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SHORT COMMUNICATION

The effect of human follicular fluid on bovine oocyte developmental competence and embryo quality

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Abstract In this study, the hypothesis that embryo development during routine IVF procedures is determined by the pre-ovulatory follicular fluid composition was tested. Follicular fluid from women with obesity ('obese') and a 'positive' or 'negative' IVF outcome was added during the in-vitro maturation of bovine oocytes. 'Negative' and 'obese' follicular fluid reduced bovine embryo development, compared with laboratory control embryo development ($P < 0.05$ or $P < 0.1$). The addition of follicular fluid also altered bovine blastocyst gene expression. Furthermore, *LDHA* and *PPARGC1B* gene expression differed between follicular fluid groups. Data suggest that pre-ovulatory follicular fluid can potentially affect oocyte developmental competence and embryo quality. Furthermore, the bovine model may be used as a screening tool.

KEYWORDS: embryo quality, follicular fluid, in-vitro maturation, IVF, oocyte developmental competence

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Introduction

Maternal metabolic disorders, such as obesity and type 2 diabetes, are associated with hormonal and biochemical changes in the serum, which are reflected in the micro-environment of the maturing oocyte, the follicular fluid (Leroy et al., 2012; Valckx et al., 2012). Such changes, such as follicular fluid free fatty acid (FFA) concentrations, hamper in-vitro bovine oocyte developmental competence as well as embryo quality and metabolism (Van Hoeck et al., 2011). Moreover, adding human follicular fluid high in triglycerides and FFAs from obese patients impaired murine oocyte maturation and caused endoplasmic reticulum stress (Yang et al., 2012). Furthermore, adding bovine follicular fluid with high concentrations of FFA during bovine in-vitro cumulus–oocyte complex (COC) maturation, resulted in a massive intracellular lipid storage in the cumulus cells, which may be seen as a protective mechanism (Aardema et al., 2013). Other parameters, such as insulin, cholesterol and total protein in the follicular fluid have also been studied (Valckx et al., 2012), but it remains unclear whether and how the composition of the pre-ovulatory follicular fluid may influence the enclosed oocyte's developmental success during in-vitro embryo growth. The aim of this study was to test whether the follicular fluid composition during final maturation *in-vivo* might influence the quality of embryos generated during human IVF procedures. To investigate this, it was essential to uncouple intrinsic oocyte quality from potential effects caused by the follicular fluid. Therefore, human follicular fluid was used in an entirely independent in-vitro setting, with the aim of studying the effect of exposing bovine in-vitro maturing oocytes to human follicular fluid from women with differential IVF outcomes or obesity, on bovine oocyte developmental competence and subsequent embryo quality. The bovine model was chosen because of multiple similarities between human and bovine ovarian physiology, oocyte maturation and early embryo development (Menezo and Herubel, 2002). To our knowledge, this is the first time this model has been implemented as a potential screening tool to study the effect of the composition of pre-ovulatory follicular fluid on oocyte and embryo quality.

Materials and methods

Collection and selection of follicular fluid samples

Samples were collected as previously described by Valckx et al. (2012), with ethical approval (FER-P0905/F18). Because numerous variables may influence IVF outcome, strict exclusion criteria were applied to eliminate known confounders: age over 38 years, body mass index (BMI) less than 18 kg/m^2, polycystic ovarian syndrome, blood sample contamination, fewer than six oocytes aspirated, less than 50% successfully fertilized oocytes and the male factors cryptozo- or azoospermia. Selected samples consequently originated from women that reacted well to ovarian stimulation, with the exclusion of major male and female subfertility risk factors. Three groups were defined using specific inclusion criteria: 'negative' (n = 6): 30% or less day 3 good-quality embryos (criteria as described in Valckx et al., 2012) originating from that oocyte retrieval session, BMI less than 30 kg/m^2 and the oocyte originating from the follicular fluid sample did not develop into a good-quality embryo; 'positive' (n = 6): 50% or more day 3 good-quality embryos originating from that oocyte retrieval session, BMI less than 30 kg/m^2 and the oocyte originating from the follicular fluid sample developed into a good-quality embryo; and 'obese' (n = 6): BMI over 30 kg/m^2.

Following these criteria, six samples per treatment group, originating from 15 women, were selected out of a total of 120 samples from 70 women.

Bovine in-vitro embryo production

Immature bovine COCs were isolated from slaughterhouse ovaries and matured in groups of 50 for 24 h in 500 µl serum-free maturation medium, supplemented with 25% heat-inactivated follicular fluid pools from the different treatments. A routine serum and follilcular fluid free laboratory control was run in parallel. Oocytes were fertilized and presumptive zygotes cultured in groups of 25–30 embryos (50 µl serum-free synthetic oviduct fluid medium droplets under oil), after routine laboratory procedures as in Van Hoeck et al. (2011) with minor modifications.

Bovine oocyte and embryo quality parameters (four replicates)

In each replicate, 100 COCs were cultured in each treatment group. Five or six oocytes and presumptive zygotes per treatment and per replicate were stained in 5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (5 min, Sigma-Aldrich, Bornem, Belgium), to determine maturation and fertilization rate, respectively. Denuded mature oocytes were collected after 24-h in-vitro maturation (10-min vortexing to remove cumulus cells) and presumptive zygotes were collected after fertilization (3-min vortexing). Cleavage rate and blastocyst formation, for the remaining presumptive zygotes, were documented on days 2 and 8 post insemination, respectively. Six or seven day-8 expanded blastocysts, per treatment and per replicate were stained with DAPI and TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (Roche Diagnostics, Vilvoorde, Belgium) as previously described (Van Hoeck et al., 2011) to study total cell number and apoptotic cell ratio, respectively.

Gene expression (four replicates)

An equal number of normal and expanded blastocysts for each treatment was snap frozen (in groups of 10) on day 7.5 of culture, and analysed for the transcript abundance of the genes presented in Figure 1. These genes were particularly chosen for their relevance in embryo development, quality and metabolism. mRNA isolation, retrotranscription and real-time polymerase chain reaction quantification were carried out as described by Van Hoeck et al. (2011), with minor modifications.

Statistical analyses

PASW 18.0 for Windows, Chicago, IL, USA was used for statistical analysis. Binary outcomes (oocyte and embryo

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development) were analysed with binary logistic regression, continuous variables (total cell number and apoptotic cell index) with a mixed model analysis of variance and post-hoc Sheffé tests. Treatment was entered as a fixed factor and replicate as random factor. Insignificant interaction terms were omitted from the model. mRNA abundance was studied with a one-way analysis of variance and post-hoc Student–Newman–Keuls tests. Statistical significance and trends were set at $P < 0.05$ and $P < 0.1$, respectively.

Results

The results showed no significant difference in maturation rate, fertilization rate, blastocyst hatching rate, total cell number and apoptotic cell index. Cleavage rate, however, was reduced for the 'negative' treatment, compared with the laboratory control treatment ($P < 0.05$). Furthermore, a trend was seen for a reduced blastocyst formation for 'negative' follicular fluid and 'obese' embryos, compared with the laboratory control embryos, and a trend for a reduced blastocyst formation from cleaved zygotes in 'obese' embryos, compared with laboratory control embryos ($P < 0.1$) (Table 1). Gene expression of *LDHA* was higher in laboratory control and 'obese' embryos, compared with 'negative' and 'positive' embryos ($P < 0.05$) (Figure 1). DNMT3A and SLC27A1 expression was higher in laboratory control embryos compared with 'positive' embryos ($P < 0.05$). TP53 and ACCA1 expression were higher in laboratory control embryos, compared with 'positive', 'negative' and 'obese' embryos ($P < 0.05$). Finally, PPARGC1B expression was higher in laboratory control embryos, compared with 'negative' and 'obese' embryos ($P < 0.05$). PPARGC1B expression was also higher in 'positive' compared with 'obese' embryos ($P < 0.05$) (Figure 1). mRNA transcript abundance of GAPDH, MNSOD, IGF2R, GLUT1, GPX1 and NRF2 was not affected by treatment.

Discussion

To our knowledge, this is the first time, a bovine oocyte maturation model was used as a screening tool to specifically investigate the effect of human pre-ovulatory follicular fluid from women with a differential IVF outcome or obesity, on in-vitro bovine oocyte developmental competence and embryo quality. Support for the validity of the bovine model, which allowed the essential uncoupling of human intrinsic oocyte quality and follicular fluid, comes from the observation that embryo development was similar between the 'positive' treatment and our laboratory control treatment. Our results showed that nuclear maturation and fertilization potential were not affected by treatment. Our data furthermore suggest that oocyte developmental competence is impaired by the 'negative' and 'obese' treatments, because the 'negative' treatment reduced bovine cleavage rate ($P < 0.05$), the 'negative' and 'obese' treatments tended to reduce blastocyst formation ($P < 0.1$) and the 'obese' treatment tended to reduce blastocyst formation from cleaved zygotes ($P < 0.1$), compared

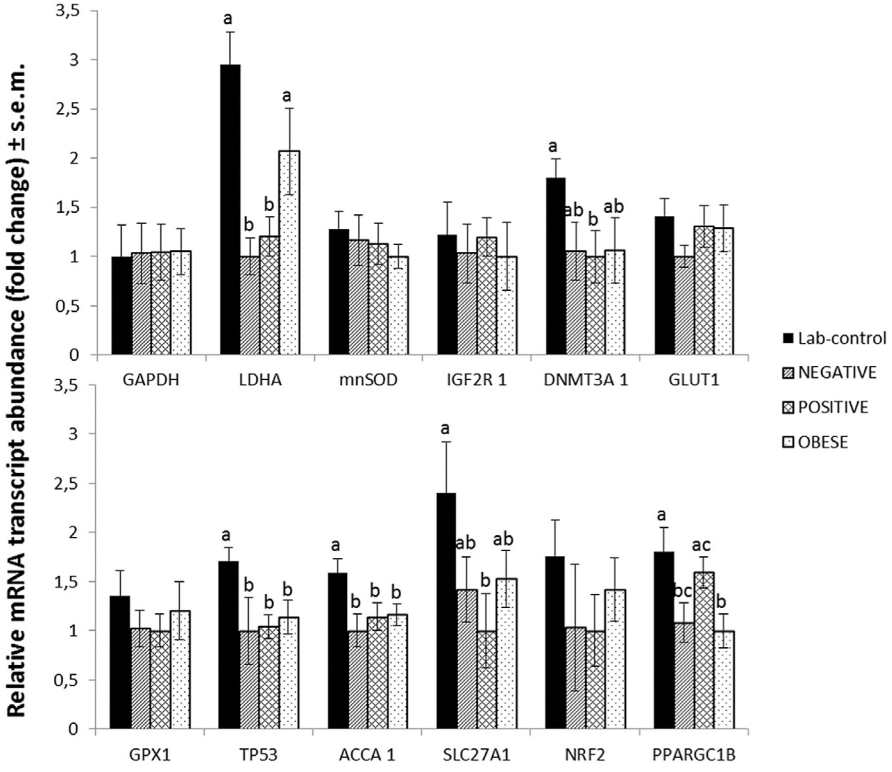

Figure 1 Gene expression analyses. Day 7.5 blastocyst transcript abundance for laboratory control, 'negative' 'positive' and 'obese' follicular fluid treatment groups. Data are presented as relative fold change (±standard error of mean), with reference to the treatment with the lowest expression level. [abc]Data with a different superscript differ significantly ($P < 0.05$).

Table 1 Oocyte and embryo outcome parameters.

	Laboratory control	*Negative*	*Positive*	*Obese*
Maturation rate (%)	74 (14/19)	92 (22/24)	78 (18/23)	62 (13/21)
Fertilization rate (%)	79 (19/24)	63 (15/24)	79 (19/24)	76 (16/21)
Cleavage rate (%)	80^{a} (263/327)	74^{b} (233/317)	77^{ab} (256/331)	76^{ab} (230/301)
Blastocyst formation (%)	31^{A} (101/327)	25^{B} (79/317)	27^{AB} (89/331)	24^{B} (72/301)
Blastocyst formation from cleaved zygotes (%)	38^{A} (101/263)	34^{AB} (79/233)	35^{AB} (89/256)	31^{B} (72/230)
Hatching rate (%)	39 (39/101)	28 (22/79)	36 (32/89)	32 (23/72)
Cell number (n)*	108 ± 25 (25)	110 ± 24 (27)	101 ± 21 (26)	110 ± 22 (26)
Apoptotic cell index (%)*	5.6 (6/108)	5.5 (6/110)	5.9 (6/101)	3.6 (4/110)

Maturation and fertilization rates were determined by 4',6-diamidino-2-phenylindole staining in a subset of oocytes/zygotes, and the remaining embryos proceeded in culture until the blastocyst stage. Data are presented as percentages or as means ± standard deviation.
[ab]Data with a different superscript differ significantly ($P < 0.05$);
[AB]Data tend to be different ($P < 0.1$).
*Numeric variables between brackets represent the number of embryos stained (cell number) and the mean counts (mean number of apoptotic cells/mean cell number) of those embryos for each treatment group (apoptotic cell index).

with the laboratory control. Most significant differences in bovine blastocyst mRNA transcript abundance were found between the laboratory control and embryos from the 'negative', 'positive' and 'obese' follicular fluid groups (*LDHA, DNMT3A, TP53, ACCA, SLC27A1, PPARGC1B*) and are as such caused by adding follicular fluid to the maturation medium. Interestingly, *LDHA* is responsible for the reversible oxidation of lactate to pyruvate with the production of reducing NADH and is overexpressed in 'obese' embryos. *LDHA* expression is also increased in oocytes exposed to elevated FFA concentrations (Van Hoeck et al., 2013), and might act as a cytosolic reductant in the defence against oxidative stress. *PPARGC1B* regulates cellular differentiation, development and oxidative metabolism and is expressed to a higher degree in 'positive' embryos, compared with 'obese' embryos, suggesting that its function might be improved in 'positive' embryos. On the basis of these results, it is possible that these pathways may be involved in the distinction between good- and bad-quality embryos during IVF procedures.

In conclusion, although the described effect sizes were small, our results suggest that changes in the composition of the follicular fluid may be responsible for decreased oocyte and embryo quality in this study and that the bovine model may be used as a screening tool to investigate the effect of human follicular fluid composition on IVF outcome.

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References

Aardema, H., Lolicato, F., Van De Lest, C.H., Brouwers, J.F., Vaandrager, A.B., Van Tol, H.T., Roelen, B.A., Vos, P.L., Helms, J.B., Gadella, B.M., 2013. Bovine cumulus cells protect maturing oocytes from increased fatty acid levels by massive intracellular lipid storage. Biol. Reprod. 88, 164.

Leroy, J.L.M.R., Rizos, D., Sturmey, R., Bossaert, P., Gutierrez-Adan, A., Van Hoeck, V., Valckx, S., Bols, P.E.J., 2012. Intrafollicular conditions as a major link between maternal metabolism and oocyte quality: a focus on dairy cow fertility. Reprod. Fertil. Dev. 24, 1–12.

Menezo, Y.J., Herubel, F., 2002. Mouse and bovine models for human ivf. Reprod. Biomed. Online 4, 170–175.

Valckx, S.D., De Pauw, I., De Neubourg, D., Inion, I., Berth, M., Fransen, E., Bols, P.E., Leroy, J.L., 2012. Bmi-related metabolic composition of the follicular fluid of women undergoing assisted reproductive treatment and the consequences for oocyte and embryo quality. Hum. Reprod. 27, 3531–3539.

Van Hoeck, V., Sturmey, R.G., Bermejo-Alvarez, P., Rizos, D., Gutierrez-Adan, A., Leese, H.J., Bols, P.E., Leroy, J.L., 2011. Elevated non-esterified fatty acid concentrations during bovine oocyte maturation compromise early embryo physiology. PLoS ONE 6, e23183.

Van Hoeck, V., Leroy, J.L., Arias Alvarez, M., Rizos, D., Gutierrez-Adan, A., Schnorbusch, K., Bols, P.E., Leese, H.J., Sturmey, R.G., 2013. Oocyte developmental failure in response to elevated

non-esterified fatty acid concentrations: mechanistic insights. Reproduction 145, 33–44.
Yang, X., Wu, L.L., Chura, L.R., Liang, X., Lane, M., Norman, R.J., Robker, R.L., 2012. Exposure to lipid-rich follicular fluid is associated with endoplasmic reticulum stress and impaired oocyte maturation in cumulus-oocyte complexes. Fertil. Steril. 97, 1438–1443.

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