Animal Science Papers and Reports vol. 34 (2016) no. 4, 339-349
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Cathepsin mRNA level in bovine cumulus cells fails to be a good marker of oocyte quality*

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(Accepted August 18, 2016)

Cathepsins (CTS) are lysosomal cysteine proteinases, of which elevated transcript content is attributed to the reduced quality of oocytes. The aim of this study was to investigate whether transcript content of three CTS genes in cumulus cells (CC) may be related to oocyte quality. The relationships between mRNA content of CTS genes in CC, the fatty acids profile in follicular fluid (FF) and selected quality traits of the corresponding oocyte (COC morphology, follicle diameter, mtDNA copy number) were investigated. Although an increased RA of the CTSB and the CTSK genes was accompanied by inferior COC morphology, it was also correlated with a higher mtDNA copy number in the oocyte and an increased content of C18.3n3 and n3 in FF. The last two traits were attributed to better quality oocytes, which contrasts with the high RA of the CTS genes. In conclusion, elevated transcript levels of CTS genes within CC are of limited value as an indicator of reduced quality in bovine oocytes. We suggest that the possible reason for the ambiguity of the obtained data could be the origin of cumulus cells within the COC, which may prove to be crucial for this type of studies.

KEY WORDS: cathepsins / mtDNA copy number / follicular fluid / fatty acids / COC morphology

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*This study was supported by the research grant from the Ministry of Science and Higher Education, Poland (Grant no. N N302 604438).
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Efforts of many scientists have been focused on finding a reliable and non-invasive marker of oocyte developmental competence. Gene expression analysis in cumulus cells (CC) is one of the most often undertaken approaches in search of reliable oocyte quality markers. Although the investigations mainly focus on human reproductive research, some interesting data is also available for cattle. Bettegowda *et al*. [2008] discovered higher mRNA abundance of cathepsins B, K, S and Z (lysosomal cysteine proteinases) in cumulus cells of prepubertal heifers than in adult cows. Elevated levels of cathepsin B, S and Z transcripts were also observed in cumulus cells surrounding oocytes of reduced quality [Bettegowda *et al*., 2008]. The presence of cathepsin B (CTSB) protein in bovine oocytes and cumulus cells has also been correlated with COC morphology [Balboula *et al*., 2010]. More recently, a suppressing effect of cathepsin B on oocyte quality has been described. The developmental competence of heat-shocked bovine COCs during IVM has been improved by the inhibition of cathepsin B activity, which was related to apoptosis pathways [Balboula *et al*., 2013]. Based on the results provided above, a negative impact of cathepsins on developmental competence of bovine COCs has been proposed.

Other traits of follicle environment (e.g. fatty acid composition in follicular fluid) present potentially valuable oocyte quality markers. Concentrations of particular fatty acids (FAs, e.g. stearic, palmitic, oleic and linoleic acids) in the follicular fluid or in IVM medium were related to COC morphology, meiotic stage, cleavage and blastocyst yield [Homa and Brown, 1992, Leroy *et al*., 2005, Sinclair *et al*., 2008, Jungheim *et al*., 2011, Marei *et al*., 2010, Van Hoeck *et al*., 2011, Aardema *et al*., 2011]. We have recently reported a multifactorial analysis based on contents of four fatty acids (C16:0, C16:1, C18:1cis9, C22:5n3) and activity of two enzymes (Δ9-desaturase (16) and elongase) in bovine follicular fluid [Warzych *et al*., 2014]. The proposed statistical model allowed a 72% prediction of oocyte morphology, whereas none of the parameters analysed separately provided a reliable estimation of COC quality. Literature sources have focused on linolenic acid (ALA; C18:3n3), which is a natural component of cellular plasma and follicular fluid. Marei *et al*. [2009] revealed a stimulatory effect of ALA supplementation of the IVM media on cleavage and blastocyst rates; however, the final effect depended on the concentration applied.

The content of mitochondrial DNA (mtDNA) is a recognised marker of oocyte quality. Reduced mtDNA copy number has been attributed to oocytes of lower quality, mainly in the human, mouse and pig [Reynier *et al*., 2001, Santos *et al*., 2006, Wai *et al*., 2010, El Shourbagy *et al*., 2006]. In cattle, however, Chiaratti *et al*. [2010] observed a compensative replication of mtDNA in early parthenogenetic embryos, thus the significance of mtDNA copy number of maternal origin for oocytes and early embryos has been disputable.

The aim of the present experiment was to investigate whether transcript contents of the three CTS genes in cumulus cells are related to selected traits attributed to oocyte quality. In order to accomplish this goal, relationships between parameters

characterising CC (RA of 3 CTS genes) and FF (FA profile) and selected quality traits of the corresponding oocyte (COC morphology, follicle diameter, mtDNA copy number) have been investigated. The most interesting result of this experiment is finding evidence for a lack of a clear relation between transcript contents of cathepsin genes in cumulus cells and in oocyte related traits. Although increased mRNA levels of the CTSB and CTSK genes in CC were accompanied by inferior COC morphology, they were also correlated with higher mtDNA copy numbers in oocytes as well as increased contents of C18.3n3 and n3 in FF. The last two traits were previously attributed to oocytes of better quality, which is in contrast with the high RA of the CTS genes. Thus, a higher RA of CTS genes within CC may not be a direct indicator of bovine oocytes of reduced quality.

Material and methods

Sample collection

Each sample analysed in this experiment comprised 3 follicular components representing individual follicles: cumulus cells, the oocyte and follicular fluid (FF). The following analyses were performed: oocyte – mtDNA copy number, CC – relative transcript abundance of 3 cathepsin genes, FF – glucose concentration and fatty acid composition.

Cumulus oocyte complexes were recovered from bovine ovaries collected at a slaughterhouse. Each follicle was measured and subjected to individual aspiration with a 1ml syringe. Individually aspirated FF was transferred to a Petri dish. Follicles of less than <6 mm in diameter were aspirated jointly from each ovary pair, thus the follicular fluid from single follicles of this category was not analysed. Each aspirated COC was transferred to 0.2% PBS/PVP, whereas FF was centrifuged and the supernatant was frozen in liquid nitrogen. At the same time, the morphology grade of each COC was assessed according to the modified criteria described by Stojkovic *et al*. [2001]: grade 1 – homogenous ooplasm, complete, compact and multilayered cumulus cell mass; grade 2 – homogeneous ooplasm with some irregular pigmentation, >5 layers of compact cumulus cells; grade 3 – heterogeneous, partially vacuolated ooplasm, 3-5 layers of cumulus cells; grade 4 – heterogeneous, pigmented ooplasm, expanded cumulus cell mass.

Prior to analyses, all cumulus cells surrounding individual oocytes were removed by vigorous pipetting in 150 µl of 0.2% PBS/PVP. Approximately half of the total number of cumulus cells were frozen in liquid nitrogen and the oocytes were washed 3x in a 0.2% PBS/PVP and carefully examined for the presence of cumulus cells. Denuded, individual oocytes were placed in 200 µl PBS and immediately frozen in liquid nitrogen. All samples were stored at -80°C.

Relative transcript abundance of 3 CTS genes in cumulus cells

RNA was extracted with the High Pure miRNA Isolation Kit (Roche) according to the manufacturer's protocol. Synthesis of complementary DNA was carried out with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The cDNA samples were stored at -20°C.

Analyses were performed on a Roche Light Cycler 2.0 and the calculations were based on the 'Second Derivative Maximum' method (Roche Diagnostics, Mannheim, Germany). The 18S rRNA and GAPDH genes were used as reference genes. The reactions were carried out in 20 µl capillaries with a Light Cycler FastStart DNA Master HybProbe (Roche Diagnostics). Detailed information concerning primers and FRET probes designed by TIB MOLBIOL (Germany) and used in the experiment is presented in Table 1.

Table 1. Sequences of primers and probes designed and applied for the present experiment

Gene	Primers and probes sequences	Annealing temperature (°C)	$MgCl_2$ concentration (mM)	Product length (bp)
18S rRNA	F: GGAGGTAGTGACGAAAAATAACAA R: CCAAGATCCAACTACGAGCTT FL: GCGGAAGGATTTAAAGTGGACTCAT-FL LC: 640-CCAATTACAGGGCCTCGAAAGAGT	62	5	185
CTSB	F: CTGGACACAACTTCTACAACGTG R: TGGATGCAGATCCGGTCA FL: CTTTCAGGCAGAACCACATCCGC-FL LC: 640-GCAAATGCATCTCTCTGGGGCAGCT p	62	5	243
CTSK	F: CTTCCAGTATGTGCAGAAGAACC R: GGTCCCACTCGGGCTAC FL: TCCATATGTTGGACAGGATGAAAATTGCA-FL LC: 640-GTACAATCCAACAGGCAAGGCAGCT p	62	5	176
CTSS	F: TGACCAGTGAAGAAGTGATATCT R: CACAAGCACCCTGGTATT FL: GGGTCTGACTTGTAAGTGACATTTCT-FL LC: 640-GGCCATTGGCTGGGAACT p	62	5	161
CTSZ	F: CACTGTTGTCGGTGCAGC R: CAGAGGGTGTAGTTCTTGATGACAT FL: GTCCCACACTGGTTAAACTTGTCACACTC-FL LC: 640-TGGTCCTTGGCCTGGTAGTTGTTGC p	62	5	216
COX1	F: AAATAATATAAGCTTCTGACTCC R: TCCTAAAATTGAGGAAACTCCT	56	2	190

Mitochondrial DNA (mtDNA) copy number in oocytes – real time PCR analysis

Extraction of genomic and mtDNA was conducted according to the producer's protocol (High Pure PCR Template Preparation Kit, Roche). The samples were incubated in binding buffer and proteinase K for 10 min at 70°C. The following steps involved the use of an inhibitor removal buffer and double washing. Finally, total DNA from individual oocytes was eluted into a fresh 1.5 ml tube in 200 µl of Elution Buffer. The DNA samples were stored at -20°C until analysis.

Real-time absolute quantification analysis was conducted using the standard curve method. All reactions were performed on the Roche Light Cycler 2.0 system with Roche reagents (Roche, Switzerland) according to the manufacturer's protocol with a set of starters for the COX1 gene according to Bermejo-Alvarez *et al*. [2008]. The reaction conditions included denaturation for 10 min at 95°C, amplification for 40 cycles at 95°C for 15 s, 56°C for 10 s and 72°C for 10 s. Each reaction was followed by melting curve analysis to verify the specificity of the amplification. All of the presented values for the mtDNA analyses are absolute and refer to the copy number.

Fatty acid composition of follicular fluid

Fatty acid (FA) composition of follicular fluid was analysed by gas chromatography according to the procedure described by Cieslak *et al*. [2009] and adapted to the follicular fluid analysis by Warzych *et al*. [2011].

Statistical analysis

The relationships between mtDNA copy number, relative abundance of the 3 cathepsin genes and fatty acid composition were evaluated using Spearman's rank correlations. Non-parametric Kruskal–Wallis one-way analysis of variance was used to evaluate differences in mtDNA copy number and to analyse mRNA levels of cathepsin genes within COC quality categories and follicle diameter classes. These calculations were performed in the R package (R Development Core Team 2011).

Results and discussion

Mitochondrial DNA in oocytes

MtDNA content was analysed in 136 individual oocytes. The average mtDNA copy number per oocyte was 2 439 346 (min. 85 400, max. 7 320 000). The content of mtDNA within the oocyte was not significantly affected either by the morphology grade of the host COC or by the diameter of the follicle of origin. The following mtDNA content (mean±SEM) in particular morphology grades was observed: grade 1 COCs – 2 292 225 ± 285 068, grade 2 COCs – 2 680 289±308 297, grade 3 COCs – 2 410 500±177 296 and grade 4 COCs – 2 425 103±178 332. When follicle diameter was considered, the following values were obtained: <6mm follicles – 2 303 186±172 050, 6-8mm follicles – 2 596 894±210 239 and >8mm follicles – 2 446 018±233 230.

Relative transcript abundance of 3 CTS genes in cumulus cells

The transcript level of cathepsin genes was analysed in approx. 70 samples of cumulus cells. With regard to COC morphology, morphology grade 3 showed the highest RA for CTSB, morphology grade 4 the highest RA for CTSK, whereas the mRNA level for CTSZ was almost identical in morphology grades 1 and 4 (Fig. 1).

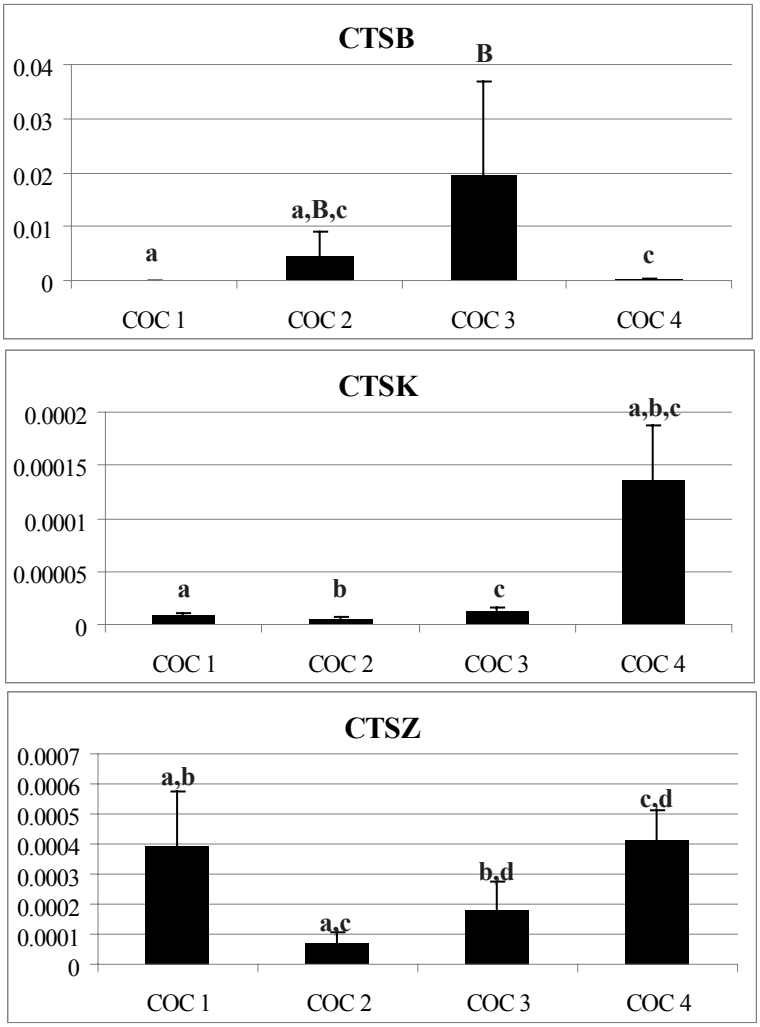

Fig. 1. Relative transcript abundance of 3 cathepsin genes (CTSB, CTSK, CTSZ) in bovine cumulus cells with regard to the morphology grade of cumulus-oocyte complexes (COC1, COC2, COC3, COC4). Bars marked with different letters differ significantly at: small letters – $P<0.05$; capitals – $P<0.01$.

When the mRNA level of CTS genes was analysed with regard to follicular diameter, only the CTSB gene showed a difference between CC originating from 6-8mm follicles and >8mm follicles (Fig. 2).

A positive correlation was observed ($r=0.27$, $P<0.05$) between the mtDNA copy number in the oocyte and the mRNA level of the CTSB gene in cumulus cells.

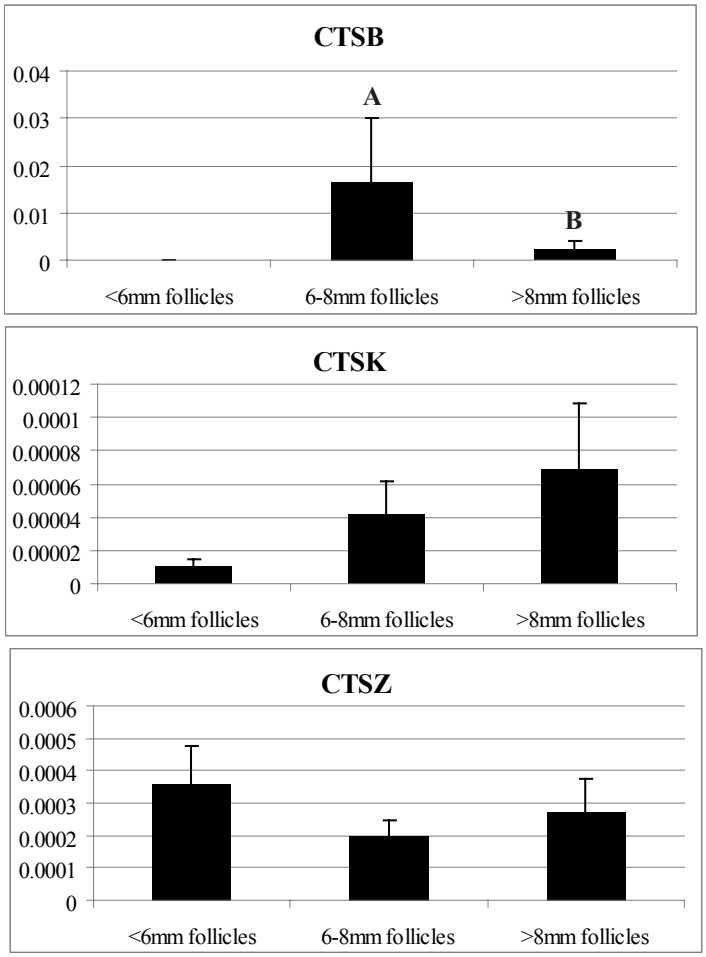

Fig. 2. Relative transcript abundance of 3 cathepsin genes (CTSB, CTSK, CTSZ) in bovine cumulus cells with regard to the diameter of host ovarian follicles. Bars marked with different letters differ significantly at P<0.01.

Fatty acid content in follicular fluid

The FA composition was investigated in 63 FF samples. The average concentration of total FA was 467.95 µg/ml. The most abundant FAs were C18:2n6 (123.37µg/ml), C16:0 (92.46µg/ml) and C18:1cis9 (84.65µg/ml) and C18:0 (66.69µg/ml).

The average concentration of C18.3n3 FA was 18.13 µg/ml (62.72µM) and it was correlated with transcript levels of the CTSB (r=0.32, P<0.05) and CTSZ genes (r=0.37, P<0.05). Moreover, RA for CTSZ was correlated with n3 FAs (r=0.34, P<0.05).

The results obtained in this study indicated that the highest level of the CTSB gene transcripts was noted in cumulus cells of morphology grade 3. However, no such relationship was observed for COCs of morphology grade 4. COCs of grades 3 and 4 displayed the lowest developmental competence. These were previously characterised by elevated mRNA levels of the cathepsin genes [Bettegowda *et al*., 2008, Balboula *et al*., 2010]. Similarly to the CTSB gene, no significant relationship between transcripts of the CTSK and the CTSZ genes was observed for the analysed quality traits. Also, when follicle size was considered, it did not provide grounds for a conclusion whether the analysed genes were differently expressed in CC from follicles of different diameter, which, according to the literature, significantly affects COC quality [Hendriksen *et al*., 2000]. Therefore the results of our study are quite difficult to interpret. However, it should be stressed that in the presented experiment only the outer layer of cumulus cells was separated for the purpose of the gene expression studies, as the oocytes with the remaining cumulus cells could potentially be subjected to the in vitro procedure. In contrast, Bettegowda *et al.* [2008] pooled 5 cumulus cells in each sample. It has been established that COCs exhibit an inside-outside gradient in gene expression from the oocyte perspective [Hussein *et al.* 2005]. Thus the origin of cumulus cells (whether they are located next to the oocyte or in the outer layers) may be crucial obtained data. It was previously pointed by Seli *et al.* [2010] that scientists should reflect on the general application of gene expression studies of cumulus cells with regard to oocyte quality. In our opinion the presented results support this statement.

Search for potential relationships between the traits analysed in this study revealed a positive rank correlation between mRNA levels of 2 cathepsin genes (CTSB, CTSZ) in cumulus cells and the concentration of the C18.3n3 fatty acid in follicular fluid. The transcript level of the CTSZ gene was further positively correlated with the total n3 FA concentration. This result represents a key finding of the present study. It was previously shown that C18.3n3 supplementation of the maturation medium provided beneficial effects on developmental competence of bovine oocytes [Marei *et al*., 2009, 2012]. The stimulatory effect was observed only for 50 μM C18.3n3 supplementation, whereas higher doses (100 μM) proved detrimental [Marei *et al*., 2009]. Also a positive correlation was observed between this FA concentration and the developmental potential of bovine COCs [Matoba *et al*. 2014]. The concentration of C18.3n3 (62.72 μM) in FF analysed in the present study was similar to the beneficial concentration of 50 μM supplemented by Marei *et al*. [2009]. Thus, it may be hypothesised that the higher C18.3n3 concentration in FF stimulates apoptosis in cumulus cells by induction of CTSB expression, which is a known stimulator of apoptosis [Vancompernolle *et al*., 1998]. However, in our latest study a negative correlation of the C18.3n3 concentration in FF and the apoptotic index in CC was observed [Warzych *et al*., 2014]. Therefore, we strongly suggest the need to further analyse this phenomenon.

Another interesting finding of the present study was a positive correlation between RA of the CTSB gene in cumulus cells and mtDNA content in the corresponding oocyte. This aspect has not been previously investigated. Elevated RA for the CTSB

gene in CC was attributed to COCs of reduced quality [Bettegowda *et al*., 2008]. Thus, a positive correlation between RA of CTSB in CC and mtDNA copy number in oocytes is rather unexpected, since a higher mtDNA content was attributed to oocytes of better quality in the human, mouse and pig [Reynier *et al*., 2001, Santos *et al*., 2006, Wai *et al*., 2010, El Shourbagy *et al*., 2006]. In cattle, however, the data on mtDNA copy number in oocytes is inconclusive. No relation between oocyte quality (revealed by oocyte origin and blastocyst rate) and mtDNA copy number was observed after IVM [Tamassia *et al*., 2004]. On the other hand, Chiaratti *et al*. [2010] showed a reduced mtDNA content in oocytes derived from small follicles (1-3mm) when compared to bigger follicles (3-6 and 6-8mm). Since follicles of 1-3 mm contain oocytes of lower quality [Lequarre *et al*., 2005], it has been suggested that a lower mtDNA content coincides with oocytes of reduced quality. The same authors suggested that the mtDNA copy number of oocytes was not related to their quality, because competent bovine embryos retained the ability to reverse mtDNA depletion during preimplantation development. Furthermore, a high variation in mtDNA copy number in oocytes has been previously reported [Chiaratti *et al*., 2010] and was also observed in this study. Chiaratti *et al*. [2010] suggested that combining the mtDNA copy number with oocyte competence may generate errors and give false conclusions. They also hypothesised that a lack of a significant rank correlation between oocyte quality and mtDNA content may be due to the extraordinary amounts of mtDNA within the oocyte and only average requirements for ATP during early cleavages. Thus, it may be summarised that the mtDNA copy number in bovine oocytes remains a controversial subject. Based on the published evidence, it is difficult to explain a positive correlation between the mRNA level of the CTSB gene in cumulus cells and the mtDNA content in the corresponding oocyte.

In summary, the most important finding of our study is the lack of an apparent relation between the expression of cathepsin genes in bovine cumulus cells and well-described markers of oocyte quality (COC morphology, follicle diameter, mtDNA copy number, FA profile in follicular fluid). Although an increase in RA of the CTSB and CTSK genes in CC was accompanied by an inferior oocyte morphology, it was also correlated with a higher mtDNA copy number in the oocyte as well as C18.3n3 and n3 contents in FF. The last two parameters have been previously attributed to oocytes of better quality, which contrasts with the high mRNA level of the CTS genes. Thus a higher transcript level of CTS genes within CC may not indicate bovine oocytes of reduced quality. We suggest that a possible reason for the ambiguity of the obtained data could be connected with the origin of cumulus cells within COC, which may prove to be crucial for this type of studies.

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