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The effect of interaction between macromolecule supplement and oxygen tension on bovine oocytes and embryos cultured *in vitro*

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Summary

Aiming to improve *in vitro* production of bovine embryos and to obtain supplements to replace serum for *in vitro* maturation (IVM), this study evaluated the effects of macromolecular supplementation of IMV medium (bovine serum albumin – BSA, polyvinyl alcohol – PVA, polyvinyl pyrrolidone – PVP, Ficoll, KnockoutSR, or fetal calf serum – FCS) and oxygen tension [5% CO_2 in air (20% O_2) or 5% CO_2, 5% O_2 and 90% N_2 (5% O_2)] on oocyte maturation and embryo development. Nuclear progression to germinal vesicle breakdown, metaphase I and metaphase II stages were evaluated and overall results revealed that undefined (FCS) and semi-defined (BSA) media gave better results at 20% O_2 and defined media (PVA, PVP and Ficoll) at 5% O_2. Independent of macromolecule supplement, IVM at 20% O_2 was considered optimal for nuclear maturation. To evaluate embryo development, oocytes matured in the previously described conditions were fertilized and cultured at the same oxygen tension used for IVM and assessed for cleavage (43.0 to 74.8%) and development to morulae (16.4 to 33.8%), blastocyst (7.7 to 52.9%) and hatched blastocyst (9.6 to 48.1%). Apart from oxygen tension, all treatments, except Knockout (22.7%), gave similar results for blastocyst development (26.5 to 38.7%). Independently of macromolecule supplement, higher development rates were obtained in an oxygen tension of 20% O_2 (67.4% cleavage, 29.2% morulae, 40.8% blastocyst and 34.0% hatched blastocyst) when compared with 5% O_2 (52.5, 21.8, 18.2 and 15.6%, respectively). This study indicates that BSA, PVA, PVP and Ficoll can replace serum during IVM and that the optimal atmospheric condition for *in vitro* production of bovine embryos is 5% CO_2 and 20% O_2.

Keywords: Cattle, Embryo, Macromolecule, Maturation, Oocyte, Oxygen

Introduction

The *in vitro* culture systems used for embryo production still do not reach the level of effectiveness obtained *in vivo*. Whilst *in vivo* embryonic development rates achieve 70% blastocyst rate, only about 85% of *in vitro* matured oocytes are fertilized and only 30 to 40% of these develop to the blastocyst stage (Gutiérrez-Adan *et al.*, 2001). For this reason, the development of strategies to improve efficiency of *in vitro* production (IVP) of bovine embryos is sought. Among several factors associated with culture media and methods that influence this procedure, great importance is given to macromolecule supplements and atmospheric conditions.

The macromolecule supplements that are frequently employed in IVP are bovine serum albumin (BSA) and fetal calf serum (FCS). These supplements are colloid particles that facilitate fluid transport through biological membranes (Webster, 1982) and improve maturation and embryo development (Carolan *et al.*, 1995). BSA and FCS quelate heavy metal ions, buffer pH and act as surfactants and reactive oxygen species

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(ROS) scavengers (Orsi & Leese, 2004; Stein, 2007). BSA also increases intracellular free amino acids after its hydrolysis (Orsi & Leese, 2004) and serum possesses more than 1000 different compounds, including growth factors and hormones (Stein, 2007). Because of those characteristics, it is difficult to obtain a suitable substitute for BSA and FCS in culture. However, as BSA and FCS are products of animal origin: (i) their composition is not completely known; (ii) they present high variation among producers and batches (Mckiernan & Bavister, 1992); and (iii) they offer the risk of disease transmission (Krisher *et al.*, 1999). Then the culture medium supplemented with BSA and FCS is semi-defined or undefined in composition and contributes to variability of the culture systems and the obtained results (Bavister, 1995; Krisher *et al.*, 1999).

Thus, aiming to standardize IVP procedures and also to avoid pathogens transmission, synthetic and defined supplement sources are frequently used, including polyvinyl alcohol (PVA; Fukui *et al.*, 2000), polyvinyl pyrrolidone (PVP; Chung *et al.*, 2007), Ficoll (Kuleshova *et al.*, 1999) and serum replacers, as synthetic serum substitute (Sagirkaya *et al.*, 2007) and KnockoutSR (Moore *et al.*, 2007). Among them, PVA and PVP are the synthetic macromolecules most used in culture medium to replace BSA and FCS. Ficoll is a polysaccharide usually employed in vitrification solutions (Checura & Seidel, 2007). KnockoutSR is a protein source used for stem-cell culture and whose defined formula is protected by fabricant, but does not have serum in its composition (Goldsborough *et al.*, 1998). However, the embryos produced in fully defined media usually present a developmental block (Camous *et al.*, 1984) and reduced viability (Wright & Bondioli, 1981) when compared with those embryos cultured in undefined or semi-defined media.

Atmospheric conditions also affect IVP because the oxygen tension usually employed in *in vitro* culture (5% CO_2 in air, which corresponds to 20% O_2) is higher than that existent in ovarian follicles, oviduct and uterus. In follicles, the oxygen from capillaries diffuses through the layers of granulosa cells and a gradient of O_2 occurs from the follicle periphery to the central oocyte (Gosden & Byatt-Smith, 1986). Early embryo development after fertilization begins in oviduct, where the O_2 tension is lower than in the atmosphere (Mass *et al.*, 1976). Thus, the higher oxygen concentration used for *in vitro* culture may be a factor in the production of ROS that provoke intracellular damage and are detrimental for embryonic development (Batt *et al.*, 1991). However, the published results using 20% or 5% O_2 during the IVP steps are still controversial. Some research in bovine demonstrates that culture in lower oxygen tension (from 5 to 10%) improves maturation and embryo development (Nakao & Nakatsuji, 1990; Thompson *et al.*, 1990; Voelkel & Hu, 1992) whilst others observed better results when using 20% O_2 (Oyamada & Fukui, 2004; Castro e Paula & Hansen, 2007). Moreover, evidences of interaction between culture medium and oxygen tension were also reported (Noda *et al.*, 1994; Castro e Paula & Hansen, 2007).

Among the steps in IVP, *in vitro* maturation (IVM) is highlighted (Brackett & Zuelke, 1993; Eppig, 1996; Sirard & Blondin, 1996) because in this phase the oocyte accumulates the mRNA and proteins that are essential to progression to the embryonic genome activation stage and to the acquisition of developmental competence (Thibault *et al.*, 1987; Sirard *et al.*, 1989).

Thus, the aim of this study was to evaluate the effects of macromolecule supplements (BSA, PVA, PVP, Ficoll, Knockout and FCS) in IVM and of oxygen tension of 5% or 20% during all IVP procedures (maturation, fertilization and development culture – IVMFC) on oocyte nuclear maturation and embryo development to hatched blastocyst stage.

Materials and methods

Reagents, media and culture conditions

Chemicals were purchased from Sigma Chemical Co., unless otherwise stated.

The medium for *in vitro* maturation (IVM) consisted of TCM-199 (Gibco BRL) supplemented with 0.2 mM sodium pyruvate, 25 mM sodium bicarbonate, 75 μg/ml kanamycin (Gibco BRL), 0.5 μg/ml FSH (Pluset®), 100 IU/ml hCG (Profasi®), 1.0 μg/ml estradiol and one macromolecular supplement according to experimental design.

In vitro fertilization (IVF) medium consisted of Tyrode's albumin lactate pyruvate (TALP) supplemented with 0.2 mM sodium pyruvate, 6 mg/ml fatty acid-free BSA, 25 mM sodium bicarbonate, 13 mM sodium lactate, 75 μg/ml kanamycin, 4 μl/ml PHE solution (2 mM penicillamine, 1 mM hypotaurine and 250 μM epinephrine) and 10 μg/ml heparin.

In vitro culture (IVC) medium was synthetic oviductal fluid (SOF) supplemented with 0.2 mM L-glutamine, 0.34 mM sodium citrate, 2.8 mM myo-inositol, 2% MEM essential amino acid solution, 1% MEM non-essential amino acid solution, 0.2 mM sodium pyruvate, 75 μg/ml kanamycin, 5 mg/ml fraction V fatty acid-free BSA and 2.5% FCS.

Cultures (IVM, IVF and IVC) were carried out at 38.5 °C with maximum humidity and under an atmosphere of 5% CO_2 in air (20% O_2) or 5% CO_2, 5% O_2 and 90% N_2 (5% O_2) depending upon the experimental design.

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Oocyte recovery and culture

Abattoir-derived ovaries were transported to the laboratory in saline solution at 30–35 °C. The follicles (2–8 mm) were aspirated using an 18-gauge needle attached to a 20 ml syringe. Oocytes with at least four layers of cumulus cells were selected for the experiments.

For IVM, oocytes were washed and cultured in IVM medium supplemented with 6 mg/ml BSA, 6 mg/ml PVA, 6 mg/ml PVP, 6 mg/ml Ficoll, 10% KnockoutSR (Gibco BRL), or 10% FCS (Gibco BRL). The IVM culture was performed in 100 µl droplets (20 oocytes per droplet) under mineral oil (Dow Corning Co.) for 24 h.

Assessment of nuclear maturation

The percentage of oocytes at germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII) stages were respectively recorded at 6, 18 and 24 h of IVM. For that purpose, oocytes were stripped from their cumulus cells by vortexing for 3 min in 0.2% hyaluronidase and then stained with 10 µg/ml Hoechst 33342 for 10 min, placed between slide and coverslip and visualized under epifluorescence microscopy (330–385 nm; at 200×magnification).

In vitro fertilization

Motile spermatozoa were obtained by centrifugation of frozen–thawed semen on a Percoll (Pharmacia) discontinuous density gradient (2 ml of 45% Percoll over 2 ml of 90% Percoll) for 30 min at 900 *g* at room temperature. The supernatant was discarded and the spermatozoa were counted on a haemocytometer and then resuspended in IVF medium to obtain a final concentration of 2×10^6 cells/ml. Finally, 4 µl of the sperm suspension were added to each droplet, for a final concentration of 1×10^6 sperm/ml. Oocytes and sperm were co-incubated for 24 h.

In vitro development culture and embryo evaluation

Following fertilization, the presumptive zygotes were transferred to IVC medium. Zygotes were incubated under mineral oil up to 48 h for assessment of cleavage rates under stereoscopic microscopy (at a 40× magnification), when 2- and 4-cell embryos were counted. Morulae, blastocyst and hatched blastocyst development rates were observed, respectively, at 144 (day 6), 168 (day 7) and 192 h (day 8) post insemination (hpi).

Experimental design

Experiment I: The effects of macromolecular supplementation of IVM medium and oxygen tension on oocyte nuclear maturation

Oocytes ($n = 4129$ in five replicates) were *in vitro* matured in medium supplemented with one of the following macromolecules: BSA (6 mg/ml), PVA (6 mg/ml), PVP (6 mg/ml), Ficoll (6 mg/ml), Knockout (10%), or FCS (10%); in two oxygen tension: 5% CO_2 in air (20% O_2) and 5% CO_2, 5% O_2 and 90% N_2 (5% O_2). Oocytes were assessed for nuclear maturation and data for GVBD, MI and MII were collected, respectively, at 6, 18 and 24 h of IVM.

Experiment II: The effects of macromolecular supplementation of IVM medium and oxygen tension during maturation, fertilization and culture on embryonic development

Oocytes ($n = 1139$ in six replicates) were *in vitro* matured for 24 h in the same conditions described for the Experiment I. After IVM, they were submitted to IVF and IVC in 20% or 5% O_2 and data for cleavage (48 hpi) and development to morulae (144 hpi), blastocyst (168 hpi) and hatched blastocyst (192 hpi) were calculated over total number of oocytes, and were recorded.

Statistical analysis

In this study, data were reported as mean ± standard error (SEM). Data were arcsine transformed and analysed by ANOVA in which the effect of macromolecule, oxygen tension and interaction between macromolecule and oxygen tension were evaluated. When a statistical significant effect was found, multiple comparisons of means were determined using Tukey's test (SAS Program V.8). A p-value <0.05 was considered to be statistically significant.

Results

Experiment I: The effects of macromolecular supplementation of IVM medium and oxygen tension on oocyte nuclear maturation

At 6 h of IVM in 20% oxygen tension, GVBD rates in oocytes treated with FCS (49.8% ± 4.9) were higher ($p < 0.05$) than in PVP (30.7% ± 5.3), Ficoll (32.0% ± 5.1) and Knockout (31.1% ± 4.9) (Table 1). At 5% O_2, the treatments PVA (43.0% ± 5.7), PVP (44.7% ± 5.9) and Ficoll (43.8% ± 7.6) were superior ($p < 0.05$) to BSA (25.2% ± 4.3), Knockout (22.3% ± 4.9) and FCS (24.1% ± 4.8) (Table 1). For all treatments, except BSA and FCS that presented higher ($p < 0.05$) results at 20% O_2, the

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Table 1 Oocyte nuclear maturation stages.

O_2 tension (%)	Group	GVBD (6 h)		MI (18 h)		MII (24 h)	
		n	% mean ± SEM	*n*	% mean ± SEM	*n*	% mean ± SEM
20	BSA	81	$40.3 \pm 5.6^{a,b}$	104	38.4 ± 5.6^{c}	95	64.9 ± 6.2^{a}
	PVA	79	$35.5 \pm 8.6^{a–c}$	104	$41.4 \pm 5.8^{b,c}$	136	67.6 ± 5.5^{a}
	PVP	83	$30.7 \pm 5.3^{b,c}$	125	34.6 ± 6.5^{c}	117	62.5 ± 5.2^{a}
	Ficoll	130	$32.0 \pm 5.1^{b,c}$	123	32.0 ± 6.5^{c}	137	60.6 ± 6.4^{a}
	Knockout	101	$31.1 \pm 4.9^{b,c}$	121	32.9 ± 6.0^{c}	135	$54.4 \pm 6.3^{a–c}$
	FCS	118	49.8 ± 4.9^{a}	86	36.4 ± 5.2^{c}	99	60.0 ± 5.8^{a}
5	BSA	86	25.2 ± 4.3^{c}	76	35.0 ± 6.1^{c}	116	36.8 ± 5.9^{d}
	PVA	104	$43.0 \pm 5.7^{a,b}$	129	$58.5 \pm 6.8^{a,b}$	111	$41.9 \pm 6.4^{b–d}$
	PVP	116	$44.7 \pm 5.9^{a,b}$	98	66.1 ± 5.6^{a}	115	$56.0 \pm 5.2^{a,b}$
	Ficoll	106	$43.8 \pm 7.6^{a,b}$	103	60.4 ± 5.9^{a}	152	$38.5 \pm 5.7^{c,d}$
	Knockout	125	22.3 ± 4.9^{c}	141	63.6 ± 7.2^{a}	149	29.3 ± 6.1^{d}
	FCS	118	24.1 ± 4.8^{c}	144	$50.5 \pm 7.2^{a–c}$	166	30.1 ± 6.8^{d}

Germinal vesicle breakdown (GVBD), metaphase I (MI) and metaphase II (MII), respectively, at 6, 18 and 24 h of *in vitro* maturation in medium supplemented with bovine serum albumin (BSA; 6 mg/ml), polyvinyl alcohol (PVA; 6 mg/ml), polyvinyl pyrrolidone (PVP; 6 mg/ml), Ficoll (6 mg/ml), KnockoutSR (10%) and fetal calf serum (FCS; 10%), in oxygen (O_2) tensions of 5% CO_2 in air (20% O_2) and 5% O_2, 5% CO_2 and 90% N_2 (5% O_2).
$^{a–d}$Values with different superscript letters within the same column differ ($p < 0.05$).

GVBD stage rates were similar ($p > 0.05$) between both oxygen tensions (Table 1).

At 18 h, MI rates (32.0 to 41.4%) were similar ($p > 0.05$) in all treatments at 20% O_2 (Table 1). In 5% oxygen tension, higher ($p < 0.05$) M I rates were observed in groups PVA (58.5% ± 6.8), PVP (66.1% ± 5.6), Ficoll (60.4% ± 5.9), Knockout (63.6% ± 7.2) and FCS (50.5% ± 7.2) (Table 1). The treatments PVP, Ficoll and Knockout presented differences ($p < 0.05$) between both oxygen tensions, and showed higher ($p < 0.05$) results at 5% O_2 (Table 1).

At 24 h, similar ($p > 0.05$) MII rates (54.4 to 67.6%) were observed among all treatments at 20% O_2 (Table 1). At 5% O_2, PVP (56.0% ± 5.2) was superior ($p < 0.05$) to all treatments, but similar ($p > 0.05$) to PVA (41.9 ± 6.4) (Table 1). PVP at 5% O_2 was similar ($p > 0.05$) to all treatments at 20% O_2. In a comparison of both oxygen tensions, PVP was the only treatment that showed similar ($p > 0.05$) MII rates at 20% and 5% O_2, while the other supplements presented better results at 20% O_2 (Table 1).

Experiment II: The effects of macromolecular supplementation of IVM medium and oxygen tension during maturation, fertilization and culture on embryonic development

Data for cleavage and embryonic development to morulae, blastocyst and hatched blastocyst after *in vitro* maturation in medium with different macromolecule supplements (BSA, PVA, PVP, Ficoll, Knockout and FCS) under two oxygen atmospheres (20% and 5% O_2) are presented in Table 2. The results of the ANOVA showed that there was no effect ($p > 0.05$) of macromolecule supplements of IVM medium (BSA, PVA, PVP, Ficoll, Knockout and FCS) in any of the variables studied, such as cleavage (43.0 to 74.8%) and development to morulae (16.4 to 33.8%), blastocyst (7.7 to 52.9%) and hatched blastocyst (9.6 to 48.1%). On the other hand, oxygen tension (20% and 5% O_2) had a significant effect ($p < 0.05$) in all the variables studied. There was no interaction between macromolecular supplements of IVM medium and oxygen tension. Hence, the data for those variables were presented separately as independent variables (Tables 3 and 4).

The macromolecule supplement used during *in vitro* maturation did not affect ($p > 0.05$) the rates of cleavage (51.0 to 67.9%) and development to morulae (20.8 to 30.5%) and hatched blastocyst (20.0 to 32.5%) (Table 3). Blastocyst development rates were higher ($p < 0.05$) in treatments PVA (35.6% ± 6.0) and FCS (38.7% ± 5.9) when compared to Knockout (22.7% ± 5.4) (Table 3).

Higher ($p < 0.05$) rates of cleavage and development to morulae, blastocyst and hatched blastocyst were obtained after *in vitro* culture in 20% O_2 (67.4, 29.2, 40.8 and 34.0%, respectively) when compared with 5% O_2 (52.5, 21.8, 18.2 and 15.6%, respectively) (Table 4).

Discussion

Oocyte nuclear maturation involves meiosis resumption (germinal vesicle breakdown–GVBD), resulting in MI, first meiotic completion, polar body emission and progression to MII stage. These steps, together with the cytoplasmic maturation, prepare oocytes for being fertilized and to acquire embryonic developmental

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Table 2 Cleavage and embryonic development to morulae, blastocyst and hatched blastocyst.

O_2 tension (%)	Group	*n*	Cleavage (% mean ± SEM)	Morulae (% mean ± SEM)	Blastocyst (% mean ± SEM)	Hatched blastocyst (% mean ± SEM)
20	BSA	86	59.0 ± 6.3	32.9 ± 4.1	36.4 ± 2.5	28.2 ± 4.6
	PVA	84	73.0 ± 5.9	30.3 ± 5.2	42.5 ± 8.2	36.1 ± 9.1
	PVP	112	74.8 ± 7.7	24.4 ± 6.0	43.8 ± 8.3	39.9 ± 7.1
	Ficoll	95	67.1 ± 4.6	25.3 ± 2.6	33.9 ± 6.1	28.9 ± 6.1
	Knockout	109	60.3 ± 5.8	29.5 ± 4.2	35.3 ± 5.8	25.3 ± 7.3
	FCS	95	70.0 ± 3.1	33.8 ± 5.9	52.9 ± 5.9	48.1 ± 5.2
5	BSA	90	43.0 ± 9.4	19.4 ± 2.6	11.6 ± 4.8	10.0 ± 5.0
	PVA	89	57.3 ± 8.5	21.9 ± 5.2	25.3 ± 6.5	19.7 ± 8.1
	PVP	97	59.7 ± 6.0	26.7 ± 9.2	17.8 ± 6.2	14.3 ± 5.7
	Ficoll	116	50.9 ± 7.9	16.4 ± 3.3	20.8 ± 8.0	16.8 ± 4.5
	Knockout	75	53.2 ± 7.1	21.7 ± 5.9	7.7 ± 2.1	9.6 ± 3.2
	FCS	91	52.3 ± 8.1	26.5 ± 2.2	24.5 ± 6.0	19.5 ± 5.3

Cleavage (48 hpi) and embryonic development to morulae (144 hpi), blastocyst (168 hpi) and hatched blastocyst (192 hpi) in bovine oocytes *in vitro* matured in medium supplemented with bovine serum albumin (BSA; 6 mg/ml), polyvinyl alcohol (PVA; 6 mg/ml), polyvinyl pyrrolidone (PVP; 6 mg/ml), Ficoll (6 mg/ml), Knockout (10%) and fetal calf serum (FCS; 10%) and submitted to oxygen (O_2) tensions of 5% CO_2 in air (20% O_2) and 5% O_2, 5% CO_2 and 90% N_2 (5% O_2) during maturation, fertilization and culture.

Table 3 Cleavage and embryonic development to morulae, blastocyst and hatched blastocyst in bovine oocytes *in vitro* matured in medium supplemented with bovine serum albumin, polyvinyl alcohol, polyvinyl pyrrolidone, Ficoll, Knockout or fetal calf serum.

Group	*n*	Cleavage (% mean ± SEM)	Morulae (% mean ± SEM)	Blastocyst (% mean ± SEM)	Hatched blastocyst (% mean ± SEM)
BSA	176	51.0 ± 5.9	26.8 ± 3.2	26.5 ± 4.7^{a,b}	20.9 ± 4.4
PVA	173	65.1 ± 5.5	25.7 ± 3.8	35.6 ± 6.0^{a}	29.5 ± 6.6
PVP	209	67.9 ± 5.3	25.5 ± 5.0	32.0 ± 6.5^{a,b}	29.7 ± 6.2
Ficoll	211	59.0 ± 5.0	20.8 ± 2.4	28.0 ± 5.1^{a,b}	24.1 ± 4.4
Knockout	184	56.7 ± 4.5	25.9 ± 3.5	22.7 ± 5.4^{b}	20.0 ± 5.5
FCS	186	61.1 ± 4.9	30.5 ± 3.5	38.7 ± 5.9^{a}	32.5 ± 5.7

Cleavage (48 hpi) and embryonic development to morulae (144 hpi), blastocyst (168 hpi) and hatched blastocyst (192 hpi) in bovine oocytes *in vitro* matured in medium supplemented with one of the following macromolecules: bovine serum albumin (BSA; 6 mg/ml), polyvinyl alcohol (PVA; 6 mg/ml), polyvinyl pyrrolidone (PVP; 6 mg/ml), Ficoll (6 mg/ml), Knockout (10%) and fetal calf serum (FCS; 10%).

^{a,b}Values with different superscript letters within the same column differ ($p < 0.05$).

Table 4 Cleavage (48 hpi) and embryonic development to morulae (144 hpi), blastocyst (168 hpi) and hatched blastocyst (192 hpi) in bovine oocytes *in vitro* matured, fertilized and cultured under oxygen tensions of 5% CO_2 in air (20% O_2) and 5% O_2, 5% CO_2 and 90% N_2 (5% O_2).

Oxygen tension (%)	*n*	Cleavage (% mean ± SEM)	Morulae (% mean ± SEM)	Blastocyst (% mean ± SEM)	Hatched blastocyst (% mean ± SEM)
20	581	67.4 ± 2.4^{a}	29.2 ± 1.9^{a}	40.8 ± 2.7^{a}	34.0 ± 2.9^{a}
5	558	52.5 ± 3.2^{b}	21.8 ± 2.1^{b}	18.2 ± 2.5^{b}	15.6 ± 2.2^{b}

^{a,b}Values with different superscript letters within the same column differ ($p < 0.05$).

competence (Thibault *et al.*, 1987; Sirard *et al.*, 1989). Then, culture conditions, which included medium composition and oxygen tension, used for IVM of mammalian oocytes influence subsequent embryonic development (Rose & Bavister, 1992; Stock *et al.*, 1997). Currently, the medium commonly used for IVM is TCM-199 supplemented with serum and hormones. However, several studies attempted to use defined macromolecule as supplements to replace serum in culture, thus aiming to avoid composition variations and the risk of disease transmission (Mckiernan & Bavister, 1992; Stock *et al.*, 1997; Krisher *et al.*, 1999).

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For that reason, undefined (supplemented with FCS), semi-defined (BSA) and defined (PVA, PVP, Ficoll and Knockout) TCM-199 media were used for IVM of bovine oocytes under two oxygen tensions (5% and 20% O_2) to assess their effects on nuclear maturation. The normal meiosis progression was evaluated as the percentage of oocytes at GVBD, MI and MII at 6, 18 and 24 h of IVM, respectively.

Under atmospheric oxygen concentration (20% O_2) the number of oocytes at GVBD stage produced in the undefined medium with FCS was higher than that in defined media with PVP, Ficoll and Knockout. This finding may be caused by meiosis acceleration promoted by FCS, as reported for blastocyst development (Mastromonaco *et al.*, 2004). However, all macromolecule supplements were similar in MI and MII rates at 20% O_2. At a lower oxygen concentration (5% O_2) the defined supplements PVA, PVP and Ficoll were superior to BSA, Knockout and FCS in GVBD rates, but all treatments, except BSA, were similar in MI rates and PVP was superior in MII rates. From these results, we can observe that, for the earliest step of nuclear progression (GVBD), undefined (FCS) and semi-defined (BSA) media were more efficient at 20% O_2, while defined media (supplemented with PVA, PVP, or Ficoll) were better at reduced oxygen tension (5% O_2). Thus, the oxygen concentration influenced the macromolecule supplement used for IVM, similar to the oxygen tension effects observed with culture medium (TCM-199 versus SOF; Castro e Paula & Hansen, 2007) and with other supplements, such as glucose (Oyamada & Fukui, 2004) and melatonin (Papis *et al.*, 2007). The positive effects of undefined/semi-defined media at 20% O_2 and of defined media at 5% O_2 became less pronounced as oocytes progressed in development.

Based on the findings of nuclear maturation (MII rates), all evaluated macromolecule supplements were suitable for use in IVM of bovine oocytes at 20% O_2, but at 5% O_2 comparable results were only obtained for PVP. Then, we support the hypothesis that IVM under the most frequently used oxygen concentration, 20% O_2 (Harvey, 2007), is optimal for nuclear maturation independent of the macromolecule supplement used (defined, semi-defined or undefined). These results are contrary to the described detrimental effect of atmospheric oxygen tension (20% O_2) during IVM on nuclear progression (Eppig & Wigglesworth, 1995; Smitz *et al.*, 1996). Moreover, we observed that reduced oxygen tension (5% O_2) during IVM was detrimental for oocyte nuclear maturation, as previously reported (Ali & Sirard, 2002).

To assess the effects of macromolecule supplement and oxygen tension on embryonic development, oocytes were matured in IVM media supplemented with BSA, PVA, PVP, Ficoll, Knockout and FCS at 5% or 20% O_2 and then submitted to *in vitro* fertilization (IVF) and culture (IVC) in the same atmospheric condition used for IVM. The zygotes were then evaluated for cleavage and development to morulae, blastocyst and hatched blastocyst stages.

Independently of macromolecule supplement and oxygen tension, the results obtained for cleavage (mean of 60.9%) and development to morulae (25.7%), blastocyst (29.4%) and hatched blastocyst (24.7%) were similar to the average results described in the literature.

All the macromolecule supplements used for IVM promoted similar rates of cleavage, morulae and hatched blastocyst. For blastocyst development, Knockout alone was inferior to IVM media supplemented with PVA or FCS, in agreement with the results that the Knockout serum replacer is not a beneficial supplement for maturation (Moore *et al.*, 2007). Few studies have evaluated the effects of different macromolecule supplements during maturation on embryonic development. Among these, it was observed that IVM in media supplemented with BSA (Fukui *et al.*, 2000) and PVP in bovine (Ali & Sirard, 2002; Chung *et al.*, 2007) and Ficoll in mouse (Kuleshova *et al.*, 1999) produced similar results in embryonic development when compared with serum. Then serum, which is the most used supplement used for IVM, can be effectively replaced by BSA, PVA, PVP and Ficoll without detrimental effects on early embryonic development.

These results are in disagreement with the observed reduction in embryonic development when PVA (Fukui *et al.*, 2000) was used for IVM. Also, we did not observe developmental block (Camous *et al.*, 1984) nor reduced viability (Wright & Bondioli, 1981) of embryos when IVM was performed in defined media.

Analysing the two atmospheric conditions employed during all IVP procedures (including IVM, IVF and IVC), independent of macromolecule supplement for IVM, we observed that utilization of atmospheric oxygen tension (20% O_2) resulted in higher rates of embryonic development (cleavage, morulae, blastocyst and hatched blastocyst) than a lower oxygen concentration (5% O_2). Therefore, these results suggest that, when adopting only one atmosphere condition for all IVP procedure, it is preferable to use 20% O_2 rather than 5% O_2.

In constrast to other reports (Yuan *et al.*, 2003; Corrêa *et al.*, 2008), we have obtained suitable development rates to blastocyst (40.8%) in IVC at 20% O_2 in the absence of co-culture.

The detrimental effects observed for 5% O_2 during IVP culture may be a consequence of the utilization of this atmospheric value, especially for IVM, as lower maturation rates were obtained under this oxygen concentration. It is well established that IVM conditions affect embryonic development (Rose & Bavister, 1992;

Stock *et al.*, 1997) and that an oxygen tension lower than atmospheric in IVM reduces cleavage and blastocyst development (Betterbed & Wright, 1985; Castro e Paula & Hansen, 2007). The superiority of a higher oxygen concentration during IVM may be provided by oxygen availability to oocyte, after its consumption by cumulus cells in cumulus–oocyte complexes (COCs; Castro e Paula & Hansen, 2007), as described for the oxygen gradient that occurs in ovarian follicles (Gosden & Byatt-Smith, 1986). However, better embryo development after IVM under lower oxygen tension was also reported (Hashimoto *et al.*, 2000), which may reflect other effects of medium composition and culture conditions.

Some studies (Papis *et al.*, 2007; Corrêa *et al.*, 2008) assumed that higher oxygen tensions during IVC resulted in ROS production that was detrimental for embryo development. However, it was previously demonstrated that physiological concentrations of ROS may, therefore, play a key role in the process of oocyte maturation (Dalvit *et al.*, 2005). Then, IVM culture of COCs under low oxygen concentration may have lead to oocyte hypoxia and impairment of a physiological concentration of ROS that is necessary for normal cellular growth and development (Dalvit *et al.*, 2005).

In summary, we observed that oxygen concentration influences the macromolecule supplement used for IVM in nuclear maturation, as beneficial results especially in earlier steps of maturation were obtained for undefined (FCS) and semi-defined media (BSA) at 20% O_2 and for defined media (PVA, PVP and Ficoll) at 5% O_2. Independent of macromolecule supplement, higher nuclear maturation rates were obtained at 20% O_2 and this atmospheric oxygen tension was found to be optimal for IVM of bovine oocytes. To promote embryo development, considering macromolecule supplement and oxygen tension as independent variables, the supplements BSA, PVA, PVP and Ficoll can replace FCS for IVM of bovine oocytes, while Knockout was not a suitable macromolecular supplement for IVM. As improved results for embryonic development were achieved at atmospheric oxygen tension, when all the steps of *in vitro* production (IVMFC) of bovine embryos were performed using only one atmosphere condition, the choice of oxygen concentration of 20% O_2 is preferred to 5% O_2.

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