Zygote **23** (August), pp. 563–572. © Cambridge University Press 2014
doi:10.1017/S0967199414000239 First Published Online 10 June 2014

Insulin influences developmental competence of bovine oocytes cultured in α-MEM plus follicle-simulating hormone

Gustavo Bruno Mota[2], *Ingrid Oliveira e Silva*[2], *Danielle Kaiser de Souza*[2,4], *Flavia Tuany*[2], *Michele Munk Pereira*[3], *Luiz Sergio de Almeida Camargo*[3] and *Alzira Amélia Martins Rosa e Silva*[1]
Laboratory for the Study of Reproduction, Biological Institute, University of Brasília; Embrapa Dairy Cattle, Juiz de Fora; and Faculty of Ceilandia, University of Brasília, Brazil

Date submitted: 6.6.2013. Date revised: 07.04.2014. Date accepted: 7.4.2014

Summary

The aim of this study was to evaluate the dose–response effect of insulin, plus follicle-simulating hormone (FSH) at a fixed concentration, in a serum-free defined culture medium (DCM) on the *in vitro* maturation of bovine cumulus–oocyte complexes (COCs). For oocyte nuclear maturation, the expression levels of *GDF9*, *GLUT1*, *PRDX1* and *HSP70.1* transcripts related to oocyte and embryo developmental competence were analysed. For *in vitro* maturation (IVM), cumulus–oocyte complexes from slaughterhouse ovaries were distributed into four groups based on insulin concentration added to serum-free DCM, which was composed of alpha minimum essential medium (α-MEM), as basal medium: (1) DCM control: 0 ng/ml; (2) DCM1: 1 ng/ml; (3) DCM10: 10 ng/ml; and (4) DCM100: 100 ng/ml. After IVM, the nuclear status of a sample of oocytes was analysed and the other oocytes were submitted for *in vitro* fertilization (IVF) and *in vitro* culture (IVC). Different concentrations of insulin did not affect significantly the nuclear maturation and cleavage rate (72 h post-insemination) across all groups. Blastocyst rate (192 h post-insemination) did not differ in DCM control (24.3%), DCM1 (27.0%) and DCM10 (26.3%) groups, but the DCM100 (36.1%) group showed a greater blastocyst rate ($P < 0.05$) than the DCM control. Insulin concentrations of 1, 10, or 100 ng/ml decreased the relative levels of *GDF9* and *HSP70-1* transcripts in oocytes at the end of IVM ($P < 0.05$). The transcripts levels of *PRDX1* decreased ($P < 0.05$) only when 10 or 100 ng/ml insulin was added to the DCM medium. No difference in levels of *GLUT1* transcripts ($P > 0.05$) was observed at the different insulin concentrations. The results indicated that insulin added to DCM influenced levels of transcripts related to cellular stress (*HSP70-1* and *PRDX1*) and oocyte competence (*GDF9*) in bovine oocytes and at higher concentrations enhanced blastocyst production.

Keywords: Bovine, *In vitro* maturation, Insulin, Oocytes, Transcripts.

Introduction

The *in vitro* production (IVP) of embryos has been largely successful in cattle and might improve their genetic background by shortening significantly the generation gap, when compared with artificial insemination and embryo transfer (ET). However, oocyte quality (Lonergan *et al.*, 1994; Otoi *et al.*, 1997) and *in vitro* maturation (IVM) conditions (Warzych *et al.*, 2007) may determine the proportion of embryos that are suitable for ET.

It is well known that oocyte competence is acquired during oocyte growth, but is only concluded during the final period of maturation *in vivo* (Ferreira *et al.*, 2009). Some ultrastructural cytoplasmic changes in the oocyte occur after 18 h and extrusion of the polar body occurs 19 h after the luteinizing hormone (LH) surge (Ferreira *et al.*, 2009). However, the maturation process *in vitro* is influenced by culture

[1]All correspondence to: Alzira A. M. Rosa e Silva. Laboratory for the Study of Reproduction, Biological Institute, University of Brasília, DF, 70910–900, Brazil. Tel: +55 61 31072914. e-mail: aamresil@unb.br
[2]Laboratory for the Study of Reproduction, Biological Institute, University of Brasília, DF, 70910–900, Brazil.
[3]Embrapa Dairy Cattle, Juiz de Fora, MG, 36038–330, Brazil.
[4]Faculty of Ceilandia, University of Brasília, DF, Brazil.

https://doi.org/10.1017/S0967199414000239 Published online by Cambridge University Press

environment; alpha minimum essential medium (α-MEM) defined medium (DCM) has been used to mimic the physiological *in vivo* conditions as it maintains oestrogen concentrations, aromatase expression in follicular cell walls (Vasconcelos *et al.*, 2013) and blockage of oocyte nuclear maturation (Oliveira e Silva *et al.*, 2011).

Several growth factors [e.g. epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), and insulin] are known to be involved in mammalian oocyte growth and maturation within ovarian follicles (van den Hurk & Zhao, 2005). These factors have been used widely for IVM of bovine cumulus–oocyte complexes (COCs) to improve the results achieved by *in vitro* procedures (Sagirkaya *et al.*, 2007; Vireque *et al.*, 2009). The combination insulin–transferrin–selenium (ITS) has also been used to improve the conditions in defined IVM systems (Quirk *et al.*, 2000). The combination of IGF1, insulin, androstenedione, transferrin, and sodium selenite, for maturation of bovine oocytes in serum-free and albumin-free minimum essential medium (DCM) in the absence of follicle-simulating hormone (FSH) could achieve similar results to those of the non-defined medium supplemented with serum in terms of blastocysts production and expression of *HSP70-1* and *BAX* genes (Vireque *et al.*, 2009).

The role of FSH in the maturation of bovine COC has also been well described in the literature and it acts to enhance cAMP intracellular levels and activate proteins that modulate gene expression (Rodriguez & Farin, 2004). Insulin interacts with FSH in the steroidogenic pathway in granulosa cells (Adashi *et al.*, 1988; Minegishi *et al.*, 2000; Spicer *et al.*, 2002).

Insulin induces the cellular uptake of glucose and presents mitogenic and anti-apoptotic activity (Augustin *et al.*, 2003). Insulin receptor mRNA expression is increased in the pre-ovulatory dominant follicles in granulosa cells (Shimizu *et al.*, 2008). Insulin also seems to be involved in modulating the response of granulosa cells to gonadotrophins (Willis & Franks, 1995) and has positive effects on the developmental potential of bovine oocytes (Ocaña-Quero *et al.*, 1998) during the IVM process. However, excess insulin may have detrimental effects (Chaves *et al.*, 2012; Grazul-Bilska *et al.*, 2012; Rhee *et al.*, 2013).

Insulin can influence the expression of several genes (O'Brien *et al.*, 2001), but the direct effect of insulin on the levels of transcripts associated with metabolic profile, cellular competence and stress in oocytes is not yet clear. The response to different energy substrates and antioxidant profile of oocytes *in vitro* might provide an insight into their metabolic requirements. Glucose transport type 1 (*GLUT1*) mRNAs have been detected in bovine oocytes. It is well known that developmental competence of oocytes decreases with low or high glucose levels, diminishing cytoplasmic maturation and impairing nuclear maturation (Sutton-McDowall *et al.*, 2010), therefore glucose uptake is imperative to the metabolic state and viability of the cell. Peroxiredoxin 1 (PRDX1) demonstrated an antioxidative action, is present in the oocyte cytoplasm and the transcript levels decrease after oocyte maturation (Neumann *et al.*, 2003; Thélie *et al.*, 2007; Pereira *et al.*, 2010).

Heat shock proteins (HSP) promote cell protection against heat damage, preventing protein denaturation (Kregel, 2002), blocking apoptosis, and its transcription rate is increased by cellular stress (Kiang & Tsokos, 1998).

Growth differentiation factor 9 (GDF9) is an oocyte-secreted factor, a member of TGF-β family of growth factors, with critical importance in the regulation of normal cumulus cell function, and thus COCs competence (Fair *et al.*, 2007; Gilchrist & Thompson, 2007). In addition, GDF9 acts in follicular cells and along with other members of the TGF-β family is crucial for follicle development (Knight & Glister, 2006).

Based on oocyte meiotic arrest (Oliveira e Silva *et al.*, 2011) and embryo development (Vireque *et al.*, 2009) promoted by the use of DCM, the aim of the present study was to evaluate the dose–response effect of insulin in DCM, without IGF1 and in the presence of FSH, by analysing oocyte nuclear maturation, the relative levels of transcripts of genes associated with metabolism, competence and cellular stress, and subsequent embryo development *in vitro.*

Materials and methods

All reagents used in the study were from Sigma Chemical Co. unless otherwise stated.

Oocyte recovery and selection

Cattle ovaries were obtained at a local slaughterhouse and shipped to the laboratory in warm saline solution (supplemented with 0.1 g/1 of streptomycin). COCs were obtained by aspiration of 3–8 mm follicles using a 21-gauge needle in a 10-ml syringe manipulated with Dulbecco's phosphate-buffered saline (D-PBS; Gibco, Grand Island, NY, USA) supplemented with 0.4% bovine serum albumin (BSA). Only oocytes showing homogeneous cytoplasm and with more than three layers of granulosa cells were used.

Oocyte culture medium composition

The composition of the defined culture medium (DCM) is reported in a patent filled by the Fundação

https://doi.org/10.1017/S0967199414000239 Published online by Cambridge University Press

Universidade de Brasília and was used with modifications to evaluate the effect of the different doses of insulin. The DCM was composed of α-MEM medium (Invitrogen-Gibco/BRL) supplemented with polyvinyl alcohol (PVA), 10 ng/ml FSH, androstenedione, non-essential amino acids (Invitrogen-Gibco/BRL), transferrin, sodium selenium, sodium bicarbonate (Invitrogen-Gibco/BRL), HEPES, and antibiotics (penicillin and streptomycin).

Experimental design

Four maturation groups were established, one insulin free (DCM control) and three with COCs matured in DCM supplemented with bovine insulin (Sigma, USA): 1 ng/ml (DCM1); 10 ng/ml insulin (DCM10); and 100 ng/ml (DCM100).

In vitro maturation and fertilization

The *in vitro* maturation and fertilization procedures were adapted from a previous study (Camargo *et al.*, 2007). Briefly, COCs were matured *in vitro* in DCM for 22–24 h in a humidified atmosphere with 5% CO_2 in air and at 38.5°C. For *in vitro* fertilization *in vitro* matured COCs were separated into groups of 25–30, washed and transferred to 100-μl drops of fertilization medium under mineral oil. Motile spermatozoa were obtained after centrifugation in a Percoll discontinuous density gradient (45–90%) and added to the fertilization drop at a final concentration of 1×10^6 ml^{-1}. IVF was performed in Fert–TALP medium (Gordon, 1994), supplemented with penicillamine, hypotaurine, epinephrine, and heparin for 22 h under same conditions used during maturation.

In vitro embryo culture

After fertilization, oocytes were partially stripped by mechanical pipetting in CR2aa medium (previously equilibrated in 5% CO_2 in air and in a humidified atmosphere at 38.5°C) until one or two layers of cumulus cells remained. Groups of 15–20 presumptive zygotes with their respective cumulus cells were subsequently cultured in 50 μl of CR2aa medium supplemented with 10% fetal calf serum (FCS) and covered with mineral oil. Embryo culture was performed in 5% CO_2 in air in a humidified atmosphere at 38.5°C. The rates of cleavage and blastocyst generation were assessed at 72 h post-insemination (hpi), and 192 hpi (day 8), respectively.

Oocyte nuclear maturation evaluation

At the end of the IVM period, 236 COCs were denuded and fixed in 3:1 ethanol:acetic acid solution for 48 h and subsequently transferred to glass slides in small drops. Vaseline and paraffin were used to maintain the coverslip in contact with the oocytes. Slides were stained with 1% lacmoid for the oocyte nuclear maturation analysis under a phase contrast microscope at ×400 or ×1000 magnification (Sirard and Coenen, 1993). Oocytes were classified in stages as immature (germinal vesicle and germinal vesicle breakdown), intermediary (metaphase I to telophase I), and mature (metaphase II).

Total RNA extraction and reverse transcription

After IVM, three pools of 10 oocytes from each group had their cumulus cells removed by repeated pipetting in PBS medium supplemented with PVA and then were frozen in liquid nitrogen. These samples were stored for 2 weeks at –80°C before RNA extraction. Total RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions, and treated with DNase I (27 units for 15 min at room temperature for every sample). The RNA samples were reverse transcribed using the SuperScript III First-Strand Synthesis Supermix (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Reactions were performed using oligo$(dT)_{20}$ primers, dNTP mix, Superscript^{TM} III RT, RNaseOUT^{TM}, $MgCl_2$, RT buffer in a final volume of 20 μl. The samples were first incubated at 65°C for 5 min and then for 50°C for 50 min. The reaction was terminated at 85°C for 5 min and then chilled on ice. After that, RNase H was added to the samples and these were incubated at 37°C for 20 min. The RNA and cDNA quantification and purity for each sample was performed using 1 μl of sample in spectrophotometer ND-100 (NanoDrop, Wilmington, DE, USA).

Relative quantification by real-time polymerase chain reaction (PCR)

Relative quantification was performed in triplicate using real-time PCR (ABI Prism 7300 Sequence Detection Systems, Foster City, CA, USA). Reactions were prepared using a mixture of SYBR Green PCR Master Mix (Applied Biosystems), primers, nuclease-free water, and cDNA. The amounts of primers and cDNA used in the reactions were previously standardized to achieve greater primer efficiency. The following cDNA amounts per reaction were used: 600 ng for the peroxiredoxin 1 (*PRDX1*) and heat shock protein 70.1 (*HSP70-1*) genes, 400 ng for the glucose transporter type 1 (*GLUT1*), and 200 ng for the *ACTB* and differentiation factor 9 (*GDF9*) genes.

The cycling conditions were 95°C for 10 min, 45 cycles at 95°C for 15 s, the gene-specific primer annealing temperature for 30 s (Table 1), and 60°C for 30 s. After each PCR run, a melting curve analysis was performed to confirm that a single specific product was

https://doi.org/10.1017/S0967199414000239 Published online by Cambridge University Press

Table 1 Sequences of target bovine gene primers and melting temperatures

Gene	Primer sequence	Melting temperature (°C)	Length (bp)	GenBank reference or literature reference
GDF9	F: GACCCCTAAATCCAACAGAA R: AGCAGATCCACTGATGGAA	53	120	NM_174681
GLUT1	F: CCAAGGATCTCTCAGAGCACAG R: TTCTTCTGGACATCACTGCTGG	53	110	Sagirkaya *et al*. (2007)
PRDX1	F: ATGCCAGATGGTCAGTTCAAG R: CCTTGTTTCTTGGGTGTGTTG	53	224	Mourot *et al*. (2006)
HSP70-1	F: AACAAGATCACCATCACCAACG R: TCCTTCTCCGCCAAGGTGTTG	59	275	NM_174550
ACTB	F: GACATCCGCAAGGACCTCTA R: ACATCTGCTGGAAGGTGGAC	53	205	NM_173979
GAPDH	F: CCAACGTGTCTGTTGTGGATCTGA R: GAGCTTGACAAAGTGGTCGTTGAG	53	237	Mourot *et al*. (2006)

F, forward primer; R, reverse primer.

generated. No-template controls (NTC), comprised of the PCR reaction mix without nucleic acid, were also run with each primer to confirm the absence of contaminations. Primer efficiency was calculated using LinRegPCR software (Ramakers *et al.* 2003) for each reaction. The primer efficiency for target genes was 1.85 ± 0.03, 1.74 ± 0.03, 1.85 ± 0.03 and 1.85 ± 0.08 for *GDF9*, *GLUT1*, *PRDX1* and *HSP70-1* genes, respectively. Expression of *ACTB* and *GAPDH* genes were analysed in order to identify a better endogenous reference gene (endogenous control) for this experiment. *ACTB* showed lower coefficient of variation of threshold cycles (1.5%) and higher primer efficiency (1.95 ± 0.06) among replicates than *GAPDH* (1.7% and 1.93 ± 2.2) gene and it was then chosen as reference gene. Relative abundance (RA) analyses were performed using the Relative expression software tool (REST) (Pfaffl *et al.*, 2002) and based on primer efficiency.

Statistical analysis

The differences in oocyte nuclear maturation, cleavage and blastocyst rates after seven replicates were assessed by the chi-squared (×2) test. The relative gene expression analyses were performed using the REST (Pfaffl *et al.*, 2002) using a pairwise fixed reallocation randomization test. Differences with $P < 0.05$ were considered to be significant.

Results

Dose–response of insulin effect on nuclear maturation of COCs *in vitro*

The nuclear maturation rates of 236 oocytes are presented in Fig. 1. The presence of insulin in the defined medium did not change the nuclear maturation rates (immature, intermediary, or mature) in all groups analysed after 24 h of IVM ($P > 0.05$) (Fig. 1).

Dose–response effect of insulin in relative quantification of transcripts in oocytes

The relative transcript levels of *GDF9*, *GLUT1*, *PRDX1*, and *HSP70-1* in oocyte were assessed on COCs matured in DCM control, DCM1, DCM10 or DCM100. These relative amounts of mRNAs were compared with the calibrator, the insulin-free group (DCM control).

The insulin concentrations used in DCM did not change the relative levels of the *GLUT1* transcript, while DCM1, DCM10, and DCM100 decreased the levels of the *GDF9* and *HSP70-1* transcripts (Figs. 2 and 3). The relative levels of *PRDX1* transcripts only decreased in the DCM10, and DCM100 groups (Fig. 3).

Dose–response effect of insulin in IVM on embryo production rate *in vitro*

No significant differences for oocyte maturation were observed in embryo cleavage rates among DCM control, DCM1, DCM10, and DCM100 groups ($P > 0.05$) (Table 2). A significant higher blastocyst formation rate was observed in the DCM100 group in comparison with the DCM control group ($P < 0.05$) (Table 2). No difference was observed in the blastocyst generation rates among DCM control, DCM1, and DCM10 groups ($P > 0.05$) (Table 2).

Discussion

This experiment evaluated the developmental competence of bovine COCs matured in α-MEM medium

https://doi.org/10.1017/S0967199414000239 Published online by Cambridge University Press

Table 2 Effect of insulin in defined culture medium (DCM) of COCs on cleavage and blastocyst production

Group	No.	Cleavage % (*n*)	Blastocyst day 8 % (*n*)
DCM control	136	57.40 (78)[a]	24.30 (33)[b]
DCM1	141	64.50 (91)[a]	27.00 (38)[a,b]
DCM10	137	67.20 (92)[a]	26.30 (36)[a,b]
DCM100	133	63.20 (84)[a]	36.10 (48)[a]

[a,b]Different letters in the same column differ statistically by chi-squared test ($P < 0.05$).
No., total number of oocytes/group.
DCM control, DCM without insulin supplementation; DCM1, DCM control supplemented with 1 ng/ml of bovine insulin; DCM10, DCM control supplemented with 10 ng/ml of bovine insulin; DCM100, DCM control supplemented with 100 ng/ml of bovine insulin.

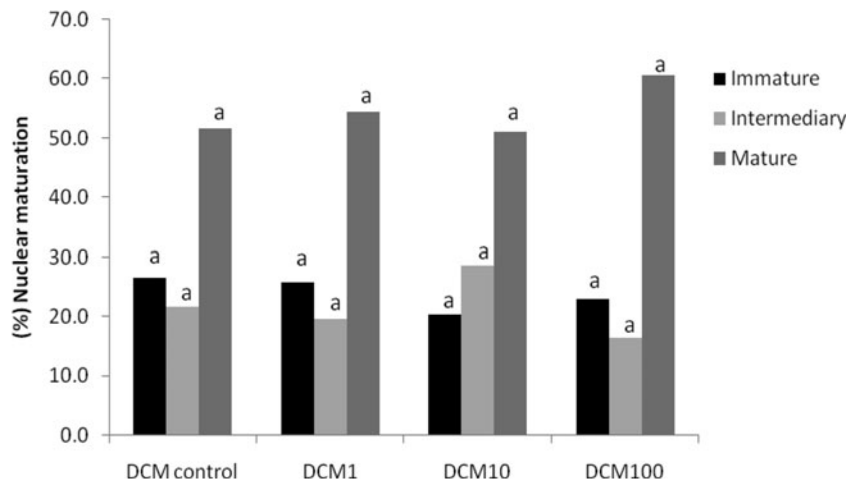

Figure 1 Nuclear maturation status of bovine COCs ($n = 236$) matured for 24 h in DCM supplemented with different doses of insulin. Results are expressed in percentage (%). [a]Indicates that there is no difference within each nuclear maturation stage among the four groups of insulin ($P < 0.05$), determined by chi-squared test. DCM control, DCM without insulin supplementation. DCM1, DCM control supplemented with 1 ng/ml of bovine insulin. DCM10, DCM control supplemented with 10 ng/ml of bovine insulin. DCM100, DCM control supplemented with 100 ng/ml of bovine insulin.

supplemented with different concentrations of insulin and with a fixed concentration of FSH. Our data show that use of α-MEM serum-free medium produced suitable bovine COC maturation and embryos. Insulin supplementation increased the *in vitro* production of embryos and was associated with changes in the levels of *GDF9*, *PRDX1*, and *HSP70-1* transcripts.

In the present study, three insulin concentrations and a fixed concentrations of FSH, plus transferrin and sodium selenium were also used, to detect any direct effect of insulin. Several studies have suggested that insulin alone (Lee *et al.*, 2005), or in combination with transferrin and selenium in IVM systems, improves the developmental competence of COCs in prepubertal bovine (Córdova *et al.*, 2011), porcine (Jeong *et al.*, 2008), and buffalo (Raghu *et al.*, 2002). Similarly, our results demonstrated that the nuclear maturation stage was not affected by the presence of different doses of insulin, however nuclear maturation was significantly lower compared with commercial medium supplemented with higher doses of FSH and serum (data not shown). Cytoplasmatic maturation progression and low rates of nuclear maturation have been described previously as a consequence of DCM culture conditions (Oliveira e Silva *et al.*, 2011) and due to the presence of low FSH concentrations in culture (de Souza *et al*, 2013).

Insulin promotes the proliferation and steroidogenic activity induced by FSH in granulosa cells (Spicer *et al.*, 2002). Insulin and IGF1 increase the activity of granulosa cells in culture in part by increasing the number of FSH receptors (rFSH) and levels of *rFSH* mRNA (Adashi *et al.*, 1988; Minegishi *et al.*, 2000). The role of FSH in the maturation of bovine COCs is well known. FSH acts on cumulus cells by increasing the levels of cAMP that is transmitted to oocytes via gap

https://doi.org/10.1017/S0967199414000239 Published online by Cambridge University Press

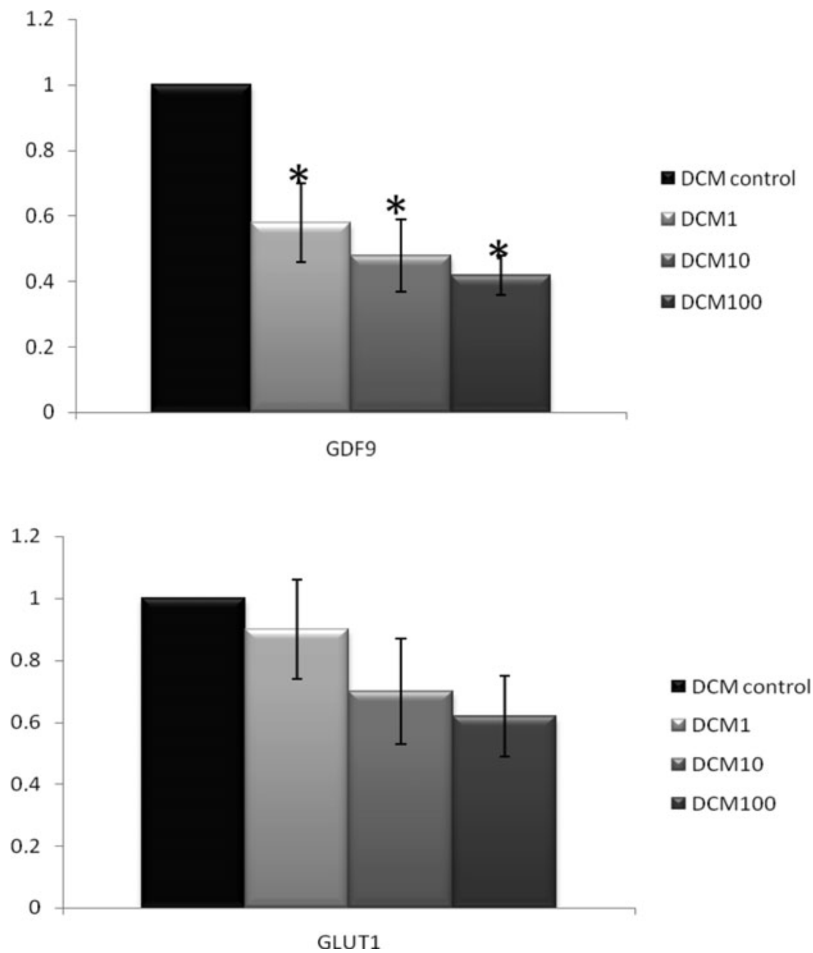

Figure 2 Relative abundance of transcripts related to maturation (*GDF9*) and glucose transporter 1 (*GLUT1*) in bovine oocytes matured in DCM supplemented with different doses of insulin. *Indicates significant difference ($P < 0.05$) in comparison with DCM 0 ng/ml insulin versus DCM with 1, 10 or 100 ng/ml. The results are shown as mean and standard error using the medium as DCM–calibrator (score 1). DCM control, DCM without insulin supplementation; DCM1, DCM control supplemented with 1 ng/ml of bovine insulin; DCM10, DCM control supplemented with 10 ng/ml of bovine insulin; DCM100, DCM control supplemented with 100 ng/ml of bovine insulin.

junctions. This temporary increase in the cAMP levels in the oocyte activates protein kinase II (PKAII), which in turn stimulates the transcription of genes necessary for maturation (Rodriguez & Farin, 2004). Therefore, it is possible that the cumulus cells also exhibit higher levels of rFSH after insulin stimulation and influence oocyte maturation, however more experiments need to be done to elucidate these physiological interactions *in vitro.*

Transcripts related to metabolic profile, cellular competence and stress were analysed in order to investigate oocyte competence after culture in different doses of insulin, bearing in mind that the maturation process per se can lead to RNA degradation compared with the immature oocyte (Thélie *et al.*, 2007).

Insulin reduced the transcript levels of *GDF9*, which is an oocyte-specific member of the TGF-β family that is involved in proliferation, differentiation, and regulation of cumulus cells (Eppig, 2001; Gilchrist & Thompson, 2007). This oocyte factor seems to be a cumulus expansion-inductor factor that enables granulosa cells to respond to FSH by producing hyaluronic acid (Eppig, 2001). It has been shown previously that *in vivo* matured oocytes have lower abundance of *GDF9* transcripts than their *in vitro* matured counterparts (Lonergan *et al.*, 2003), despite their higher developmental competence (Humblot *et al.*, 2005). As the transcription activity in oocytes during meiotic maturation is low (Bettegowda & Smith, 2007) and transcripts may be degraded during maturation (Thélie *et al.*, 2007), the decrease of *GDF9* transcripts in oocytes with high competence may be due to their translation, favoring further development, coupled to RNA degradation.

Insulin in the defined medium did not alter the abundance of *GLUT1* transcripts. Analysis of *GLUT1*

https://doi.org/10.1017/S0967199414000239 Published online by Cambridge University Press

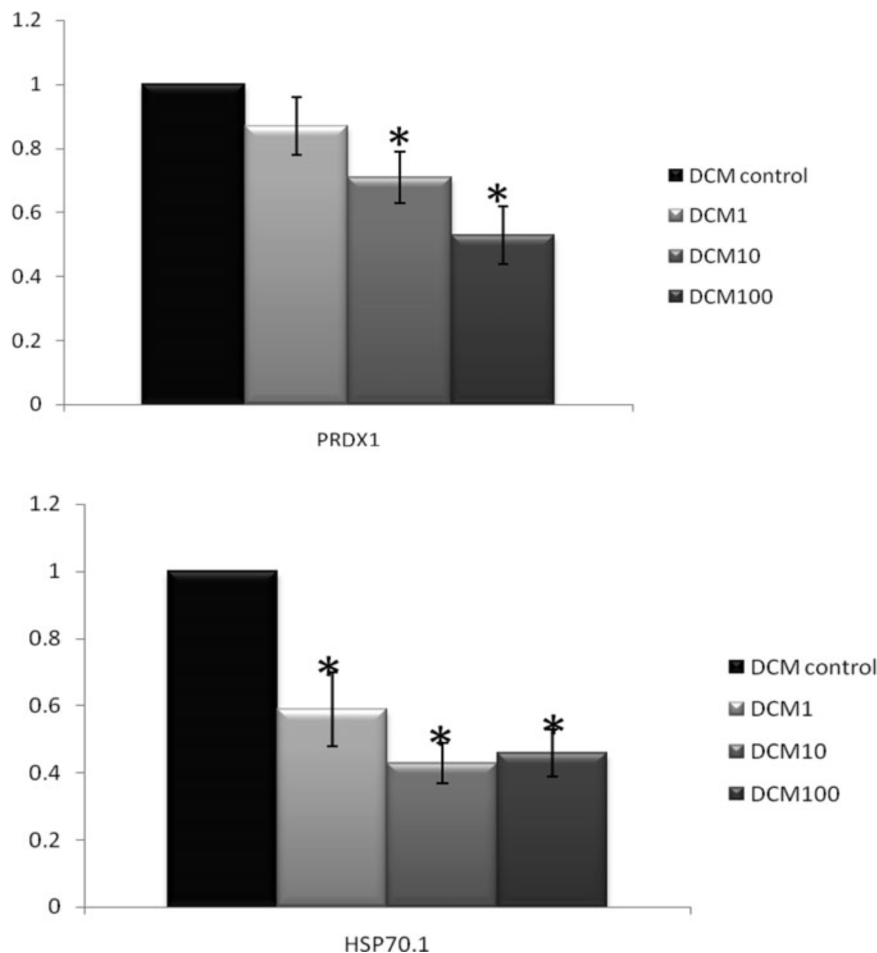

Figure 3 Relative abundance of transcripts related to cell stress (*PRDX1* and *HSP70-1*) in bovine oocytes matured in DCM supplemented with different doses of insulin. *Indicates difference ($P < 0.05$) in comparison with DCM 0 ng/ml insulin versus DCM with 1, 10 or 100 ng/ml. The results are shown as mean and standard error using the medium as DCM–calibrator (score 1). DCM control, DCM without insulin supplementation; DCM 1, DCM control supplemented with bovine insulin at 1 ng/ml; DCM 10, DCM control supplemented with bovine insulin at 10 ng/ml; DCM 100, DCM control supplemented with bovine insulin at 100 ng/ml.

transcripts in oocytes and embryos has been used to better understand the metabolic process in these structures (Lequarre *et al.*, 1997, Lopes *et al.*, 2007). The levels of *GLUT1* mRNA in bovine oocytes reduces by half during maturation, perhaps because of the natural degradation of mRNAs during this period (Lequarre *et al.*, 1997). The RA of *GLUT1* in mature oocytes does not change with the source of protein used, being oestrous cow serum or PVA (Wrenzycki *et al.*, 1999). In addition, studies have indicated that oocytes make use of substrates of glucose metabolism from cumulus cells (Sutton *et al.*, 2003), and glucose metabolism is low in the oocyte during maturation (Rieger & Loskutoff 1994; Sutton-McDowall *et al.*, 2010). These findings indicate that *GLUT1* transcript levels in oocytes are largely reduced during maturation and slightly influenced by the maturation medium, and suggest that the positive effect of insulin during oocytes maturation on further embryo development is not associated with an increase in glucose uptake via GLUT1 transporters.

Insulin may play a role in oxidative stress through regulation of antioxidant enzymes (Wang *et al.*, 2012), but no information is available about the effect of insulin on expression of peroxiredoxins (*PRDX*) and *HSP* genes in oocytes. The *PRDX1* and *HSP70-1* genes have demonstrated antioxidant proprieties in several cells and are present in bovine oocytes (Christians *et al.*, 2003, Leyens *et al.*, 2004, Pereira *et al.*, 2010). In this study, the addition of 10 or 100 ng/ml of insulin decreased the abundance of *PRDX1* and *HSP70-1* transcripts. In addition, just 1 ng/ml of insulin induced the downregulation of the *HSP70-1* gene. Pereira and collaborators (Pereira *et al.*, 2010) have observed that, at the end of IVM, oocytes showed relatively higher levels of *PRDX1* and *HSP70-1* transcripts in medium

https://doi.org/10.1017/S0967199414000239 Published online by Cambridge University Press

in which the source of the macromolecule was PVA instead of serum. Our results showed that the addition of 100 ng/ml of insulin in the serum-free defined maturation medium reduced the amount of transcripts associated with protection against oxidative stress and increased the production of embryos. These findings may suggest that insulin may stimulate the translation of transcripts associated with cellular stress during oocyte maturation, increasing protection against stress and, consequently, generating oocytes with low amounts of antioxidant transcripts but with high competence.

The present results provide evidence that the addition of 100 ng/ml of insulin in the DCM increased the bovine blastocyst rate compared with the other groups and independent of FSH action. In addition, the cleavage and blastocyst generation rates were similar to those reported by Vireque and collaborators (Vireque *et al.*, 2009). In present study, insulin supplemented at 100 ng/ml increased the bovine blastocyst rate when compared with the insulin-free medium, whereas no significant differences was found for lower insulin concentrations. A previous study on antral follicles cultured in the presence of insulin has reported cytoplasmic changes associated with oocyte maturation. Many of these changes occurred before nuclear maturation (Fouladi-Nashta & Campbell, 2006). Therefore, we speculate that insulin at appropriated concentrations might contribute to cytoplasmic maturation, making oocytes that reached metaphase II more competent for the production of embryos, as also observed by Oliveira e Silva and collaborators (Oliveira e Silva *et al.*, 2011).

The present study indicates the possibility of using insulin in a chemically defined medium, as a factor that is able to reduce the cellular stress response promoted by *in vitro* conditions. It highlights DCM as an option for serum-free oocyte maturation. However, the results of transcript expression related to oocyte development, metabolism and cellular stress, need to be confirmed at the protein level and additional studies are being conducted by our research group in this regard. In conclusion, our findings indicate that bovine COCs matured in serum-free DCM presented better blastocyst rates when 100 ng/ml insulin is added to the IVM. Furthermore, the use of insulin during maturation influences the levels of *GDF9*, *HSP 70.1*, and *PRDX1* transcripts in bovine oocytes.

Acknowledgements

This study was supported financially by FAP-DF/CNPq (PRONEX 193.000.577/2009). The authors thank Embrapa Dairy Cattle, Fapemig and FAP-DF/CNPq for financial support for molecular analysis. We also thank CAPES for providing a scholarship for the first author.

References

Adashi, E.Y., Resnick, C.E., Hernandez, E.R., Svoboda, M.E. & Van Wyk, J.J. (1988). *In vivo* regulation of granulosa cell somatomedin-C/insulin-like growth factor I receptors. *Endocrinology* **122**, 1383–9.

Augustin, R., Pocar, P., Wrenzycki, C., Niemann, H. & Fischer, B. (2003). Mitogenic and anti-apoptotic activity of insulin on bovine embryos produced *in vitro*. *Reproduction* **126**, 91–99.

Bettegowda, A. & Smith, G.W. (2007). Mechanisms of maternal mRNA regulation: implications for mammalian early embryonic development. *Front. Biosci.* **12**, 3713–26.

Camargo, L., Viana, J., Ramos, A., Serapião, R., de Sa, W., Ferreira, A., Guimarães, M. & do Vale Filho, V. (2007). Developmental competence and expression of the Hsp 70.1 gene in oocytes obtained from *Bos indicus* and *Bos taurus* dairy cows in a tropical environment. *Theriogenology* **68**, 626–32.

Chaves, R.N., Duarte, A.B., Rodrigues, G.Q., Celestino, J.J., Silva, G.M., Lopes, C.A., Almeida, A.P., Donato, M.A., Peixoto, C.A., Moura, A.A., Lobo, C.H., Locatelli, Y., Mermillod, P., Campello, C.C. & Figueiredo, J.R. (2012). The effects of insulin and follicle-simulating hormone (FSH) during *in vitro* development of ovarian goat preantral follicles and the relative mRNA expression for insulin and FSH receptors and cytochrome P450 aromatase in cultured follicles. *Biol. Reprod.* **87**, 69–79.

Christians, E.S., Zhou, Q., Renard, J. & Benjamin, I.J. (2003). Heat shock proteins in mammalian development. *Semin. Cell. Dev. Biol.* **14**, 283–90.

Córdova, B., Morató, R., de Frutos, C., Bermejo-Álvarez, P., Paramio, T., Gutiérrez-Adán, A. & Mogas, T. (2011). Effect of leptin during *in vitro* maturation of prepubertal calf oocytes: embryonic development and relative mRNA abundances of genes involved in apoptosis and oocyte competence. *Theriogenology* **76**, 1706–15.

de Souza, D.K., Tuany, F., Nobre, C.S., Sousa, K.L.G. & Rosa a Silva, A.A.M. (2013). Blockage of phosphatidylinositol 3-kinase (PI3K) pathway reverts the inhibition of oocyte nuclear maturation promoted by a defined medium (MIV B). SSR 46th Annual Meeting: Reproductive Health: Nano to Global 22–26 July 2013, Abstract 424. *Biol. Reprod.* **89**(Suppl.), 201–2.

Eppig, J.J. (2001). Oocyte control of ovarian follicular development and function in mammals. *Reproduction* **122**, 829–38.

Fair, T., Carter, F., Park, S., Evans, A. & Lonergan, P. (2007). Global gene expression analysis during bovine oocyte *in vitro* maturation. *Theriogenology* **68**(Suppl. 1), S91–7.

Ferreira, E.M., Vireque, A.A., Adona, P.R., Meirelles, F.V., Ferriani, R.A. & Navarro, P.A. (2009). Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. *Theriogenology* **71**, 836–48.

Fouladi-Nashta, A.A. & Campbell, K.H. (2006). Dissociation of oocyte nuclear and cytoplasmic maturation by the

https://doi.org/10.1017/S0967199414000239 Published online by Cambridge University Press

addition of insulin in cultured bovine antral follicles. *Reproduction* **131**, 449–60.
Gilchrist, R. & Thompson, J. (2007). Oocyte maturation: emerging concepts and technologies to improve developmental potential *in vitro*. *Theriogenology* **67**, 6–15.
Gordon, I. (1994). *Laboratory Production of Cattle Embryo.* London: CAB International, Cambridge University Press.
Grazul-Bilska, A.T., Borowczyk, E., Bilski, J.J., Reynolds, L.P., Redmer, D.A., Caton, J.S. & Vonnahme, K.A. (2012). Overfeeding and underfeeding have detrimental effects on oocyte quality measured by *in vitro* fertilization and early embryonic development in sheep. *Domest. Anim. Endocrinol.* **43**, 289–98.
Humblot, P., Holm, P., Lonergan, P., Wrenzycki, C., Lequarré, A.S., Joly, C.G., Herrmann, D., Lopes, A., Rizos, D., Niemann, H. & Callesen, H. (2005). Effect of stage of follicular growth during superovulation on developmental competence of bovine oocytes. *Theriogenology* **63**, 1149–66.
Jeong, Y.W., Hossein, M.S., Bhandari, D.P., Kim, Y.W., Kim, J.H., Park, S.W., Lee, E., Park, S.M., Jeong, Y.I., Lee, J.Y., Kim, S. & Hwang, W.S. (2008). Effects of insulin–transferrin–selenium in defined and porcine follicular fluid supplemented IVM media on porcine IVF and SCNT embryo production. *Anim. Reprod. Sci.* **106**, 13–24.
Kiang, J.G. & Tsokos, G.C. (1998). Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol. Ther.* **80**, 183–201.
Knight, P.G. & Glister, C. (2006). TGF-β superfamily members and ovarian follicle development. *Reproduction* **132**, 191–206.
Kregel, KC. (2002). Heat shock proteins: modifying factors in physiological stress responses and acquired thermotolerance. *J. Appl. Physiol.* **92**, 2177–86.
Lee, M.S., Kang, S.K., Lee, B.C. & Hwang, W.S. (2005). The beneficial effects of insulin and metformin on *in vitro* developmental potential of porcine oocytes and embryos. *Biol. Reprod.* **73**, 1264–8.
Lequarre, A.S., Grisart, B., Moreau, B., Schuurbies, N., Massip, A. & Dessy, F. (1997). Glucose metabolism during bovine preimplantation development: analysis of gene expression in single oocytes and embryos. *Mol. Reprod. Dev.* **48**, 216–26.
Leyens, G., Knoops, B. & Donnay, I. (2004). Expression of peroxiredoxins in bovine oocytes and embryos produced *in vitro*. *Mol. Reprod. Dev.* **69**, 243–51.
Lonergan, P., Monaghan, P., Rizos, D., Boland, M. & Gordon, I. (1994). Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture *in vitro*. *Mol. Reprod. Dev.* **37**, 48–53.
Lonergan, P., Gutiérrez-Adán, A., Rizos, D., Pintado, B., de la Fuente, J. & Boland, M.P. (2003). Relative messenger RNA abundance in bovine oocytes collected *in vitro* or *in vivo* before and 20 hr after the preovulatory luteinizing hormone surge. *Mol. Reprod. Dev.* **66**, 297–305.
Lopes, A.S., Wrenzycki, C., Ramsing, N.B., Herrmann, D., Niemann, H., Lovendahl, P., Greve, T. & Callesen, H. (2007). Respiration rates correlate with mRNA expression of *G6PD* and *GLUT1* genes in individual bovine *in vitro*-produced blastocysts. *Theriogenology* **68**, 223–36.
Minegishi, T., Hirakawa, T., Kishi, H., Abe, K., Abe, Y., Mizutani, T. & Miyamoto, K. (2000). A role of insulin-like growth factor I for follicle-stimulating hormone receptor expression in rat granulosa cells. *Biol. Reprod.* **62**, 325–33.
Mourot, M., Dufort, I., Gravel, C., Algriany, O., Dieleman, S. & Sirard, M.A. (2006). The influence of follicle size, FSH-enriched maturation medium, and early cleavage on bovine oocyte maternal mRNA levels. *Mol. Reprod. Dev.* **73**, 1367–79.
Neumann, C.A., Krause, D.S., Carman, C.V., Das, S., Dubey, D.P., Abraham, J.L., Bronson, R.T., Fujiwara, Y., Orkin, S.H. & Van Etten, R.A. (2003). Essential role for the peroxiredoxin Prdx1 in erythrocyte antioxidant defence and tumour suppression. *Nature* **424**, 561–5.
O'Brien, R.M., Streeper, R.S., Ayala, J.E., Stadelmaier, B.T. & Hornbuckle, L.A. (2001). Insulin-regulated gene expression. *Biochem. Soc. Trans.* **29**(Pt 4), 552–8.
Ocaña-Quero, J.M., Pinedo-Merlin, M., Ortega- Mariscal, M. & Moreno-Millan, M. (1998). Influence of human and bovine insulin on *in vitro* maturation, fertilization and cleavage rates of bovine oocytes. *Arch. Zootec.* **47**, 85–93.
Oliveira e Silva, I., Vasconcelos, R.B., Caetano, J.V.O., Gulart, L.V.M., Camargo, L.S.A., Báo, S.N. & Rosa e Silva, A.A. (2011). Induction of reversible meiosis arrest of bovine oocytes using a two-step procedure under defined and nondefined conditions. *Theriogenology* **75**, 1115–24.
Otoi, T., Yamamoto, K., Koyama, N., Tachikawa, S. & Suzuki, T. (1997). Bovine oocyte diameter in relation to developmental competence. *Theriogenology* **48**, 769–74.
Pereira, M.M., Machado, M.A., Costa, F.Q., Serapião, R.V., Viana, J.H.M. & Camargo, L.S.A. (2010). Effect of oxygen tension and serum during IVM on developmental competence of bovine oocytes. *Reprod. Fertil. Dev.* **22**, 1074–82.
Pfaffl, M.W., Horgan, G.W. & Dempfle, L. (2002). Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Res.* **30**, e36.
Quirk, S.M., Harman, R.M. & Cowan, R.G. (2000). Regulation of Fas antigen (Fas, CD95)-mediated apoptosis of bovine granulosa cells by serum and growth factors. *Biol. Reprod.* **63**, 1278–84.
Raghu, H., Nandi, S. & Reddy, S. (2002). Effect of insulin, transferrin and selenium and epidermal growth factor on development of buffalo oocytes to the blastocyst stage *in vitro* in serum-free, semidefined media. *Vet. Rec.* **151**, 260–5.
Ramakers, C., Ruijter, J.M., Deprez, R.H. & Moorman, A.F. (2003). Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci. Lett.* **339**, 62–6.
Rhee, Y.H., Choi, M., Lee, H.S., Park, C.H., Kim, S.M., Yi, S.H., Oh, S.M., Cha, H.J., Chang, M.Y. & Lee, S.H. (2013). Insulin concentration is critical in culturing human neural stem cells and neurons. *Cell Death Dis.* **4**:e766.
Rieger, D. & Loskutoff, N.M. (1994). Changes in the metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes *in vitro*. *J. Reprod. Fertil.* **100**, 257–62.

https://doi.org/10.1017/S0967199414000239 Published online by Cambridge University Press

Rodriguez, K.F. & Farin, C.E. (2004). Gene transcription and regulation of oocyte maturation. *Reprod. Fertil. Dev.* **16**, 55–67.

Sagirkaya, H., Misirlioglu, M., Kaya, A., First, N., Parrish, J. & Memili, E. (2007). Developmental potential of bovine oocytes cultured in different maturation and culture conditions. *Anim. Reprod. Sci.* **101**, 225–40.

Shimizu, T., Murayama, C., Sudo, N., Kawashima, C., Tetsuka, M. & Miyamoto, A. (2008). Involvement of insulin and growth hormone (GH) during follicular development in the bovine ovary. *Anim. Reprod. Sci.* **106**, 143–52.

Sirard, M.A. & Coenen, K. (1993). The co-culture of cumulus-enclosed bovine oocytes and hemi-sections of follicles: Effects on meiotic resumption. *Theriogenology* **40**, 933–42.

Spicer, L.J., Chamberlain, C.S. & Maciel, S.M. (2002). Influence of gonadotropins on insulin- and insulin-like growth factor-I (IGF-I)-induced steroid production by bovine granulosa cells. *Domest. Anim. Endocrinol.* **22**, 237–54.

Sutton, M.L., Gilchrist, R.B. & Thompson, J.G. (2003). Effects of *in vivo* and *in vitro* environments on the metabolism of the cumulus–oocyte complex and its influence on oocyte developmental capacity. *Hum. Reprod. Update* **19**, 35–48.

Sutton-McDowall, M.L., Gilchrist, R.B. & Thompson, J.G. (2010). The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* **139**, 685–95.

Thélie, A., Papillier, P., Pennetier, S., Perreau, C., Traverso, J.M., Uzbekova, S., Mermillod, P., Joly, C., Humblot, P. & Dalbiès-Tran, R. (2007). Differential regulation of abundance and deadenylation of maternal transcripts during bovine oocyte maturation *in vitro* and *in vivo*. *BMC Dev. Biol.* **7**, 7:125.

Van den Hurk, R. & Zhao, J. (2005). Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. *Theriogenology* **63**, 1717–51.

Vireque, A., Camargo, L., Serapião, R., Rosa E Silva, A., Watanabe, Y., Ferreira, E., Navarro, P., Martins, W. & Ferriani, R. (2009). Preimplantation development and expression of *Hsp-70* and *Bax* genes in bovine blastocysts derived from oocytes matured in alpha-MEM supplemented with growth factors and synthetic macromolecules. *Theriogenology* **71**, 620–7.

Vasconcelos, R.B., Salles, L.P., Oliveira e Silva, I., Gulart, L.V., Souza, D.K., Torres, F.A., Bocca, A.L. & Rosa e Silva, A.A. (2013). Culture of bovine ovarian follicle wall sections maintained the highly estrogenic profile under basal and chemically defined conditions. *Braz. J. Med. Biol. Res.* **46**, 700–7.

Wang, X., Tao, L. & Hai, C.X. (2012). Redox-regulating role of insulin: the essence of insulin effect. *Mol. Cell. Endocrinol.* **349**, 111–27.

Warzych, E., Peippo, J., Szydlowski, M. & Lechniak, D. (2007). Supplements to *in vitro* maturation media affect the production of bovine blastocysts and their apoptotic index but not the proportions of matured and apoptotic oocytes. *Anim. Reprod. Sci.* **97**, 334–43.

Willis, D. & Franks, S. (1995). Insulin action in human granulosa cells from normal and polycystic ovaries is mediated by the insulin receptor and not the type-I insulin-like growth factor receptor. *J. Clin. Endocrinol. Metab.* **80**, 3788–90.

Wrenzycki, C., Herrmann, D., Carnwath, J.W. & Niemann, H. (1999). Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. *Mol. Reprod. Dev.* **53**, 8–18.

https://doi.org/10.1017/S0967199414000239 Published online by Cambridge University Press