—Original Article—

# Multifactorial Analysis of the Follicular Environment is Predictive of Oocyte Morphology in Cattle

## Ewelina WARZYCH[1], Adam CIESLAK[2], Zofia E. MADEJA[1], Piotr PAWLAK[1], Anna WOLC[3,4] and Dorota LECHNIAK[1]

[1]*Department of Genetics and Animal Breeding, Poznan University of Life Sciences, 60-637 Poznan, Poland*
[2]*Department of Animal Nutrition and Feed Management, Poznan University of Life Sciences, 60-637 Poznan, Poland*
[3]*Department of Animal Science and Center for Integrated Animal Genomics, Iowa State University, IA 50011-3150, USA*
[4]*Hy-Line International, Dallas Center, IA 50063, USA*

**Abstract.** Numerous attempts have been recently made in the search for a reliable, fast and noninvasive assay for selection of oocytes suitable for *in vitro* embryo production. Potential markers have been described in the follicle such as follicular fluid (FF) or cumulus cells (CCs). However, the reported findings are contradictory, which may reflect the complexity of metabolism of the ovarian follicle. In the present experiment, a data set from individual follicles of known diameter was obtained: cumulus-oocyte complex (COC) morphology, fatty acid composition and glucose concentration in FF as well as apoptotic index in CCs. The obtained data was statistically analyzed either separately (univariate analysis) or simultaneously (multivariate analysis) to examine its predictive value in morphology assessment of bovine COCs. Although the univariate analysis yielded a complex relation system of the selected parameters, no clear outcome could be established. In multivariate analysis, the concentration of the four fatty acids (C16:0, C16:1, C18:1cis9, C22:5n3) and $\Delta^9$-desaturase (16) as well as elongase activities were selected as covariates. This allowed prediction of the morphology of a COC with an accuracy of 72%, which is the most interesting finding of the experiment. The present study indicates that the multifactorial model comprising of selected parameters related to the follicle appeared more effective in predicting the morphology of a bovine COC, which may improve the effectiveness of *in vitro* production systems.
**Key words:** Cumulus cells, Fatty acids, Follicular fluid, Oocyte,

*In vitro* embryo production dates back to the 1950s/1960s, which is when the first convincing reports on the rabbit, mouse, hamster, rat and humans were published [1]. Since then, *in vitro* production (IVP) effectiveness has been significantly improved; however, still only 30–40% of *in vitro* fertilized bovine oocytes reach the blastocyst stage. It is claimed that one of the problems is a deficient strategy for oocyte selection, which, despite significant progress, is still mainly based on morphology evaluation of the cumulus-oocyte complex (COC). An alternative way seems to be prediction of oocyte quality basing on evaluation of other follicular components – mainly follicular fluid (FF) or cumulus cells (CC), which have an important influence on oocyte developmental competence [2]. The first experiments relating cumulus cells analysis to human oocyte quality showed that COCs containing less than 50% of luteinized cumulus cells and no vacuoles yielded the highest fertilization outcome [3]. Nowadays, mainly follicular fluid is described as a valuable source of data on oocyte quality. FF is a product of both transfer of blood plasma constituents and of secretory activity of granulosa and thecal cells [4]. It protects the oocyte against proteolysis and extrusion and acts as a buffer against negative influences of blood [5]. Since it has an essential impact on the oocyte, follicular fluid has emerged as one of the most promising sources of data predicting oocyte quality, which in turn directly affects the blastocyst yield [6]. Some experiments have also indicated cumulus cells as elements of the follicular environment, which may indirectly reflect oocyte quality.

In discussions of follicular fluid, the emphasis is placed on some metabolites, e.g., fatty acids or amino acids. According to recent human and bovine studies, the concentrations of particular fatty acids (FAs, e.g., stearic, palmitic, oleic, linoleic acids) in follicular fluid or in IVM medium were related to COC morphology, meiotic stage as well as cleavage and blastocysts yield [7–13]. On the other hand, Sinclair *et al.* [8] observed correlation between the concentrations of palmitic and stearic fatty acids (FAs) and morphology of the bovine COC; however, no relation with blastocyst rate has been found. A recent manuscript of Matoba *et al.* [14] described relations among FF composition (e.g., fatty acids, amino acids, steroids), mRNA relative abundance in cumulus, granulose and theca cells and blastocyst rate. It emphasized the metabolomic profile of follicular tissues as a source of information on oocyte quality. Other approaches focused on cumulus cell analysis revealed associations between CC apoptosis and COC morphology [15], meiotic stage [16] or cleavage rate [17]. On the other hand, Yuan *et al.* [18] showed no relationship between COC morphology and the apoptotic index (AI) in bovine cumulus cells. We have recently shown a lack of correlation between incidence

of apoptosis in the CC and two oocyte-related parameters (COC morphology, meiotic stage) [19]. The studies referred to above present inconsistent data, which may reflect the complexity of the follicular metabolism, since interactions between the oocyte and somatic cells have an undoubted impact on oocyte growth [20]. Therefore, investigation based on individual parameters appears insufficient to predict oocyte quality, which requires a more complex approach.

In this manuscript, we proposed a multivariate statistical model based on data describing follicular fluid components (FF, CC) of individual bovine follicles. The experiment aimed at describing a set of parameters characterizing the individual follicle: fatty acid composition and glucose content in FF as well as incidence of apoptosis in CCs. The obtained set of data was statistically analyzed either separately (univariate analysis) or simultaneously (multivariate analysis) to examine its predictive value in morphology assessment of bovine COCs. Although the univariate analysis yielded a complex relation system between analyzed parameters, no clear outcome could be established. More essential information was provided by the multivariate analysis. The concentrations of four fatty acids (C16:0, C16:1, C18:1cis9, C22:5n3) and $\Delta^9$-desaturase (16) activity were selected as covariates for the prediction of COC morphology, whereas the remaining parameters were excluded. This allowed prediction of the morphology of a COC with a probability of 0.72, which is the most interesting finding of this experiment. The present study indicates that the multifactorial model comprising selected parameters related to follicular components appeared more effective in predicting the morphology of the bovine COC.

## Material and Methods

### Sample collection

Cumulus oocyte complexes were recovered from bovine ovaries of slaughterhouse origin. The diameter of each follicle was measured, and three size categories were defined: small (<6 mm, 70 follicles), medium (6–8 mm, 60 follicles) and large (>8 mm, 56 follicles). Each follicle was aspirated separately with 1 ml syringe. Individually aspirated FF was transferred to a Petri dish. The morphological grade of each COC was assessed according to a modified criteria described by Stojkovic *et al.* [21]: 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. Each sample analyzed in this experiment comprised of 3 follicular components – CCs, oocytes and FF – and represented an individual follicle. Analyses of apoptosis in CCs, glucose concentration in FF and fatty acid concentration were performed.

### Fatty acids composition in follicular fluid

The fatty acid composition of the follicular fluid was analyzed using gas chromatography according to the procedure described by Cieslak *et al.* [22] and modified by Warzych *et al.* [23]. The activities of $\Delta^9$-desaturase (16), $\Delta^9$-desaturase (18) and elongase were calculated according to Bender *et al.* [24].

### Cumulus cells apoptosis

A terminal TUNEL assay kit was used to detect apoptosis in cumulus cells (DeadEnd™ Fluorometric TUNEL System, Promega Biosciences, Madison, WI, USA) with minor modifications described by Warzych *et al.* [19]. The apoptotic index was calculated as a percentage of FITC-positive cells within all DAPI-positive cells.

### Glucose concentration

Glucose concentration was measured in 2 µl of aspirated FF with an Accu-Chek glucometer (Roche) according to the manufacturer's recommendations. Statistical analysis included the physiological concentration of glucose in bovine FF, which ranges from 1.4 to 5 mM [25].

### Statistical analysis

Both univariate analysis, which evaluates the effect of one factor at a time, and multivariate analysis, in which several factors are investigated simultaneously, were used to analyze the data. The effect of follicle size and COC morphology grade on the concentration of fatty acids was tested by Kruskal-Wallis rank sum test, followed by nonparametric Tukey-type multiple comparisons using the Miller (1981) test. Spearman's rank correlation coefficients were used to relate apoptotic index and fatty acids concentration in follicular fluid. Logistic regression in SAS PROC LOGISTIC with backward variable selection was used to find a set of variables predictive of oocyte morphology. All calculations were done using the R package (R Development Core Team, 2009) and SAS (SAS Institute, 2002–2003).

## Results

### Scope of the study

The apoptotic index was evaluated in 174 samples of CCs. A CC sample was excluded from the analysis when fewer than 50 cells could be evaluated. The average AI for the analyzed CC samples was 6.71 and varied from 0 to 97.62.

FA composition was investigated in 132 samples of FF. Samples originating from small follicles could not be analyzed due to insufficient FF volume (<100 µl). The average concentration of total FA was 460.29 µg/ml. The most abundant FAs were C18:2n6 (121.97 µg/ml), C16:0 (91.73 µg/ml), C18:1cis9 (82.45 µg/ml) and C18:0 (64.92 µg/ml).

Glucose concentration was analyzed in 101 FF samples, and the mean value was 3.51 mM (min. <0.6 mM, max. 6.55 mM). Samples originating from small follicles could not be analyzed. In the case of three FF samples, the glucose concentration was lower than the detection threshold of the glucometer (0.6 mM).

Although the collected set of data was not complete for all samples (due to, e.g., insufficient FF volume), the multivariate model was employed only for samples with a complete set of parameters.

### Univariate analysis

Follicle size in relation to other parameters: The following follicular components of the ovarian follicles were analyzed with regard to follicle size: follicular fluid (fatty acid composition and glucose content) and cumulus cells (apoptotic index). Significant differences were observed only for fatty acid composition. FF originating from

**Table 1.** Follicle size in relation to apoptotic index in cumulus cells, glucose concentration and fatty acid composition of follicular fluid

| | Small follicles | Medium follicles | Large follicles | P |
|---|---|---|---|---|
| Apoptotic index in CC | | | | |
| | 8 ± 1.84 | 6.47 ± 1.8 | 5.27 ± 1.49 | NS |
| Glucose concentration (mM) in FF | | | | |
| | n.a. | 3.15 ± 0.22 | 3.84 ± 0.21 | NS |
| Fatty acid composition (μg/ml) of FF | | | | |
| TOTAL FA | n.a. | 480.87 ± 18.14 | 439.71 ± 18.93 | NS |
| C14:0 | n.a. | 15.3 ± 1.07 | 17.4 ± 1.22 | NS |
| C15:0 | n.a. | 3.57 ± 0.18 | 3.48 ± 0.16 | NS |
| C16:0 | n.a. | 90.47 ± 4.22 | 92.99 ± 4.73 | NS |
| C16:1 | n.a. | 7.58 ± 0.43 | 7.19 ± 0.45 | NS |
| C18:0 | n.a. | 61.62 ± 2.53 | 68.23 ± 3.06 | NS |
| C18:1cis9 | n.a. | 86.46 ± 4.08 | 78.43 ± 4.2 | <0.05 |
| $\Sigma$C18:1trans | n.a. | 9.83 ± 0.76 | 8.54 ± 1.18 | NS |
| $\Sigma$C18:1cis | n.a. | 94.61 ± 4.3 | 84.87 ± 4.44 | <0.01 |
| C18:2n6 | n.a. | 137.95 ± 6.5 | 105.99 ± 4.51 | <0.01 |
| C18:3n3 | n.a. | 19.67 ± 1.09 | 15.31 ± 0.74 | NS |
| C20:4n6 | n.a. | 2.54 ± 0.25 | 2.19 ± 0.15 | NS |
| C22:5n3 | n.a. | 1.9 ± 0.21 | 1.7 ± 0.3 | <0.05 |
| SFA | n.a. | 193.79 ± 8.14 | 193.79 ± 0.69 | NS |
| UFA | n.a. | 287.08 ± 11.53 | 237.81 ± 10.06 | <0.01 |
| MUFA | n.a. | 117.44 ± 4.89 | 108 ± 5.27 | NS |
| PUFA | n.a. | 169.63 ± 7.63 | 129.81 ± 5.46 | <0.01 |
| n6 | n.a. | 141.69 ± 6.61 | 109.18 ± 4.6 | <0.01 |
| n3 | n.a. | 22.76 ± 1.19 | 17.83 ± 0.97 | <0.01 |
| $\Delta^9$-des. (16) activity | n.a. | 7.9 ± 0.33 | 7.4 ± 0.33 | NS |
| $\Delta^9$-des. (18) activity | n.a. | 57.74 ± 0.89 | 53.53 ± 0.90 | <0.01 |
| Elongase activity | n.a. | 60.42 ± 0.59 | 59.68 ± 0.59 | NS |

Values are presented as means ± SEM. n.a. – not analyzed. NS – not significant. SFA=saturated fatty acid (C14:0, C15:0, C16:0, C18:0, C20:0, C21:0, C22:0). UFA=unsaturated fatty acid (C16:1, C17:1, C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, C18:1 *trans*-12, C18:1 *trans*-13–14, C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-12, C20:1 *cis*-9, C18:2n-6, C18:3n-3, C20:3n-3, C20:4n-6, C20:5n-3). MUFA=monounsaturated fatty acid (C16:1, C17:1, C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, C18:1 *trans*-12, C18:1 *trans*-13–14, C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-12, C20:1 *cis*-9). PUFA= polyunsaturated fatty acid (C18:2n-6, C18:3n-3, C20:3n-3, C20:4n-6, C20:5n-3). C18:1trans=C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, C18:1 *trans*-12, C18:1 *trans*-13–14. C18:1cis=C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-12, C20:1 *cis*-9.

medium follicles contained more of the selected FAs (C18.1cis9, $\Sigma$C18.1cis, C18.2n6 or C22.5n3). This, in consequence, affected the level of certain groups of FAs such as UFA, PUFA, n6 or n3. The highest apoptotic index was noted for cumulus cells originating from small follicles, however, it was not statistically significant due to a big variation within each group.

The glucose concentration was similar in medium and large follicles (Table 1). The rate of FF samples with a physiological concentration of glucose (from 1.4 to 5 mM) was similar in medium (30/46, 65%) and large follicles (36/50, 72%).

COC morphological grade in relation to other parameters: Although the highest AI was observed in the grade 4 COCs, the difference was not significant. FF accompanying the grade 3 COCs had a significantly higher glucose concentration when compared with follicles having grade 4 COCs (Table 2). The rate of FF samples with a physiological concentration of glucose (from 1.4 to 5 mM) was not affected by the COC morphology [grade 1 COCs (9/14, 64%), grade 2 COCs (20/25, 80%), grade 3 COCs (24/40, 60%) and grade 4 COCs (14/18, 78%)]. Also, the FA composition in FF was not related to COC morphology (Table 2).

Fatty acid composition of FF in relation to apoptotic index in CC: Statistical analysis showed negative correlation between the apoptotic index in CCs and the concentrations of some FAs in FF (Fig. 1). It concerned the following fatty acids or FA groups: C15:0, C16:0, C16:1, C18:0, $\Sigma$C18.1cis, C18:2n6, C18:3n3 or total FA, SFA, UFA, MUFA, PUFA and n6.

Fatty acid composition of FF in relation to glucose: The glucose concentration was negatively correlated with the concentrations of the following FAs: C16:0, C16:1, C18:3n3 and n3 (Fig. 2).

Additional analysis showed that the concentration of C18:0 FA

**Table 2.** COC morphology in relation to apoptotic index in cumulus cells, glucose concentration and fatty acid concentrations in follicular fluid

| | COCs morphological grade | | | | |
| | 1st | 2nd | 3rd | 4th | P |
| | Apoptotic index in CC | | | | |
| | 6 ± 1.58 | 4.35 ± 1.31 | 5.44 ± 1.82 | 13.24 ± 4.47 | NS |
| | Glucose concentration (mM) in FF | | | | |
| | 3.44 ± 0.42 | 3.2 ± 0.24 | 3.99ᵃ ± 0.26 | 2.9ᵇ ± 0.29 | <0.05 |
| | Fatty acid composition (µg/ml) of FF | | | | |
| TOTAL FA | 486.01 ± 30.86 | 462.68 ± 36.17 | 451.85 ± 23.67 | 469.5 ± 29.03 | NS |
| C14:0 | 17.22 ± 2.03 | 19.83 ± 2.13 | 15.77 ± 1.46 | 16.54 ± 1.74 | NS |
| C15:0 | 3.67 ± 0.27 | 3.85 ± 0.31 | 3.51 ± 0.23 | 3.46 ± 0.26 | NS |
| C16:0 | 94.71 ± 6.79 | 93.11 ± 7.0 | 91.04 ± 6.21 | 96.04 ± 6.83 | NS |
| C16:1 | 8.61 ± 0.83 | 7.89 ± 1.15 | 7.24 ± 0.51 | 6.66 ± 0.48 | NS |
| C18:0 | 71.3 ± 4.8 | 64.43 ± 4.71 | 63.93 ± 3.53 | 66.66 ± 4.45 | NS |
| C18:1cis9 | 86.61 ± 6.34 | 84.46 ± 7.53 | 81.39 ± 4.88 | 77.58 ± 5.63 | NS |
| ΣC18:1trans | 7.51 ± 0.91 | 7.7 ± 1.19 | 9.61 ± 1.12 | 11.61 ± 2.19 | NS |
| ΣC18:1cis | 93.98 ± 6.74 | 91.18 ± 8.14 | 88.77 ± 5.2 | 85.03 ± 6.07 | NS |
| C18:2n6 | 125.78 ± 9.67 | 120.71 ± 13.09 | 118.52 ± 6.96 | 126.79 ± 9.11 | NS |
| C18:3n3 | 18.51 ± 1.58 | 17.87 ± 1.9 | 17.7 ± 1.27 | 17.62 ± 1.47 | NS |
| C20:4n6 | 2.95 ± 0.37 | 2.63 ± 0.55 | 2.31 ± 0.25 | 1.93 ± 0.24 | NS |
| C22:5n3 | 2.44 ± 0.65 | 1.56 ± 0.48 | 1.42 ± 0.21 | 1.94 ± 0.33 | NS |
| SFA | 211.88 ± 14.7 | 203.06 ± 14.83 | 193.42 ± 11.42 | 205.7 ± 14.02 | NS |
| UFA | 274.13 ± 18.39 | 259.62 ± 23.71 | 258.43 ± 13.66 | 263.8 ± 16.93 | NS |
| MUFA | 117.1 ± 7.99 | 111.06 ± 9.68 | 112.37 ± 6.62 | 110.4 ± 6.92 | NS |
| PUFA | 157.03 ± 12.27 | 148.56 ± 16.08 | 146.05 ± 8.05 | 153.4 ± 10.69 | NS |
| n6 | 129.98 ± 10.01 | 124.24 ± 13.25 | 122.03 ± 7.06 | 129.83 ± 9.27 | NS |
| n3 | 22.45 ± 2.05 | 20.5 ± 2.39 | 20.04 ± 1.38 | 20.3 ± 1.63 | NS |
| $\Delta^9$-des. (16) activity | 8.35 ± 0.57 | 7.5 ± 0.7 | 7.76 ± 0.47 | 6.77 ± 0.35 | NS |
| $\Delta^9$-des. (18) activity | 54.58 ± 1.26 | 56.15 ± 2.02 | 55.62 ± 1.06 | 53.15 ± 1.97 | NS |
| Elongase activity | 60.6 ± 1.01 | 59.73 ± 0.8 | 60.23 ± 0.62 | 58.73 ± 0.96 | NS |

Values are presented as means ± SEM. NS – not significant. Values with different superscripts differ significantly (P<0.05). SFA=saturated fatty acid (C14:0, C15:0, C16:0, C18:0, C20:0, C21:0, C22:0). UFA=unsaturated fatty acid (C16:1, C17:1, C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, C18:1 *trans*-12, C18:1 *trans*-13–14, C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-12, C20:1 *cis*-9, C18:2n-6, C18:3n-3, C20:3n-3, C20:4n-6, C20:5n-3). MUFA=monounsaturated fatty acid (C16:1, C17:1, C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, C18:1 *trans*-12, C18:1 *trans*-13–14, C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-12, C20:1 *cis*-9). PUFA=polyunsaturated fatty acid (C18:2n-6, C18:3n-3, C20:3n-3, C20:4n-6, C20:5n-3). C18:1trans=C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, C18:1 *trans*-12, C18:1 *trans*-13–14. C18:1cis=C18:1 *cis*-9, C18:1 *cis*-11, C18:1 *cis*-12, C20:1 *cis*-9.

was significantly lower in FF from follicles with a physiological glucose concentration of 1.4 to 5 mM when compared with FF samples having a glucose concentration outside that range (63.7 *vs.* 66.0 µg/ml respectively, P<0.05).

*Prediction of COC morphology based on the multivariate model*

In the present experiment, we decided to apply a statistical multivariate model, which was based on parameters characterizing FF as well as CCs from the same follicle.

Using logistic regression with backward variable selection, the model with variables included in Table 3 was chosen to describe the probability of oocytes falling into one of two selected categories: 1) low-quality oocytes (morphology grade 3 or 4) and 2) high-quality oocytes (morphology grade 1 or 2).

The P value for the overall model test of all regression coefficients equal to 0 was 0.006 with all individual regression coefficients significant at P=0.05. Seventy-two percent of the observations were concordant with the predicted probabilities. Thus, based on FA composition, it was possible to determine in 72% of the analyzed samples, whether the obtained COC was of proper morphology. Low-quality oocytes were characterized by a combination of high activity of the two enzymes ($\Delta^9$-desaturase (16) and elongase), high C16:0 content and lower concentrations of C16:1, C18:1cis9 and C22:5n3. According to supplementary analysis, non of the individual FAs was a good predictor of COC morphology, and it is impossible to set an individual threshold for each FA, since it is a combination of them that creates a predictable outcome (data not shown). Besides, 72% of predictions being correct seems fairly good for a complex trait.
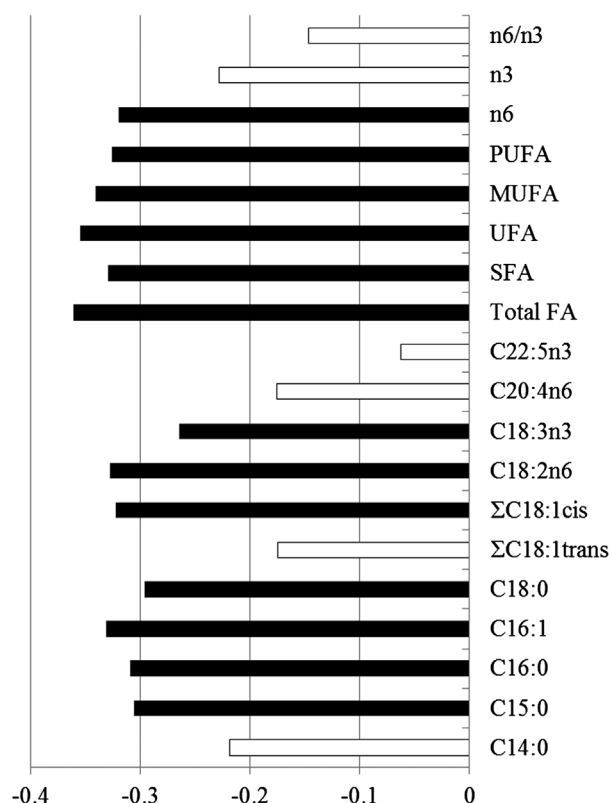
**Fig. 1.** Spearman's rank correlations between apoptotic index in the CC and fatty acids concentrations in follicular fluid. Correlations marked with black bars are significantly different from 0 (P<0.05).
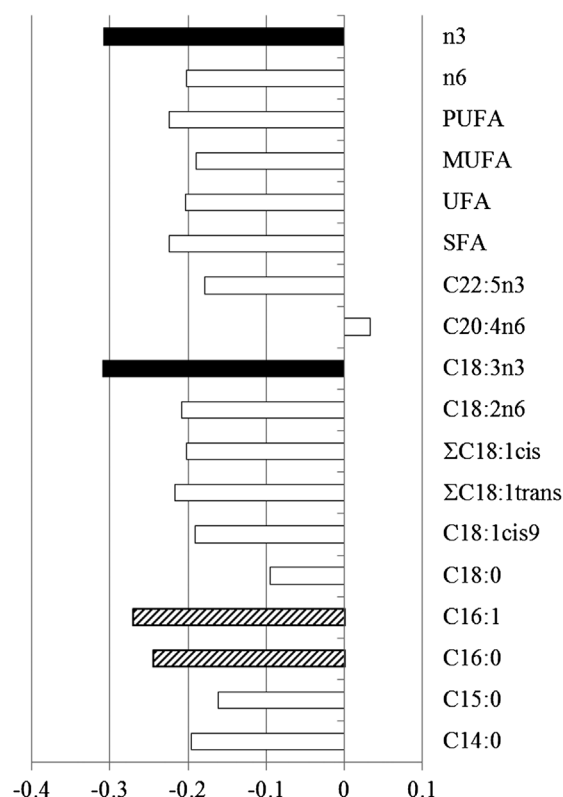
**Fig. 2.** Correlations between glucose (mM) and fatty acid (μg/ml) concentrations in follicular fluid (striped bars – P<0.05, black bars – P<0.01).

**Table 3.** Parameter estimates from the multivariate logistic regression model with backwards variable selection

| Parameter | Estimate | SE | Chi-square | Odds ratio | 95% CI Lower | 95% CI Upper | P |
|---|---|---|---|---|---|---|---|
| Intercept | −33.8553 | 11.6608 | 8.4294 | | | | 0.0037 |
| $\Delta^9$ –des. (16) activity | 0.7378 | 0.3105 | 5.646 | 2.091 | 1.138 | 3.843 | 0.0175 |
| Elongase activity | 0.3966 | 0.1524 | 6.773 | 1.487 | 1.103 | 2.004 | 0.0093 |
| C16:0 | 0.1278 | 0.0352 | 13.1843 | 1.136 | 1.061 | 1.218 | 0.0003 |
| C16:1 | −0.7883 | 0.2959 | 7.0965 | 0.455 | 0.255 | 0.812 | 0.0077 |
| C18:1cis9 | −0.0628 | 0.0241 | 6.78 | 0.939 | 0.896 | 0.985 | 0.0092 |
| C22:5n3 | −0.3155 | 0.1397 | 5.097 | 0.729 | 0.555 | 0.959 | 0.024 |

Estimate – the log odds change per unit of change in the explanatory variable, its standard error (SE) and Chi-square statistics were applied for significance testing. Odds ratio – the ratio of probabilities of falling into the low-quality oocytes category (*vs.* high quality) after increasing a given enzyme or fatty acid level by one unit. CI – confidence interval. P – Pr>ChiSq.

## Discussion

A lot of attempts have been made in the search for a reliable, fast and noninvasive assay for selection of oocytes suitable for *in vitro* embryo production. Potential markers have been found in the follicular components such as follicular fluid or cumulus cells. The reported findings are, however, contradictory, which may reflect the complexity of metabolism of the ovarian follicle. In the present experiment, two statistical models were applied in order to search

for the most accurate approach for predicting the morphological grade of bovine oocytes: 1) the univariate model showed relations between particular parameters of the follicular environment and oocyte morphology or follicular diameter, which did not give evident conclusions, and 2) the multivariate model including FF parameters like concentrations of the four FAs (C16:0, C16:1, C18:1cis9, C22:5n3) and the activities of two enzymes ($\Delta^9$-desaturase (16) and elongase), enabled prediction of the morphology of an oocyte with an accuracy of 72%. Therefore, the multivariate model may be a

promising tool for selecting good quality COCs and thus improving the efficiency of the IVP protocol in cattle.

The univariate model referred the individual follicular components (FF, CC) to either COC morphology or follicular diameter, which are approved components of oocyte morphology. It revealed that the most promising marker of oocyte morphology is the fatty acids composition of the FF. The following traits were of particular interest: concentrations of C18:1cis9 (oleic acid), ∑C18:1cis, C18:2n6 (linoleic acid), C22:5n3 (EPA – eicosapentaenoic acid), UFA, PUFA, n6 and n3 and the $\Delta^9$-desaturase (18) activity. These parameters were present at higher concentrations in medium follicles (the source of better quality COCs) than in large follicles. However, the applied model did not enable the ability to distinguish a parameter that would be correlated with the COCs of the preferred morphology (grades 1 and 2). Glucose content in FF was the only parameter distinguishing follicles with COCs of reduced quality (grades 3 and 4). Therefore, we did not select a parameter with a distinct impact on COC quality. Besides, FF from medium follicles was enriched with respect to two FAs (oleic and linoleic acids) exerting opposite effects on oocyte maturation and embryo development [12], whereas linoleic acid negatively affected the rates of MII oocytes and blastocysts [10]. Hence, simultaneous high concentrations of the two FAs oppositely affected oocyte morphology in medium follicles, which was confusing.

Another aspect of the relations between COC morphology and FA composition was that the glucose concentration or CC apoptosis could be affected by gap junctions signalling. However, only limited data is available in the literature. In human white blood cells, PUFAs inhibit gap junctional communication [26], whereas oleic acid inhibits gap junctions permeability and increases glucose uptake in cultured rat astrocytes [27]. Glucose is transferred to the growing oocyte through gap junctions [28]. Also, it has been suggested that cell death signals are transmitted through gap junctions in many cell types [29]. Thus, it may be anticipated that the follicular factors analyzed in our experiment may influence COC morphology via gap junctional signalling pathways modifications.

Since investigating the follicular environment in relation to oocyte morphology may be deceptive when a single parameter is considered, we made an attempt to create a complex model, which included several parameters characterizing follicular complements. The principles of such approach were described by Aardema *et al.* [12], who observed interesting interactions among three fatty acids – oleic acid, palmitic acid and stearic acid. Oleic acid showed the ability to adverse the negative effects of palmitic and stearic acids in bovine FF. In similar analyses previously performed on human oocytes, multivariate analysis of follicular components was shown to be a promising predictor of pregnancy [30]. Such approach was also suggested by Arya *et al.* [31], who described the concentrations of selected biomolecules from follicular fluid in a multivariate model as a reliable predictor of oocyte quality.

In the present experiment, the multivariate logistic regression with backward variable selection allowed for the accurate (72% correct) prediction of the oocyte morphology. The initial analysis included 3 components: glucose and FA concentrations in FF as well as apoptotic index in cumulus cells. A significant contribution to the prediction model was observed for the following parameters: the activities of the two enzymes [$\Delta^9$-desaturase (16) and elongase] and the

concentrations of four FAs (C16:0, C16:1, C18:1cis9, C22:5n3). The selected fatty acids have been previously described as FF components affecting oocytes and embryos. Palmitic acid (C16:0) exerted a negative effect on oocyte quality. Also, C16:0 supplemented into IVM medium negatively affected the postfertilization development [12, 13]. C16:0 was also described as a potential marker of reduced quality of immature bovine COCs [8, 31] and reduced blastocyst rate [14]. Another FA included into the multivariate model (oleic acid – C18:1cis9) exerted a positive effect on lipid accumulation, oocyte maturation and embryo development in cattle. Also, it showed an ability to adverse the negative effects of palmitic and stearic acids on oocyte maturation [12]. Although palmitoleic acid (C16:1) has not yet gained special attention as FF component, it was shown to inhibit the proapoptotic effect of saturated fatty acids in the rat pancreatic β-cells [32], thus, this particular FA seems to be worthy of attention. Eicosapentaenoic acid (EPA, C22:5n3) was the last FA included into the multivariate model. This FA has not yet been characterized with regard to follicle metabolism; however, as a precursor of prostanoids and inhibitor of the PGF$_{2\alpha}$ secretion [33], it may play a significant role in reproduction.

Energy source is another important issue when discussing the follicular environment. Accumulation of fatty acids is an integral part of energy storage in oocytes, whereas glucose may be an alternative source of energy. Although the oocyte has a limited capacity to utilize glucose [34], the accompanying cumulus cells metabolize glucose and convert it into the pyruvate/lactate [25]. Although the glucose concentration was correlated with some of the analyzed traits (e.g. COC morphological grade, concentration of particular FA) in the present study, the results were contradictory. Both the highest and the lowest concentrations were observed in follicles containing COCs of reduced quality (grade 3 and 4). Also, negative correlation was observed with the concentrations of C16.0 FA (potential marker of reduced quality oocytes) and n3 FAs which have beneficial effects on COCs. Thus, we decided to distinguish two groups of FF samples – those containing a physiological concentration of glucose (1.4 to 5 mM) [25] and those that did not contain a physiological concentration of glucose. It appeared that neither COC morphology nor follicle diameter was related to physiological concentration of glucose. A similar observation was reported by Matoba *et al.* [14], who excluded glucose from the set of candidate markers of embryo quality in cattle.

Besides the follicular fluid, cumulus cells provide information that can be also used for oocyte assessment. Although a COC accompanied by a compact cumulus cell mass is considered healthy, early signs of atresia were correlated with higher quality for the oocyte [35]. An interesting result of the present study is the lack of correlations between AI in CC and COC morphology or follicle diameter, which shows the low utility of the data concerning apoptosis in oocyte quality prediction. This confirms the published evidence, which shows that the correlation between the extent of apoptosis in the CC and the quality of an oocyte is not consistent. Human studies revealed negative correlation between CC apoptosis and meiotic stage of the oocyte [16] as well as fertilization rate [17]. On the other hand, a positive correlation [36] or lack of correlations [37, 38] has also been observed. The data available for cattle is very limited and ambiguous. The results of our study indicate that the

apoptotic index of cumulus cells was of limited value for predicting oocyte quality. Although the AI was not related to follicle diameter or COC morphology, a negative correlation with the fatty acid concentration in follicular fluid was observed. It is possible that FAs affect oocyte quality by inhibiting apoptosis in cumulus cells. However, it cannot be excluded that the high variation in apoptotic index significantly affected statistical analysis. A big variation in AI within CCs was previously reported [39]. The authors suggested a theoretical threshold for CC apoptosis that could trigger the process of oocyte degeneration; however, to our knowledge, it has not yet been defined. This may cause problems with interpretation of data on apoptosis in CCs.

In this study, we mainly focused on prediction of the morphology of bovine COCs by applying a multivariate model that included several follicular parameters. However, the experiment provided us with a set of data describing individual follicles; thus, we decided to point out some interesting interactions between FF components (FAs, glucose) and apoptosis in cumulus cells. An interesting, negative correlation between glucose and the majority of FA concentrations in follicular fluid was observed. There were significant differences with respect to the C16:0, C16:1, C18:3n3 and n3 FAs. Recently, Arya *et al.* [31] underlined energy pathways as possible essential factors affecting the fecundity. Glucose and fatty acids metabolic pathways were discussed in particular. They hypothesized that reduced glucose metabolism provokes other alternative energy pathways, which may utilize, e.g., fatty acids to cover the energy requirement. This suggests possible utilization of the C16:0, C16:1 and C18:3n3 FAs as an alternative energy source for the oocyte. This would explain the fact that C16:0 is the most abundant FA in cattle oocytes [40]. Moreover, interesting negative correlations were observed between the apoptotic index in the CC and the concentrations of the majority of FAs in FF. This was contrary to the experiments of Valholder *et al.* [41, 42], in which FAs supplementation (palmitic, stearic and oleic acid) during *in vitro* culture induced apoptosis in granulosa cells and theca cells.

In conclusion, the present experiment allowed us to obtain a complex set of data from individual bovine ovarian follicles. Statistical analysis showed that traditional univariate analysis, which is based on interpretation of a single follicular parameter, is not precise with regard to oocyte morphology estimation. Based on our results, the multivariate model in our study allowed prediction of the morphology of COCs with an accurancy of 72%. It should be noticed that the model included only selected data – the concentrations of the C16:0, C16:1, C18:1cis9 and C22:5n3 FAs and activities of two enzymes, $\Delta^9$ –desaturase (16) and elongase. The obtained data suggest that it is still possible to improve the selection of oocytes for *in vitro* embryo production; however, complex studies on follicular metabolism should be performed.

## Acknowledgments

## References:

1. **Bavister BD.** Early history of *in vitro* fertilization. *Reproduction* 2002; **124**: 181–196. [Medline] [CrossRef]
2. **Ola SI, Sun QY.** Factors influencing the biochemical markers for predicting mammalian oocyte quality. *J Reprod Dev* 2012; **58**: 385–392. [Medline] [CrossRef]
3. **Leung PC, Lopata A, Kellow GN, Johnston WI, Gronow MJ.** A histochemical study of cumulus cells for assessing the quality of preovulatory oocytes. *Fertil Steril* 1983; **39**: 853–855. [Medline]
4. **Fortune JE.** Ovarian follicular growth and development in mammals. *Biol Reprod* 1994; **50**: 225–232. [Medline] [CrossRef]
5. **Gosden RG, Hunter RH, Telfer E, Torrance C, Brown N.** Physiological factors underlying the formation of ovarian follicular fluid. *J Reprod Fertil* 1988; **82**: 813–825. [Medline] [CrossRef]
6. **Rizos D, Ward F, Duffy P, Boland MP, Lonergan P.** Consequences of bovine oocyte maturation, fertilization or early embryo development *in vitro* versus *in vivo*: implications for blastocyst yield and blastocyst quality. *Mol Reprod Dev* 2002; **61**: 234–248. [Medline] [CrossRef]
7. **Homa ST, Brown CA.** Changes in linoleic acid during follicular development and inhibition of spontaneous breakdown of germinal vesicles in cumulus-free bovine oocytes. *J Reprod Fertil* 1992; **94**: 153–160. [Medline] [CrossRef]
8. **Sinclair KD, Lunn LA, Kwong WY, Wonnacott K, Linforth RS, Craigon J.** Amino acid and fatty acid composition of follicular fluid as predictors of *in-vitro* embryo development. *Reprod Biomed Online* 2008; **16**: 859–868. [Medline] [CrossRef]
9. **Jungheim ES, Macones GA, Odem RR, Patterson BW, Lanzendorf SE, Ratts VS, Moley KH.** Associations between free fatty acids, cumulus oocyte complex morphology and ovarian function during *in vitro* fertilization. *Fertil Steril* 2011; **95**: 1970–1974. [Medline] [CrossRef]
10. **Marei WF, Wathes DC, Fouladi-Nashta AA.** Impact of linoleic acid on bovine oocyte maturation and embryo development. *Reproduction* 2010; **139**: 979–988. [Medline] [CrossRef]
11. **Van Hoeck V, Sturmey RG, Bermejo-Alvarez P, Rizos D, Gutierrez-Adan A, Leese HJ, Bols PE, Leroy JL.** Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. *PLoS One* 2011; **6**: e23183. [Medline] [CrossRef]
12. **Aardema H, Vos PL, Lolicato F, Roelen BA, Knijn HM, Vaandrager AB, Helms JB, Gadella BM.** Oleic acid prevents detrimental effects of saturated fatty acids on bovine oocyte developmental competence. *Biol Reprod* 2011; **85**: 62–69. [Medline] [CrossRef]
13. **Leroy JL, Vanholder T, Mateusen B, Christophe A, Opsomer G, de Kruif A, Genicot G, Van Soom A.** Non-esterified fatty acids in follicular fluid of dairy cows and their effect on developmental capacity of bovine oocytes *in vitro*. *Reproduction* 2005; **130**: 485–495. [Medline] [CrossRef]
14. **Matoba S, Bender K, Fahey AG, Mamo S, Brennan L, Lonergan P, Fair T.** Predictive value of bovine follicular components as markers of oocyte developmental potential. *Reprod Fertil Dev* 2014; **26**: 337–345. [CrossRef]. [Medline]
15. **Liu S, Jiang JJ, Feng HL, Ma SY, Li M, Li Y.** Evaluation of the immature human oocytes from unstimulated cycles in polycystic ovary syndrome patients using a novel scoring system. *Fertil Steril* 2010; **93**: 2202–2209. [Medline] [CrossRef]
16. **Høst E, Gabrielsen A, Lindenberg S, Smidt-Jensen S.** Apoptosis in human cumulus cells in relation to zona pellucida thickness variation, maturation stage, and cleavage of the corresponding oocyte after intracytoplasmic sperm injection. *Fertil Steril* 2002; **77**: 511–515. [Medline] [CrossRef]
17. **Lee KS, Joo BS, Na YJ, Yoon MS, Choi OH, Kim WW.** Cumulus cells apoptosis as an indicator to predict the quality of oocytes and the outcome of IVF-ET. *J Assist Reprod Genet* 2001; **18**: 490–498. [Medline] [CrossRef]
18. **Yuan YQ, Van Soom A, Leroy JL, Dewulf J, Van Zeveren A, de Kruif A, Peelman LJ.** Apoptosis in cumulus cells, but not in oocytes, may influence bovine embryonic developmental competence. *Theriogenology* 2005; **63**: 2147–2163. [Medline] [CrossRef]
19. **Warzych E, Pers-Kamczyc E, Krzywak A, Dudzińska S, Lechniak D.** Apoptotic index within cumulus cells is a questionable marker of meiotic competence of bovine oocytes matured *in vitro*. *Reprod Biol* 2013; **13**: 82–87. [Medline] [CrossRef]
20. **Senbon S, Hirao Y, Miyano T.** Interactions between the oocyte and surrounding somatic cells in follicular development: lessons from *in vitro* culture. *J Reprod Dev* 2003; **49**: 259–269. [Medline] [CrossRef]
21. **Stojkovic M, Machado SA, Stojkovic P, Zakhartchenko V, Hutzler P, Gonçalves PB, Wolf E.** Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after *in vitro* maturation: correlation with morphological criteria and developmental capacity after *in vitro* fertilization and culture. *Biol Reprod* 2001; **64**: 904–909. [Medline] [CrossRef]
22. **Cieslak A, Machmuller A, Szumacher-Strabel M, Scheeder MRL.** A comparison of two extraction methods used to quantify the C18 fatty acids in feed and digesta of rumi-

nants. *J Anim Feed Sci* 2009; **18**: 362–367.

23. **Warzych E, Cieslak A, Pawlak P, Renska N, Pers-Kamczyc E, Lechniak D.** Maternal nutrition affects the composition of follicular fluid and transcript content in gilt oocytes. *Vet Med* 2011; **56**: 156–167.

24. **Bender K, Walsh S, Evans AC, Fair T, Brennan L.** Metabolite concentrations in follicular fluid may explain differences in fertility between heifers and lactating cows. *Reproduction* 2010; **139**: 1047–1055. [Medline] [CrossRef]

25. **Sutton-McDowall ML, Gilchrist RB, Thompson JG.** The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* 2010; **139**: 685–695. [Medline] [CrossRef]

26. **Hii CS, Ferrante A, Schmidt S, Rathjen DA, Robinson BS, Poulos A, Murray AW.** Inhibition of gap junctional communication by polyunsaturated fatty acids in WB cells: evidence that connexin 43 is not hyperphosphorylated. *Carcinogenesis* 1995; **16**: 1505–1511. [Medline] [CrossRef]

27. **Lavado E, Sanchez-Abarca LI, Tabernero A, Bolaños JP, Medina JM.** Oleic acid inhibits gap junction permeability and increases glucose uptake in cultured rat astrocytes. *J Neurochem.* 1997; **69**: 721–728. [Medline] [CrossRef]

28. **Eppig JJ.** Intercommunication between mammalian oocytes and companion somatic cells. *Bioessays* 1991; **13**: 569–574. [Medline] [CrossRef]

29. **Cusato K, Ripps H, Zakevicius J, Spray DC.** Gap junctions remain open during cytochrome c-induced cell death: relationship of conductance to 'bystander' cell killing. *Cell Death Differ* 2006; **13**: 1707–1714. [Medline] [CrossRef]

30. **O'Gorman A, Wallace M, Cottell E, Gibney MJ, McAuliffe FM, Wingfield M, Brennan L.** Metabolic profiling of human follicular fluid identifies potential biomarkers of oocyte developmental competence. *Reproduction* 2013; **146**: 389–395. [Medline] [CrossRef]

31. **Arya BK, Haq AU, Chaudhury K.** Oocyte quality reflected by follicular fluid analysis in poly cystic ovary syndrome (PCOS): a hypothesis based on intermediates of energy metabolism. *Med Hypotheses* 2012; **78**: 475–478. [Medline] [CrossRef]

32. **Welters HJ, Tadayyon M, Scarpello JH, Smith SA, Morgan NG.** Mono-unsaturated fatty acids protect against beta-cell apoptosis induced by saturated fatty acids, serum withdrawal or cytokine exposure. *FEBS Lett* 2004; **560**: 103–108. [Medline] [CrossRef]

33. **Mattos R, Guzeloglu A, Badinga L, Staples CR, Thatcher WW.** Polyunsaturated fatty acids and bovine interferon-tau modify phorbol ester-induced secretion of prostaglandin F2 alpha and expression of prostaglandin endoperoxide synthase-2 and phospholipase-A2 in bovine endometrial cells. *Biol Reprod* 2003; **69**: 780–787. [Medline] [CrossRef]

34. **Sutton ML, Cetica PD, Beconi MT, Kind KL, Gilchrist RB, Thompson JG.** Influence of oocyte-secreted factors and culture duration on the metabolic activity of bovine cumulus cell complexes. *Reproduction* 2003; **126**: 27–34. [Medline] [CrossRef]

35. **de Wit AA, Wurth YA, Kruip TA.** Effect of ovarian phase and follicle quality on morphology and developmental capacity of the bovine cumulus-oocyte complex. *J Anim Sci* 2000; **78**: 1277–1283. [Medline]

36. **Raman RS, Chan PJ, Corselli JU, Patton WC, Jacobson JD, Chan SR, King A.** Comet assay of cumulus cell DNA status and the relationship to oocyte fertilization via intracytoplasmic sperm injection. *Hum Reprod* 2001; **16**: 831–835. [Medline] [CrossRef]

37. **Abu-Hassan D, Koester F, Shoepper B, Schultze-Mosgau A, Asimakopoulos B, Diedrich K, Al-Hasani S.** Comet assay of cumulus cells and spermatozoa DNA status, and the relationship to oocyte fertilization and embryo quality following ICSI. *Reprod Biomed Online* 2006; **12**: 447–452. [Medline] [CrossRef]

38. **Corn CM, Hauser-Kronberger C, Moser M, Tews G, Ebner T.** Predictive value of cumulus cell apoptosis with regard to blastocyst development of corresponding gametes. *Fertil Steril* 2005; **84**: 627–633. [Medline] [CrossRef]

39. **Hussein TS, Thompson JG, Gilchrist RB.** Oocyte-secreted factors enhance oocyte developmental competence. *Dev Biol* 2006; **296**: 514–521. [Medline] [CrossRef]

40. **Kim JY, Kinoshita M, Ohnishi M, Fukui Y.** Lipid and fatty acid analysis of fresh and frozen-thawed immature and *in vitro* matured bovine oocytes. *Reproduction* 2001; **122**: 131–138. [Medline] [CrossRef]

41. **Vanholder T, Leroy JL, Van Soom A, Opsomer G, Maes D, Coryn M, de Kruif A.** Effect of non-esterified fatty acids on bovine granulosa cell steroidogenesis and proliferation *in vitro*. *Anim Reprod Sci* 2005; **87**: 33–44. [Medline] [CrossRef]

42. **Vanholder T, Leroy J, Van Soom A, Maes D, Coryn M, Fiers T, de Kruif A, Opsomer G.** Effect of non-esterified fatty acids on bovine theca cell steroidogenesis and proliferation *in vitro*. *Anim Reprod Sci* 2006; **92**: 51–63. [Medline] [CrossRef]