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

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Abstract

We hypothesized that embryo development during routine IVF procedures is determined by the pre-ovulatory follicular fluid (FF) composition. Therefore, FF 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 FF reduced bovine embryo development, compared to the lab-control ($P < 0.05$ or $P < 0.1$). Adding FF also altered bovine blastocyst gene expression. Additionally, *LDHA* and *PPARGC1B* gene expression differed between FF groups. Our data suggest that pre-ovulatory FF can potentially affect oocyte developmental competence and embryo quality. Furthermore, the bovine model may be used as a screening tool.

Key words: follicular fluid, oocyte developmental competence, embryo quality, IVF, *in vitro* maturation

Introduction

Maternal metabolic disorders, like 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 (Leroy et al., 2012, Valckx et al., 2012), the follicular fluid (FF). Such changes, like high FF 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 FF high in triglycerides and FFAs from obese patients impaired murine oocyte maturation and caused endoplasmic reticulum stress (Yang et al., 2012). Furthermore, adding bovine FF 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 FF have also been studied (Valckx et al., 2012), but it remains unclear whether and how the composition of the pre-ovulatory FF may influence the enclosed oocyte's developmental success during *in vitro* embryo growth. Therefore, we hypothesized that the FF composition during final maturation *in vivo* might influence the quality of embryos generated during human *in vitro* fertilization (IVF) procedures. To investigate this, it was essential to uncouple intrinsic oocyte quality from potential effects caused by the FF. Therefore, we used human FF in an entirely independent *in vitro* setting, where we aimed to study the effect of exposing bovine *in vitro* maturing oocytes to human FF from women with differential IVF outcomes or obesity, on bovine oocyte developmental competence and subsequent embryo quality. We chose the bovine model because of multiple similarities between human and bovine ovarian physiology, oocyte maturation and early embryo development (Menezo and Herubel, 2002) and we aimed to implement this model for the first time as a potential screening tool to study the effect of the composition of pre-ovulatory FF on oocyte and embryo quality.

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Methods

Collection and selection of FF 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, we adopted very strict exclusion criteria to eliminate known confounders: age >38 years, body mass index (BMI) <18kg/m², polycystic ovarian syndrome, blood sample contamination, <6 oocytes aspirated, <50% successfully fertilized oocytes and the male factors cryptozo- or azoospermia. Selected samples consequently originated from women with a good response to

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ovarian stimulation, with the exclusion of major male and female subfertility risk factors. Using specific inclusion criteria 3 groups were defined:

(1) NEGATIVE (n=6): ≤30% Day 3 good quality embryos (criteria as described in Valckx *et al*., 2012) originating from that ovum pick-up (OPU) session, BMI<30kg/m² and the oocyte originating from the FF sample did not develop into a good quality embryo;

(2) POSITIVE (n=6): ≥50% Day 3 good quality embryos originating from that OPU session, BMI<30kg/m² and the oocyte originating from the FF sample developed into a good quality embryo;

(3) OBESE (n=6): BMI>30kg/m².

Following these criteria, 6 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 24h in 500µl serum-free maturation medium, supplemented with 25% heat-inactivated FF pools from the different treatments. A routine serum/FF free lab-control was run in parallel. Oocytes were fertilized and presumptive zygotes cultured in groups of 25-30 embryos (50µl serum free SOF medium droplets under oil), following routine laboratory procedures as in Van Hoeck *et al.* (2011) with minor modifications.

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Bovine oocyte and embryo quality parameters (4 replicates). In each replicate, 100 COCs were cultured in each treatment group. Five or 6 oocytes and presumptive zygotes per treatment and per replicate were stained in 5µg/ml DAPI (5min, Sigma-Aldrich, Bornem, Belgium), to determine maturation and fertilization rate, respectively. Denuded mature oocytes were collected after 24h *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 (p.i.), respectively. Six or 7 Day 8 expanded blastocysts, per treatment and per replicate were stained with DAPI and TUNEL (Roche Diagnostics, Vilvoorde, Belgium) as previously described (Van Hoeck *et al.*, 2011) to study total cell number and apoptotic cell ratio, respectively.

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Gene expression (4 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 analyzed 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 PCR quantification were performed as described by Van Hoeck *et al.*(2011), with minor modifications.

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Statistical analyses (PASW 18.0 for Windows, Chicago, IL, USA). Binary outcomes (oocyte/embryo development) were analyzed with binary logistic regression, continuous variables (total cell number/apoptotic cell index) with a mixed model ANOVA 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 ANOVA 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. However, cleavage rate was reduced for the NEGATIVE treatment, compared with the Lab-control ($P < 0.05$). Furthermore, there was a trend for a reduced blastocyst formation for NEG FF and OBESE embryos, compared with the lab-control and a trend for a reduced blastocyst formation from cleaved zygotes in OBESE embryos, compared with lab-control embryos ($P < 0.1$, **Table 1**). Gene expression of LDHA was higher in lab-control and OBESE embryos, compared with NEGATIVE and POSITIVE embryos ($P < 0.05$, **Figure 1**). DNMT3A and SLC27A1 expression was higher in lab-control, compared to POSITIVE embryos ($P < 0.05$). TP53 and ACCA expression were higher in lab-control embryos, compared to POSITIVE, NEGATIVE and OBESE embryos ($P < 0.05$). Finally, PPARGC1B expression was higher in lab-control embryos, compared with NEGATIVE and OBESE embryos ($P < 0.05$). PPARGC1B expression was also higher in POSITIVE, compared to OBESE embryos ($P < 0.05$, **Figure 1**). mRNA transcript abundance of GAPDH, MNSOD, IGF2R, GLUT1, GPX1 and NRF2 was not affected by treatment.

Discussion

For the first time, we used a bovine oocyte maturation model as a screening tool to specifically investigate the effect of human pre-ovulatory FF, 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 FF, comes from the observation that embryo development was similar between the POSITIVE treatment and our lab-control. Our results showed that nuclear maturation and fertilization potential were not affected by treatment. However, the data do suggest that oocyte developmental competence is impaired by the NEGATIVE and OBESE treatments, because (1) the NEGATIVE treatment reduced bovine cleavage rate ($P<0.05$), (2) the NEGATIVE and OBESE treatments tended to reduce blastocyst formation ($P<0.1$) and (3) the OBESE treatment tended to reduce blastocyst formation from cleaved zygotes ($P<0.1$), compared with the lab-control. Most significant differences in bovine blastocyst mRNA transcript abundance were found between lab-control and embryos from the NEGATIVE, POSITIVE and OBESE FF groups: *LDHA, DNMT3A, TP53, ACCA, SLC27A1, PPARGC1B* and are as such caused by adding FF 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 NEFA 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. Based on 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 (1) changes in the composition of the FF may be responsible for decreased oocyte and embryo quality in this study and (2) the bovine model may be used as a screening tool to investigate the effect of human FF composition on IVF outcome.

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SDMV and JLMRL declare that they were responsible for the conception and design of the study, as well as writing the manuscript. JDB declares that she performed the gene expression analyses and aided to draft the manuscript. SDMV and EDGM performed the *in vitro* work and were responsible for the collection and interpretation of data. IGFG and UP collected the follicular fluid samples. PRI and AGA declare that they supervised the gene expression analyses and performed the specific statistical analyses. PEJB critically revised the work and aided to draft the manuscript. All authors approved the final version of the manuscript. None of the authors has any conflict of interest to declare.

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Table 1: **Oocyte and embryo outcome parameters**.

	Lab-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)

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).

Figure 1: Gene expression analyses. Day 7.5 blastocyst transcript abundance for lab-control, NEGATIVE, POSITIVE and OBESE FF treatment groups. Data are presented as relative fold change (± S.E.M.), with reference to the treatment with the lowest expression level. ^{ab}Data with a different superscript differ significantly ($P < 0.05$).