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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

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Abstract

In vitro culture conditions affect both the maternal and embryonic expression of genes and is likely to alter both oocyte and embryo developmental competence. The search for better and less variable culture conditions simulating those in vivo has led to the development of defined culture media, with lower impact on the molecular reprogramming of oocytes and embryos. We evaluated embryo development and relative abundance (RA) of Hsp-70 and Bax transcripts in bovine blastocysts produced from oocytes matured in a chemically defined IVM system with synthetic polymers. Immature cumulus oocyte complexes (COCs) were matured for 22–24 h in alpha-MEM supplemented with IGF-1, insulin, 0.1% polyvinyl alcohol (PVA), or 0.1% polyvinylpyrrolidone (PVP), but without FSH or LH. The control group consisted of COCs matured in TCM plus FSH and 10% estrous cow serum. After fertilization, presumptive zygotes were co-cultured with cumulus cells until 224 h post-insemination. Total RNA was isolated from embryo pools, reverse transcribed into cDNA, and subjected to transcript analysis by real-time PCR. Cleavage rate was higher ($P < 0.05$) for the control group (68.3%) than for the PVA (54.4%) and PVP-40 (58.3%) groups. Nevertheless, there was no difference among the PVA, PVP-40 and control groups in blastocyst or hatching rates. Similarly, no difference in relative abundance of Hsp-70 and Bax transcripts was detected in comparison to the control group. We inferred that bovine oocytes can be matured in serum- and gonadotrophin-free medium supplemented with PVA or PVP, enriched with IGF-I and insulin, without altering post-cleavage development and relative abundance of some genes associated with stress and apoptosis.

Keywords: Bovine oocytes; In vitro maturation; Chemically defined medium; Embryogenesis; Hsp-70 and Bax genes

1. Introduction

The developmental competence of in vitro matured (IVM) oocytes is determined by factors such as hormonal stimulation protocols, the follicle of origin, culture conditions. Furthermore, developmental competence is

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associated with transcript accumulation during oocyte growth, which is important for initial development and for development up to the activation of the embryonic genome. Several supplements must be added to the IVM medium. Serum and bovine serum albumin (BSA) are commonly used as protein supplements to culture media for mammalian oocytes and embryos. However, these compounds may contain various molecules such as steroids, cholesterol, and peptides, to which oocytes and embryos are not exposed in vivo [1], in addition to bacterial and viral agents [2–4]. Additionally, supplementation with serum can induce changes in transcription of genes related to development, regardless of culture stage (IVM, IVF, or IVC) [5,6]. Sagirkaya et al. [2] demonstrated that the levels of certain transcripts were altered under various conditions during IVM of bovine oocytes in medium supplemented with fetal calf serum (FCS) or synthetic serum substitute (SSS). Similarly, changes in the level of maternal transcripts (in bovine oocytes and embryos) associated with supplementation of IVM medium were reported by other investigators [6,7].

Polyvinyl alcohol (PVA) and polyvinylpyrrolidone (PVP) are synthetic polymers, with a molecular weight of 30,000–70,000, used in cell culture media for the stabilization of osmotic pressure and as surfactants and heavy metal chelating agents [8]. There is evidence that PVA and PVP-40 can replace proteins [9] in IVM of bovine oocytes without impairing subsequent development [7,10–14]. Previous studies have demonstrated that PVP is also beneficial for IVM and is associated with better embryo quality [15,16]. Hormonal supplementation has been routinely used in IVM, although its use is still controversial, with wide variations among protocols [17–20]. Some studies using defined culture media have clearly demonstrated, without artifacts related to serum, the function of gonadotrophins and growth factors in oocyte maturation [9,11,21]. However, there is no consensus regarding the most appropriate supplementation. Several studies have reported that IGF-I and insulin enhanced the IVM of oocytes of various mammals [22–27] and improved embryo quality. The establishment of culture systems that can mimic the conditions of in vivo oocyte maturation can prevent or minimize the interference of culture conditions with oocyte and embryo development. Thus, the objective of the present study was to investigate the influence of chemically defined IVM systems using alpha-MEM supplemented with IGF-I, insulin and various synthetic macromolecules (PVA and PVP-40), on preimplantation development, and on the relative transcript level of Hsp-70 and Bax genes in bovine embryos.

2. Materials and methods

2.1. Reagents and culture media for in vitro embryo production

All chemicals used during the study were from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

2.1.1. Culture media for in vitro maturation

Two basic culture media were used. The first medium was minimum essential medium alpha (alpha-MEM; Invitrogen-Gibco/BRL, Grand Island, NY, USA), supplemented with 0.1% PVA or PVP-40, 100 ng/mL bovine insulin, 10 ng/mL human recombinant IGF-1 (Invitrogen Life Technologies, Grand Island, NY, USA), 10^{-7} M androstenedione, 11 mM nonessential amino acids (Invitrogen-Gibco/BRL); 5 μg/mL human transferrin, 1.4 ng/mL sodium selenium (Acros Organics, Fair Lawn, NJ, USA), 10 mM sodium bicarbonate (Invitrogen-Gibco/BRL), 0.02 M HEPES, and antibiotics (10,000 IU penicillin and 10,000 IU streptomycin. The second medium (control medium) was TCM-199 (Invitrogen-Gibco/BRL), supplemented with 10% estrous cow serum (ECS) inactivated at 56 °C for 30 min and 20 μg/mL FSH (Folltropin-Vetrepharm Canada, Belleville, ON, Canada).

2.2. Collection and in vitro maturation of bovine oocytes

Bovine ovaries were collected at an abbatoir and transported to the laboratory in 0.9% physiological saline supplemented with 0.05 g/L streptomycin at 33–37 °C. The ovaries were washed in 0.9% physiological saline at 33–37 °C, and follicles 3–8 mm in diameter were aspirated with 21 gauge needles adapted to 20 mL syringes. Cumulus–oocyte complexes recovered from the follicular fluid were washed in TALP-HEPES medium [28], containing NaCl, KCl, $NaHCO_3$, NaH_2PO_4, Na-lactate, $CaCl_2{\cdot}2H_2O$, $MgCl_2{\cdot}6H_2O$, HEPES, Pen-Strep, Na-pyruvate, and PVA or BSA (pH 7.4), and were evaluated morphologically under a stereomicroscope. Only oocytes with homogeneous cytoplasm and with more than three layers of granulosa cells were used.

2.3. Experimental design

The pool of immature COCs selected for IVM was divided into three experimental groups: (1) alpha-MEM supplemented with IGF-1, insulin and 0.1% PVA; (2)

alpha-MEM supplemented with IGF-1, insulin and 0.1% PVP; or (3) TCM-199 containing FSH and ECS (control). The three oocyte groups were cultured for 22–24 h in a humid atmosphere containing 5% CO_2 at 38.5 °C on four-well NUNC plates containing 400 μL medium and 40 oocytes per well. Twelve replicates per treatment were performed.

2.4. In vitro embryo production

Mature oocytes from the three treatments were inseminated with frozen–thawed, motile sperm separated by swim-up [29] using Sperm TALP HEPES medium [28] supplemented with 6 mg/mL BSA fraction V. Fertilization was performed in 100 μL drops of Fert TALP medium [28], supplemented with 20 μg/mL heparin and 6 mg/mL fatty acid-free BSA fraction V under mineral oil. Sperm concentration during fertilization was approximately 2×10^6 spermatozoa/mL and the sperm were co-incubated with 20 COCs for 18 h in a humid 5% CO_2 atmosphere at 38.5 °C. After fertilization, oocytes were partially denuded by pipetting in TALP HEPES medium [28] and 20 presumptive zygotes with up to two or three layers of cumulus cells were cultured in 50 μL drops of CR2aa medium [30], supplemented with 10% FCS (Invitrogen Life Technologies) and 1 mg/mL BSA under mineral oil in a humid 5% CO_2 atmosphere at 38.5 °C. The culture medium was replaced by fresh medium every 48 h. Cleavage rate was evaluated 72 h post-insemination (hpi), blastocyst rate was evaluated 168–192 hpi, and hatching rates at 224 hpi. Cleavage and blastocyst rates were calculated on the basis of number of presumptive zygotes, and hatching rates were calculated from the number of blastocysts.

2.5. RNA extraction, reverse transcription and amplification by real-time PCR

Day 8 blastocysts (168 hpi) from each group were removed from the culture drop, washed three times in TALP HEPES medium supplemented with BSA, and divided into groups of 10 embryos each. The blastocysts were quickly frozen in liquid nitrogen, stored frozen at −80 °C and thawed for RNA extraction. Total RNA was extracted from pools of 10 embryos (three replicates) using the RNeasy Micro Kit (Quiagen, Valencia, CA, USA) and the first strand was synthesized using the Superscript™ III First Strand Synthesis kit (Invitrogen, Chicago, IL, USA). Relative quantitation was performed in duplicate using real-time PCR (ABI PRISM® 7000; Applied Biosystems, Foster City, CA, USA). The reactions consisted of N iTaq™ SYBR® Green Supermix mixture with ROX (Bio-Rad, Waltham, MA, USA) containing cDNA equivalent to 0.8 blastocysts and specific gene primers (Table 1). The primers were designed using the Primer3 software [31]. The cDNA template was denatured at 95 °C for 3 min, followed by 40 cycles at 95 °C for 15 s, annealing at a specific temperature for each primer for 15 s, and extension at 72 °C for 45 s. After PCR, the melting curve was constructed for each sample to determine that a single specific product had been generated and the size of the amplicon was confirmed on 2% agarose gel stained with 2% ethidium bromide. Negative controls consisted of the PCR mix without nucleic acid. The transcript relative abundance of the H2a gene was used as an endogenous reference. The Ct values of reference gene (H2a) among treatments had a coefficient of variance of 2.06%, with an average of 30.27 ± 0.62. Calculations of relative quantitation were performed by the comparative Ct method, using the sample with the lowest value in the TCM-199 group as a control group. Data are reported as relative *n*-times difference, in relation to the control sample.

2.6. Statistical analysis

Cleavage and 8–16 cell embryos at 72 hpi, and blastocyst rate were assessed by Chi-square. Hatching rates were evaluated by Fisher's exact test. Relative quantitation was evaluated by ANOVA, and means

Table 1
Sequence, annealing temperature and size of the pairs of primers used in real-time PCR.

Gene	Sequence 5′ to 3′	Annealing temperature (°C)	Fragment size (bp)	Genbank accession no.
Hsp-70.1	5′-AACAAGATCACCATCACCAAACG 3′-TCCTTCTCCGCCAAGGTGTTG	59	275	NM174550
Bax	5′-TTTTGCTTCAGGGTTTCATCCAGGA 3′-CAGCTGCGATCATCCTCTGCAG	64	174	NM173894
H2A	5′- GCCATCCTGGCGTACCTCAC 3′- TGGATGTGTGGAATGACACC	52	176	NM174809

Table 2
Embryonic development of bovine oocytes matured in vitro in alpha-MEM supplemented with PVA (PVA), PVP-40 (PVP), or in TCM 199 plus estrous cow serum (TCM).

Treatment	Total no. zygotes	% cleavage 68–72 hpi[a] (*n*)			% blastocysts[a] (*n*)	% hatching[b] (*n*)
		2–8 cells	9–16 cells	Total	168–192 hpi	
TCM	401	34.9 (140)	32.6 (131)	68.3a (271)	42.6 (169)	40.8 (69)
PVP	353	34.4 (111)	28.8 (93)	58.3b (204)	44.6 (156)	34.6 (54)
PVA	410	29.7 (122)	24.4 (101)	54.4b (223)	40.0 (164)	36.0 (59)

Hpi: hours post-insemination. a and b: within a column, values without a common letters differed ($P < 0.05$).
[a] Chi-square test.
[b] Fischer's test.

compared by the Student–Neuman–Keuls test. Data are reported as mean ± S.E.M., with the level of significance set at $P < 0.05$.

3. Results

3.1. In vitro embryo production

A total of 1164 zygotes were obtained in 12 replicates. The total cleavage rate was higher ($P < 0.05$) in the group with oocytes matured in TCM-119 medium with ECS (68.3%) than in the groups with oocytes matured in alpha-MEM supplemented with PVA (54.4%) or PVP (58.3%). However, the proportion of embryos with more than eight blastomeres was similar ($P > 0.05$) for all groups, indicating that the rate of development during the first 72 hpi was similar for oocytes matured in chemically defined medium and for oocytes matured in medium containing serum. Similarly, maturation in alpha-MEM medium enriched with IGF-I/insulin and supplemented with PVA or PVP-40, in the absence of gonadotrophins and serum, did not affect the rate of blastocysts produced or hatching rates ($P > 0.05$) compared to maturation in TCM with ECS (Table 2).

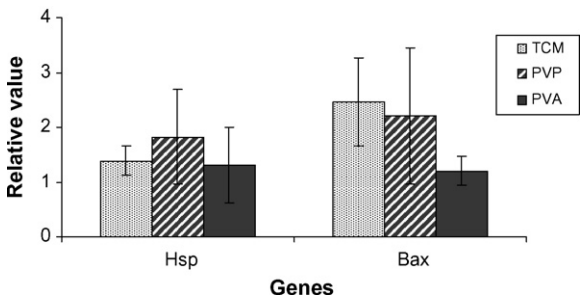

Fig. 1. Relative abundance of specific transcripts (mean ± S.D.) in bovine blastocysts produced in vitro from oocytes matured in various culture media: TCM-199 plus estrus cow serum (TCM), alpha-MEM supplemented with PVA (PVA), or PVP-40 (PVP).

3.2. Gene expression

The transcript relative abundance of the Hsp-70 and Bax genes determined by real-time PCR is shown (Fig. 1). The RA of Hsp-70 and Bax gene transcripts was similar for day 8 blastocysts originating from all IVM groups. Conditions of oocyte culture in the IVM system with alpha-MEM supplemented with PVA or PVP, in the absence of serum and gonadotrophins, did not interfere with transcripts levels of these genes after fertilization and embryo culture, compared to culture in TCM-199 supplemented with ECS.

4. Discussion

The present study was conducted to assess the effect of IVM in a chemically defined medium (alpha-MEM) enriched with IGF-I/insulin and PVA or PVP, in the absence of serum and gonadotrophins, on post-fertilization development of bovine oocytes. Oocytes matured under these culture conditions had the same potential to transform into blastocysts as oocytes matured in standard, non-defined medium (TCM-199, supplemented with serum and FSH). Therefore, the absence of serum and gonadotrophins during IVM did not compromise post-cleavage development of the oocyte. Ali and Sirard [9] demonstrated that the rate of development of oocytes matured without proteins was equivalent or superior to that of oocytes matured in the presence of serum and hormones such as FSH, LH, and estradiol. Although in the present study the cleavage rate was superior in the control group, embryos derived from oocytes matured in a defined medium had the same proportion of embryos in 9–16 cell stage at day 3 post-insemination compared to embryos derived from oocytes matured in the presence of serum. This stage is associated with maternal-embryonic transition, when the epigenetic regulation of the expression of the embryonic genome is particularly sensitive to external factors [32,33]. The hatching rate, based on the total

number of blastocysts, was also similar for embryos resulting from oocytes matured in medium with and without serum or gonadotrophins. Perhaps embryonic development was stimulated after 9–16 cells (at the morulae or blastocyst stages) in embryos derived from oocytes matured in medium supplemented with PVA or PVP-40. In that regard, Ali and Sirard [9] reported that the supplementation of IVM medium with PVP-40 stimulated the development of morulae and blastocysts. In contrast to maturation in TCM with ECS, no expansion of cumulus cells was observed in alpha-MEM containing PVA or PVP, probably due to the absence of gonadotrophins that act on hyaluronic acid secretion [34–36]. The expansion of cumulus cells appearance is not essential for the success of IVM, as reported in other studies, which observed that the expansion of these cells was not associated with cytoplasmic competence [9] or with an oocyte's ability to develop after fertilization [21].

Polyvinyl alcohol and PVP-40 have been added to the medium as substitutes of serum [8] or BSA during IVM of bovine oocytes [7,9–15]. Serum is commonly used as a protein source in culture media, but it may be contaminated with various defined and undefined molecules such as steroids, cholesterol, peptides, antibiotics, and proteases, and with extrinsic agents such as bacteria, viruses, antibodies, and prions [2–4]. In addition, the serum originates from a non-physiological clotting process [37] and its use in embryo culture has been associated with phenotypic disorders, e.g. increased birth weight [38]. Polyvinyl alcohol and PVP-40 are synthetic polymers used in the cell culture medium for the stabilization of osmotic pressure and as protective agents and chelants of heavy metal ions. Although there are few reports regarding their physicochemical properties, these polymers affect the properties of cell adhesion, protein adsorption and diffusion of paracrine and autocrine factors in the culture medium [8,39]. Polyvinylpyrrolidone seems to differ from PVA in some properties such as molecular weight, solubility and pH. Indeed, Wang et al. [40] stated that specific polymers may have different properties including molecular weight, charge type and density, structure and sequence, and conformational flexibility of the molecule, which are associated with the differences in cytotoxicity. Ali and Sirard [9] reported that replacing PVP-40 with PVA in IVM medium reduced embryonic development, especially at the morulae and blastocyst stages. Pinyopummintr and Bavister [41] also demonstrated that post-cleavage embryonic development was reduced when BSA or serum were replaced with PVA. However, in the present study, we did not detect distinct effects of IVM medium supplementation with PVA or PVP-40 on blastocyst or hatching rates. Similarly, Chung et al. [15] also reported that PVP used for maturation had no deleterious effect on subsequent embryonic development compared to FCS. Conversely, Warzych et al. [42] reported significantly lower rates of blastocysts resulting from oocytes matured in TCM supplemented with PVP-40. However, a higher concentration of PVP-40 (4%) and FSH and LH supplementation were used in maturation medium, which may have influenced the developmental competence of oocytes.

Growth factors may be necessary for successful IVM in the presence of PVA or PVP. Oyamada et al. [43] reported that EGF in maturation medium supplemented with PVA stimulated embryonic development compared to the control group. In the present study, IGF-1 and insulin were added to the maturation media with PVA and PVP, and may have contributed to the success of maturation and embryonic development, even in the absence of gonadotrophins. The biological effects of these peptides start with the activation of tyrosine kinase receptors, resulting in the activation of the two major signaling molecules of the insulin receptor (IR), i.e., the substrate of the insulin receptor and IGF-I 1 (IRS-1 or IRS-2) and the enzyme phosphatidylinositol-3 kinase (PI3K) linked to the MAP kinase pathway (MAPK mitogen-activate protein kinase), also called extracellular regulated kinase (ERK) [44,45], one of the regulators of the cell cycle and of oocyte maturation. Several studies have reported that IGF-I and insulin enhanced IVM of bovine [25,26], porcine [27], ovine [23], murine [24], and human [22] oocytes. Because of its importance for follicular development, FSH is frequently employed in IVM media to increase the effectiveness of the maturation process. The use of gonadotrophins in IVM has been reported to be important for embryonic development [46–48] and has been indicated for maturation media [49], although their use remains controversial [9,17]. Previous studies did not show a beneficial effect of FSH or LH on IVM and subsequent embryonic development [50,51]. Conversely, the effects of FSH and LH on oocyte maturation can be modulated by the presence of serum. Kito and Bavister [52] observed that the effect of FSH and LH on the rate of meiotically matured hamster oocytes was greater in the presence of FCS than in its absence. Thus, gonadotrophins may not be essential for maturation in the absence of serum, as observed in the present experiment.

In the present study, there were no significant differences among the three IVM systems in the relative

abundance of Hsp-70 and Bax gene transcripts in the blastocyst groups. Since the expression of genes related to the cell response to stress such as heat shock proteins (Hsp) and Bax may be altered during oocyte and embryo culture [42,53], the expression of these genes may be a marker of culture conditions. The Hsp-70 gene is a modulator of the cellular response to thermal stress. Heat shock proteins play a protective role inside the cell and have an anti-apoptotic function [54]. Bax is a pro-apoptotic gene; its role is associated with bcl-2 activity. The proportion of Bax:bcl-2 proteins predetermined the cellular response to an apoptotic stimulus [55]. It has been suggested that interaction in the expression of the Hsp-70 and Bax genes may occur under stress conditions, with suppression of Bax activation in cells with high Hsp-70 levels [56].

Although there was no difference among experimental groups in the level of Hsp-70 gene transcripts, some authors have reported up-regulated expression of this gene in embryos derived from oocytes matured in TCM-199 medium supplemented with 10% serum, compared to defined or semi-defined maturation media, or media containing a lower concentration of serum [5,6,42]. Sagirkaya et al. [2] reported that RA for Hsp-70 gene was altered under different IVM media supplemented with FCS or SSS. Warzych et al. [42] suggested that serum in the culture medium acted as a stressor, inducing the expression of the Hsp-70 gene. However, conflicting results have been reported [57,58] and the potential effects of various serum sources (FCS, oestrous cow serum, etc.) and/or effects of different serum lots, have not been clarified. Supplements added to the IVM medium in the present study also did not affect the level of Bax gene transcripts in the blastocysts produced. However, we observed a trend for down-regulation of the Bax gene in embryos resulting from oocytes matured in alpha-MEM medium supplemented with PVA, although no significant difference was detected. Warzych et al. [42] also did not observe changes in the relative abundance of Bax gene transcripts in day 9 blastocysts after IVM of bovine oocytes with PVP-40, BSA or FCS. However, up-regulation of the Bax gene in embryos produced in vitro has been reported in several studies [59,60]. It was noteworthy that the increase in the RA of this gene was detected in blastocysts after in vitro culture (IVC) with serum [59], but not in resulting preimplantational bovine blastocysts from oocytes matured with FCS. Unfortunately, there are few data regarding the influence of IVM media composition on the expression of the Bax gene in bovine embryos. Warzych et al. [42] were the first to publish data regarding the expression of this gene in embryos resulting from oocytes matured under various culture conditions. This author showed that IVM medium supplemented with PVP-40, fatty acid-free BSA (fafBSA) or FBS did not affect RA for Bax and bcl-2 genes in hatched blastocysts, but increased Bax to Bcl-2 ratio when oocytes were matured with fafBSA. It is noteworthy that embryo culture conditions can also affect gene expression in blastocysts and mask possible effects of IVM systems on embryos.

In conclusion, bovine oocytes were matured in serum- and gonadotrophin-free medium, supplemented with PVA or PVP-40 and growth factors, with no changes in post-cleavage development or in the RA of stress-associated genes such as Hsp-70 and Bax. However, a better understanding of the basic factors that control the biofunctionality of these synthetic polymers in culture systems is needed, and the risk of immunogenic and cytotoxic risks for oocytes and embryos, should also be investigated.

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