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Alterations in aquaporin 4 expression in the chicken ovary following tamoxifen treatment

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Short communication

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Previously, we demonstrated the follicular size- and a layer of the follicular wall-dependend expression of aquaporin 4 (AQP4) in the chicken ovary. In this study, we aimed to examine the mRNA and protein expression of aquaporin 4 (AQP4) in the chicken ovary during a pause in laying induced by a tamoxifen (TMX, oestrogen receptor modulator) treatment. Ovarian white and yellowish follicles and the granulosa and theca layers of the largest yellow preovulatory follicles were harvested from control and TMX-treated (daily until the cessation of egg-laying) hens. It was found that the TMX treatment lowered the AQP4 transcript abundance in the white follicles, but increased it in the theca layer of the F3-F1 follicles. Moreover, the TMX caused a decrease in AQP4 protein abundance in the theca layer of the F2 follicle. We propose that AQP4 plays a role in the ovarian follicle development and that there is a relationship between oestradiol action and the AQP4 gene and protein expression in chicken ovarian follicles.

Key words: hen, ovarian follicles, AQPs, TMX, mRNA, protein.

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The ovary of the mature laying hen is composed of follicles at various stage of development. These range from slow growing, prehierarchical follicles that are white (1-4 mm in diameter; WFs) and yellowish (4-8 mm; YFs); to fast growing follicles arranged into a preovulatory hierarchy of small yellow (8-12 mm; SYFs) and large yellow (12-35 mm; F_n-F1). The largest follicle (F1) is the most developed and is the first to ovulate (Hrabia 2022).

The ovarian follicle's growth, development and ovulation are accompanied by a series of events, such as selection into the preovulatory hierarchy or atresia, reorganisation of the follicular wall and a deposition of a large amount of the yolk into the oocyte. It has been proven that the yolk consists of 48% water (Etches 1996). The growth of the follicle requires a large amount of water passing through the follicular wall. The proteins responsible for transporting water across the membrane under an osmotic gradient are aquaporins (AQPs). So far, 13 AQPs (AQP0 to AQP12) have been found in many organs including the female and male reproductive systems

in mammals, where they play a crucial role in maintaining water flow between the tissue components (Kordowitzki *et al*. 2020; Ferre-Dolcet & Rivera del Alamo 2023). Some AQPs have been demonstrated to be present in different cells and tissues of avian species (Sugiura *et al*. 2008; Skowronski *et al*. 2009; Yoshimura *et al*. 2011; Nowak *et al*. 2017; Socha *et al*. 2018). However, little is known about the role or regulation of AQP family members in the ovaries of birds. So far, differentially expressed AQP5 has been reported in hen ovarian tumour cells (Tiwari *et al*. 2014). The distribution of the AQP4 transcript and protein in chicken ovarian compartments was also demonstrated, and it was suggested that AQP4 may take part in the regulation of follicle growth and development (Nowak *et al*. 2017); however, there is no information concerning the hormonal regulation of AQP4 in the chicken ovary. Since oestradiol plays a crucial role in various biological events in the ovary, in the present study we hypothesised that it is involved in the regulation of APQ4 expression in this organ. Accordingly, the gene and protein expression of AQP4 was examined in hen ovarian follicles during a pause in laying induced by a tamoxifen (TMX; oestrogen receptor modulator) treatment. TMX is a well-known synthetic oestrogen antagonist in birds.

Materials and Methods

Animal experiments were conducted according to the research protocol approved by the Local Animal Ethics Committee in Krakow, Poland (Approval No. 218/2015). Hy-Line Brown laying hens, aged 34 weeks, were assigned to two groups: the control (n = 6), injected subcutaneously with a vehicle (ethanol); and the experimental group (n = 6), which was treated with TMX at a dose of 6 mg/0.3 ml ethanol/kg of body weight, as precisely described by Socha *et al*. (2018). The hens were treated daily until a pause in egg laying occurred in all the TMX-treated birds. The chickens of both groups were euthanised on Day 8 of the experiment. The following ovarian tissues were isolated: WFs, YFs, and three of the largest yellow preovulatory F3-F1 follicles. From the preovulatory follicles, the theca (T) and granulosa (G) layers were separated.

Total RNA extraction, reverse transcription (RT) and a quantitative real time PCR were performed, as previously described by Socha *et al*. (2018). In brief, two µg of RNA from each sample was reverse-transcribed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommendations. The obtained cDNA was used in a duplex real-time qPCR for AQP4 and 18S rRNA, in a 10 µl volume containing 5 µl of TaqMan Gene Expression Master Mix (Applied Biosystems), 0.5 µl TaqMan Gene Expression Assays with a specific TaqMan MGB-probe and one pair of primers (AQP4 Assay ID: Gg03346640_m1, GenBank Accession No. NM_001004765.1, amplicon size: 87 bp; Applied Biosystems), 0.5 µl of Eucaryotic 18S rRNA Endogenous Control (pair of primers and TaqMan probe-labelled VIC/TAMRA, amplicon size: 187 bp; Applied Biosystems), 3 µl of water and 1 µl of cDNA (10 x diluted samples after the RT). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels (RQ) of the AQP4 gene after normalisation to 18S rRNA, and a calibration to expression in the tissue of the control group.

Protein extraction and a Western blot analysis were performed as described in detail by Nowak *et al*. (2017). A primary rabbit polyclonal anti-chicken AQP4 antibody (custom-made by Operon Biotechnologies, Tokyo, Japan; dilution 1:2500) and a secondary horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Advansta, USA; 1:5000) were used. The membranes were stripped and were reprobed with mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) HRP-conjugated IgG (Invitrogen, USA; 1:1000). Chemiluminescent detection employing ChemiDoc-It 410 (VisionWorks Life Science software) was performed. The AQP4 protein levels were normalised against the GAPDH.

The variables were examined for normality using the Shapiro-Wilk test. The significance of differences between the two means (TMX vs. control) was analysed using the Student's *t*-test. Differences in values were considered to be significant at $p < 0.05$. The results are presented as the mean ±SEM.

Results and Discussion

The present investigation demonstrated the relative abundances of AQP4 mRNA transcript and protein in the chicken ovarian follicles following the TMX treatment. The response to the oestrogen receptor blockage by the TMX depended on the stage of the follicle development and the layer of the follicular wall (Figs 1 and 2). In the control hens, the expression of AQP4 mRNA in the granulosa layer of the largest follicles was very low, or not detected in some samples; thus, these results were not shown. Moreover, in the theca layer, we did not find signifi-

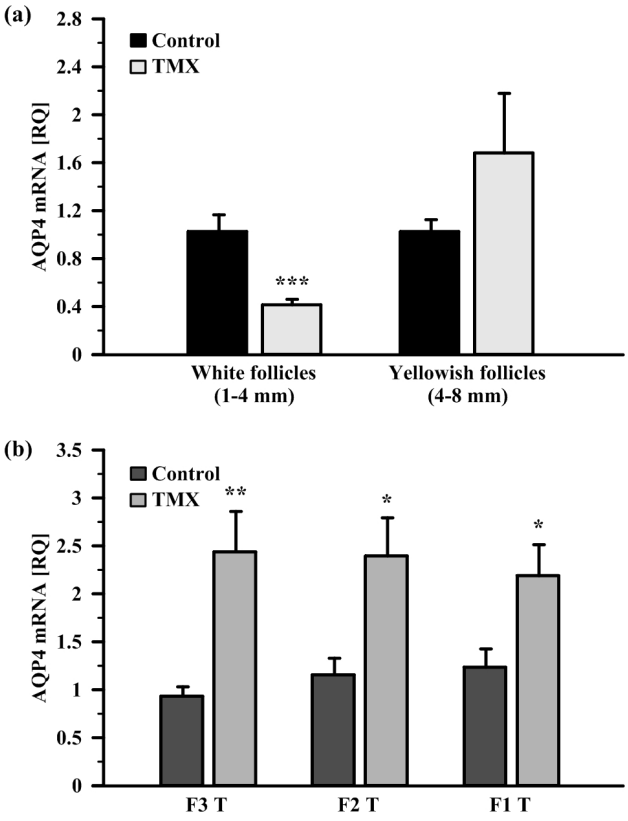

Fig. 1. Relative expression of AQP4 mRNA in chicken ovarian follicles following the tamoxifen (TMX) treatment (a and b). Each value represents the mean of the relative quantity (RQ) ±SEM from six chickens normalised to the expression of 18S rRNA and standardised to expression in the control tissue. Asterisks denote differences between the control and TMX treated birds for each type of tissue (*p < 0.05, **p < 0.01, ***p < 0.001). Abbreviations: F3-F1, three of the largest yellow preovulatory follicles (F3 < F2 < F1); T, theca layer.

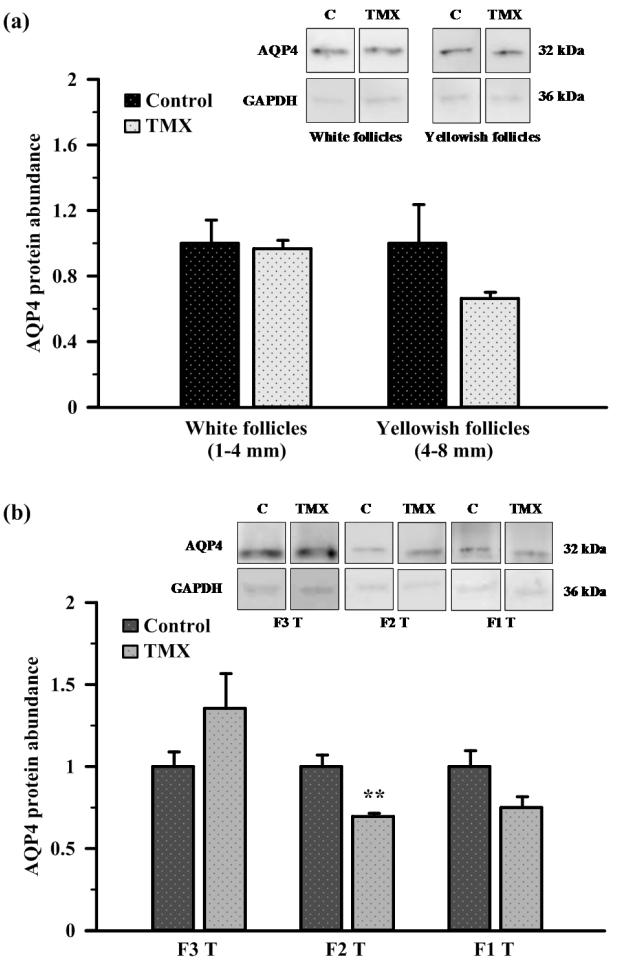

Fig. 2. Protein abundance of AQP4 in chicken ovarian follicles following the tamoxifen (TMX) treatment (a and b). The upper panels show representative blots of AQP4 protein in the ovarian follicles. The graphs show the relative protein abundance of AQP4 in the ovarian follicles, expressed as the ratio relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and compared to the control (C) value that is set as 1. Asterisks denote differences between the control and the TMX treated birds for each type of tissue (**p = 0.002). Abbreviations are as in Fig. 1.

cant differences in AQP4 transcript abundances between the F3-F1 follicles (RQ values: 0.93 ±0.10, 0.83 ±0.29 and 0.71 ±0.10, respectively). The presence of the AQP4 transcript and protein in the ovarian compartments of the control hens was in agreement with our previous observations (Nowak *et al.* 2017). These results further indicate that AQP4 may be involved in the regulation of folliculogenesis in birds, as has also been strongly suggested in the ovaries of different species (for a review, see Sales *et al.* 2013; Kordowitzki *et al.* 2020; Ferre-Dolcet & Rivera del Alamo 2023). Besides the involvement of AQP4 in water movement within the follicular tissues and the regulation of cellular functions, AQP4 may influence water transport to the egg yolk in birds. The enlargement of the follicle during the final phase of development is accompanied by the rapid accumulation of a huge amount of yolk, i.e. on average, up to 2 g of yolk per day into a domestic hen's preovulatory oocytes (Johnson 2015). Finally, each egg contains about 9.2 g of water (Etches 1996). Such a huge addition needs rapid movement of the water. Thus, it is plausible that the AQP4 present in the follicle wall participates in water transport into the yolk. The results of the constant AQP4 expression in the wall of the largest preovulatory follicles may indicate a similar rate of water passage into the oocyte during the final stage of follicle development.

The growth and development of avian follicles until ovulation are accompanied by changes in the steroid hormone production and their receptor abundances (Hrabia 2022). It is well established in hens that the secretion of oestradiol decreases, while progesterone increases during the ovarian follicle's maturation. Steroids, as key regulators of the ovarian

processes, may control the AQPs expression as has been suggested for the mammalian reproductive system (for a review, see Kordowitzki *et al*. 2020) as well as for the chicken oviduct (Yang *et al*. 2016). For example, in the mouse uterus, oestradiol induced the expression of AQP3, AQP4, AQP5 and AQP8, while it inhibited the AQP1 and AQP11 expression. Interestingly, progesterone inhibited the oestradiol-stimulated AQP3 and AQP4 expression (de Oliveira *et al*. 2020). Treatment of chicks with diethylstilbestrol, a synthetic oestrogen, caused an increase in the expression of AQP3 mRNA in the magnum and isthmus (Yang *et al*. 2016). In addition, the pattern of the AQP3 mRNA expression changed in an oestrogen-dependent manner during the moulting period in hens (Yang *et al*. 2016). Thus, in the present study, the potential involvement of oestradiol in the regulation of AQP4 expression in the chicken's ovarian compartments was evaluated. For this purpose, laying hens were treated with TMX to block the oestrogen receptors and, consequently, the action of oestradiol. This leads to a disruption of follicle development and the cessation of egg laying (Socha *et al*. 2018). The treatment of the hens with TMX decreased the AQP4 mRNA expression by 60% in WFs ($p < 0.001$), but did not change the AQP4 transcript level in YFs compared to the control group (Fig. 1a), as well as the AQP4 protein abundances in both classes of prehierarchical follicles (Fig. 2a). Within the group of large yellow follicles, TMX caused an increase in the AQP4 mRNA abundance in the theca layer of the F3, F2 and F1 follicles, by 160% ($p = 0.001$), 110% ($p = 0.007$) and 77% ($p = 0.034$), respectively (Fig. 1b). Moreover, TMX decreased the AQP4 protein abundance in the theca layer of the F2 follicle by 31% ($p = 0.002$). A reduced expression of AQP4 mRNA and protein was also observed in the chicken oviduct during a pause in laying induced by a TMX treatment (Socha *et al*. 2018), and by fasting (Hrabia *et al*. 2020) when the concentrations of steroids were very low. The results obtained indicate a relationship between the action of oestradiol and the expression of the AQP4 gene and protein in chicken ovarian follicles. Thus, oestradiol may regulate follicular development, at least in part, by influencing the AQP4 expression.

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Author Contributions

Research concept and design: A.H.; Collection and/or assembly of data: D.W., N.S.; Data analysis and interpretation: D.W., A.H.; Writing the article: D.W.; Critical revision of the article: N.S., A.H.; Final approval of article: D.W., N.S., A.H.

Conflict of Interest

The authors declare no conflict of interest.

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