstrated mRNA expression of ERα in the chicken CAM, whereas in the chorioallantoic membrane of American alligator (*Alligator mississippiensis*) mRNA expression of ERα (CRUZE *et al*. 2012) and in the Turtle *(Pseudemys nelsoni)* mRNA expression of ERα as well as ERβ were detected (CRUZE *et al*. 2013). The expression of ERα mRNA decreased 2-fold between embryonic stages (19 and 25), whereas ERβ expression increased 12.3-fold between embryonic stages 19 and 25 (CRUZE *et al.* 2013). In the present study, such differences in mRNA expression of estrogen receptor mRNA among analyzed stages of embryogenesis were not observed, however parallel to the reptilian CAM, in the chicken CAM mRNA expression of estrogen receptor α was significantly higher than expression of ERβ. A similar relation was previously observed in the laying hen ovary (HRABIA *et al.* 2008). This may be the result of the different physiological roles of the two isoforms of estrogen receptors. ERα activity may be associated with proliferation, whereas ERβ with stimulation of apoptosis and differentiation of cells (ZHAO *et al*. 2008; PATERNI *et al*., 2014). Moreover, the CAM of the chicken shares the capability of chorioallantoic placentae to synthesize and respond to the signaling of progesterone (P4), indicating that steroidogenic and steroid responsive extraembryonic membranes are not exclusive characteristics of viviparous amniotes (ALBERGOTTI *et al.* 2009). In addition, it was also demonstrated by CRUZE *et al*. 2013 that the CAM of the American alligator exhibits mRNA expression of steroidogenic enzymes critical in the synthesis of progestins, androgens and estrogens. They also detected mRNA expression of androgen receptor and glucocorticoid receptor suggesting that oviparous extraembryonic membranes have the potential to respond to steroid hormone signaling and to synthesize steroid hormones, which are mainly produced in gonads and adrenal glands (BRUGGEMAN *et al*. 2002; JOHNSON *et al*. 2002).

The last aim of this study was to assess the expression of mRNA encoding caspase-1 and -3 in the chicken CAM. The mRNA expression of caspase-1 and -3 was detected in chicken CAM at all three analyzed stages of embryogenesis. Expression of caspase-1 was significantly lower than that of caspase-3. Apoptotic cells have been previously detected during the early phase of chicken embryogenesis at sites of active growth, morphogenesis and active cell migration (HIRATA & HALL 2000). Studies on cell death in chicken embryos have been frequently focused on individual organ systems (JEFFS *et al*. 1992; HURLE *et al*. 1995; MILLER & BRIGLIN 1996; SANDERS 1997). Apoptotic cells have been previously localized three hours after the onset of incubation (HIRATA & HALL 2000), as well as in the gastrulating embryo (SANDERS & WRIDE 1997). Our results demonstrate that mRNA expression of caspase-1 and caspase-3 does not change among the analyzed stages of embryogenesis. This may suggest the participation of apoptosis and/or pyroptosis in the process of CAM development in the chicken embryo.

In summary, leptin receptor was immunolocalized, and leptin and estrogen receptors and caspase-1 and -3 mRNA was detected in the chicken CAM at all examined stages of embryogenesis. The results show the potential of this membrane to respond to different stimuli (leptin, estrogens) during early chicken embryogenesis. Additionally, the presence of caspase-1 and -3 mRNA expression in CAM tissue at E12, E15 and E18 indicates that chicken CAM may be used as a model in analysis of the mechanism of cell death after *in ovo* manipulations.

These results suggest that the CAM is a target tissue for leptin and estrogens and additionally, that the development of this membrane is associated with apoptosis and/or pyroptosis. Our results should be taken into consideration in studies with the CAM as an experimental model, in tests of endocrine disrupting potential of chemicals, and analysis of therapeutic methods and metastatic potential of cancers.

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