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Oxygen tension during *in vitro* culture of bovine embryos: Effect in production and expression of genes related to oxidative stress

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Abstract

In vitro bovine embryos production and quality was evaluated in two culture systems, which utilize different oxygen tension. After IVM/IVF presumptive zygotes were cultured in either one of the two systems. The culture systems evaluated were—high O_2: SOFaaci medium and culture for 7 d under 5% CO_2 in air, at 39 °C in the presence of cumulus cells (control); low O_2: SOFaaci medium and culture for 7 d under 5% CO_2 and 5% O_2 at 39 °C. In low O_2 system the zygotes were denuded by successive pipetting before being transferred to culture medium, while in the high O_2 zygotes kept the cumulus cells that remained after IVF. Cleavage rates were evaluated 48 h post-insemination (hpi) and the blastocyst rates at D6 and D7 post-insemination (pi). From both groups a total of 94 expanded blastocysts, from D7 of culture, were fixed and stained with aceto-orcein to evaluate cell numbers. Seven pools of 15 embryos from each treatment were frozen for gene expression evaluation. The abundance of transcripts for genes related to oxidative stress, superoxide dismutase (Mn-SOD), catalase, gluthatione peroxidase (GPX) and for embryo quality, interferon-τ (IFN-τ) were determined using a semi-quantitative RT-PCR. Cleavage rate was similar ($P > 0.05$) for both groups. The blastocyst rate at D6 pi was greater ($P < 0.05$) in the group cultured under low O_2 tension (37.4%) than in the high O_2 tension (21.9%). However, blastocyst rate and total cell number at D7 were similar ($P > 0.05$) between groups. No change ($P > 0.05$) in transcript amount between treatments was observed for GPX, catalase and IFN-τ genes. However, the relative abundance of transcripts for Mn-SOD gene was greater ($P < 0.05$) for embryos cultured in high O_2 tension system. The results suggest that bovine embryos can be

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cultured either in SOFaaci medium under greater O_2 tension in the presence of cumulus cells, or in SOFaaci medium under less O_2 tension, without affecting embryo production or quality.

Keywords: Bovine; Embryos; Gene expression; Oxygen tension; Oxidative stress

1. Introduction

Although substantial progress has been made in procedures for *in vitro* maturation, fertilization and culture of bovine oocytes, the percentage of embryos that are able to normally development is still less in the *in vitro* produced than in those *in vivo* produced (Khurana and Niemann, 2000; Takahashi et al., 2000; Lonergan et al., 2003a). Several differences have been shown between those two types of embryos such as cell number, lipid contend, tolerance to cryopreservation and chromossomal abnormalities (Viuff et al., 2000; Ward et al., 2001; Rizos et al., 2003).

Abundant evidence has been presented over the last decade indicating that the developmental potential of embryos *in vitro* is, ultimately, dependent on the quality of the oocyte from which it originates (Lonergan et al., 2003a; Sirard et al., 2003; Rodriguez and Farin, 2004). However, there is also evidence that the culture environment which the embryos are exposed can affect their quality (Lonergan et al., 1999; Van Soom et al., 2002; Rizos et al., 2003; Yuan et al., 2003).

A number of factors can influence the culture environment such as media composition, protein supplementation, number of embryos present in the culture drop and gas atmosphere (Carolan et al., 1996; Hendricksen et al., 1999; Fukui et al., 2000; Khurana and Niemann, 2000). Among those factors, oxidative stress induced by greater oxygen tension has received special attention in the last few years (Ali et al., 2003; Bedaiwy et al., 2004; Fatehi et al., 2005). One of the differences between *in vivo* and *in vitro* environments is the O_2 tension. Oxygen concentration in air (20%), which was generally employed in embryo culture systems is considerable greater than the O_2 tension in the oviduct and uterus of most mammals (Fischer and Bavister, 1993). Greater *in vitro* embryo development is observed when the O_2 tension is reduced from 20% to 5% in various species (Takahashi et al., 2000; Guérin et al., 2001; Yuan et al., 2003; Kitagawa et al., 2004). Those results suggested that greater O_2 tension during culture is detrimental for embryo development, probably due to the accumulation of reactive oxygen species (ROS).

Currently, the majority of culture systems for IVP embryos utilized SOFaaci medium with 5% O_2 (Lonergan et al., 1999; Hashimoto et al., 2000; Van Soom et al., 2002; Ali et al., 2003; Luciano et al., 2005). This system eliminates the requirement for co-culture, but, increases manipulation requirements by removing cumulus cells after IVF, exposing the zygotes to a stress that could also affect their developmental potential (Holm et al., 1999).

SOFaaci medium can be used in greater O_2 tension-environments without removing cumulus cells after IVF improving blastocyst development (Pereira et al., 2005). Cumulus cells which are kept with the zygote after IVF, therefore might be functioning as a scavenger by removing toxic substances from the culture medium (Fujitani et al., 1997). Therefore, oxidative stress caused by greater O_2 tension could be minimized by the presence of these cells, in such a way that the embryo production would be similar in both systems (Lonergan et al., 1999; Wrenzycki et al., 2001; Fatehi et al., 2005). The present study compared two culture systems, which employ different O_2 tension, evaluating the *in vitro* production of bovine embryos and their quality.

2. Materials and methods

2.1. Chemicals

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated.

2.2. Oocyte recovery and in vitro maturation (IVM)

Ovaries from crossbred cows (*Bos indicus* × *Bos taurus)* were collected immediately after slaughter and transported to the laboratory in saline solution (NaCl 0.9%) supplemented with penicillin G (100 IU/ml) and streptomycin sulfate (100 μg/ml) at 35 °C. Cumulus oocyte complexes (COC) were aspirated from 2 to 8 mm diameter follicles with a 18 gauge needle and pooled in a 15 ml conical tube. After sedimentation, the COC were recovered and selected by using a stereomicroscope. The greater quality COC, with a homogenous cytoplasm and at least three layers of cumulus cells, were used. The selected COC were washed three times in maturation medium and transferred to a 200 μl drop of maturation medium under silicone oil and incubated for 22 h at 39 °C in 5% of CO_2 in air. The maturation medium consisted of TCM-199 (Invitrogen, CA, USA) supplemented with 10% FCS (v/v) (Invitrogen, CA, USA), 24 IU/ml of LH, 0.01 IU/ml of FSH and antibiotics (100 IU/ml of penicillin and 50 μg/ml of streptomycin), with 5% CO_2 in air at 39 °C.

2.3. In vitro fertilization (IVF)

For IVF, COC were separated in groups of 25–35, washed and transferred to the 200 μl drop of fertilization medium. Fertilization medium used was TALP (Parrish et al., 1988) supplemented with penicillamine (2 mM), hypotaurine (1 mM), epinephrine (250 mM) and heparin (10 μg/ml). Frozen semen from one ejaculate of a single superior fertility bull was used for all treatments and replicates. Motile spermatozoa were obtained by the Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradient method (Parrish et al., 1995) and were added into the fertilization drop in a final concentration of 1×10^6 spermatozoa/ml. Spermatozoa and oocytes were co-incubated for 22 h at 39 °C with 5% of CO_2 in air, being the day of *in vitro* insemination considered as D0.

2.4. In vitro culture

After co-incubation, the presumptive zygotes were washed in embryo culture medium and randomly distributed into the culture systems with different O_2 tension. For both systems, the culture medium was SOFaaci (Holm et al., 1999), supplemented with 2.77 mM of myo-inositol and 5% FCS. In the system with greater O_2 tension (High O_2) the zygotes were removed from the IVF drop and placed in PBS without calcium and magnesium supplemented with 0.03% BSA, while the other group was prepared. Then, they were washed and transferred to a 200 μl drop of SOFaaci media and cultured at 39 °C under an atmosphere of 5% CO_2 in air. In the lesser O_2 tension system (low O_2) the zygotes were placed in PBS without calcium and magnesium and denuded by repeated pippeting. After denudation the zygotes were washed and transferred to 200 μl drops of SOFaaci medium and cultured at 39 °C in an atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Embryos were evaluated on D2 post-insemination (pi) for cleavage and D6 and D7 pi for blastocyst rates. Only grade 1 and 2 blastocyst were considered for all evaluations. Embryo quality was graded according to the IETS guidelines (Robertson and Nelson, 1998).

2.5. Determination of total cell number

Total cell number of D7 blastocysts was determined according to the protocol describe by Vajta and collaborators (2000). Briefly, blastocysts were placed in sodium citrate solution (0.9%) for 5–10 min, then fixed in etanol:acetic acid:water (3:2:1). After air dried the slides they were fixed again in ethanol:acetic acid (3:1) for 12 h. Fixed embryos were stained with a 2% acetic orcein for 10 min. The cell number was determined under a light microscope (100×).

2.6. Determination of the relative abundance of gene transcripts

The abundance of transcripts for genes related to oxidative stress, manganese-superoxide dismutase (Mn-SOD), catalase, gluthatione peroxidase (GPX) and related to embryo quality, interferon-τ (IFN-τ) were evaluated using a semi-quantitative RT-PCR (Siebert and Larrick, 1992; Jang et al., 2005; Arnold et al., in press). Total RNA was isolated from seven pools of 15 embryos, per treatment, using Trizol Reagent (Invitrogen, CA, USA) according to the manufacturer's protocol, with modifications. Briefly, 100 μl of Trizol and 25 μg of glycogen per pool of embryos was used. Following, the samples were treated with 0.5 IU DNAse (Amersham Biosciences AB, Uppsala, Sweden) and were immediately used for reverse transcription (RT) that was conducted in a total volume of 20 μl using 0.5 μg oligo (dT) primers (Invitrogen, CA, USA). The reaction mix consisted of 200 μM of each dNTP (Invitrogen, CA, USA), 1× RT buffer, 2 μl DTT 0.1 M, 40 IU RNase inhibitor (Invitrogen, CA, USA), and 200 IU SuperScript II (Invitrogen, CA, USA). The RT reaction was performed at 42 °C for 52 min, with a final incubation at 70 °C for 15 min. Polymerase chain reaction (PCR) was performed using 1 μl cDNA, 2 IU Taq DNA polymerase (Invitrogen CA, USA), 0.5 μM of each specific primer, 200 μM of each dNTP, 2.0 mM $MgCl_2$, 1× PCR buffer, using a PTC-100 thermocycler (MJ Research, Watertown, MA). The sequences of the primers and size of amplified products for each gene are presented in Table 1. The PCR program used an initial step of 93 °C for 5 min following 29 cycles (GPX, Mn-SOD, interferon-τ), 31 cycles (gapdh), 33 cycles (catalase) at 93 °C for 40 s, 54 °C for 40 s, and 72 °C for 1 min. The final incubation was done at 72 °C for 5 min. The exponential phase of the PCR was determined by using the same reaction conditions as described above testing 20–35 cycles for each gene (data not shown). As negative control, reactions were performed using total RNA in PCR or omitting reverse transcriptase during RT. After amplification, the amplicons were eletrophoresed in the same 1.5% agarose gel, stained with ethidium bromide (10 mg/ml), and photographed under UV illumination. All amplicons were run in the same agarose gel, and the amount of gene expression was quantified by densitometry using ImageJ Software version 1.36b, National Institutes of Health, USA. The relative quantities of mRNA of each gene were determined by the $IOD_{specific\ gene}/IOD_{gapdh}$ ratio, where IOD is the integrated optical density.

2.7. Statistical analysis

The data for cleavage and blastocyst rates were evaluated by the chi-square analysis and the data for total cell number were examined by analysis of variance Prophet program, version 5.0 (1996). Gene expression data were compared by using the *t*-test or Mann–Whitney rank sum

Table 1
Primers sequences and size of amplicons for each specific gene used in the gene expression evaluation

Gene	Sequences	Amplicons size (bp)
Catalase		
Sense	5′-GTTCGCTTCTCCACTGTT-3′	454
Antisense	5′-GGCCATAGTCAGGATCTT-3′	
Mn-SOD		
Sense	5′-CCCATGAAGCCTTTCTAATCCTG-3′	307
Antisense	5′-TTCAGAGGCGCTACTATTTCCTTC-3′	
Interferon-τ		
Sense	5′-GCCCTGGTGCTGGTCAGCTA-3′	564
Antisense	5′-CATCTTAGTCAGCGAGAGTC-3′	
GPX		
Sense	5′-CGCCGAGTGTGGTTTAC-3′	315
Antisense	5′-AGGTCCTTCTCTATCACCAG-3′	
GAPDH		
Sense	5′-CCCATCACCATCTTCCAGG-3′	471
Antisense	5′-AGTGAGCTTCCCGTTCAGC-3′	

test, performed with the Prophet program, version 5.0 (1996). The results are expressed as the mean ± S.E.M. P values <0.05 were considered significant.

3. Results

3.1. Embryo development

A total of 1118 COC were used in a seven replicate experiment. Cleavage and blastocyst rates were evaluated at D2, D6 and D7 post-insemination (pi) or both systems, and results are presented in Table 2. No differences ($P > 0.05$) between treatments were observed in the cleavage rate. The culture system with less O_2 tension had a stimulatory effect on the extent of embryo development having a greater ($P < 0.05$) percentage of embryos at the blastocyst stage on D6 than the system with greater O_2 tension. However, when D7 embryos were evaluated the blastocyts rate (Table 2) and the total cell number (Table 3) were similar ($P > 0.05$) for both systems.

Table 2
Cleavage (D2) and blastocyst rates at D6, D7 for embryos cultured in SOF medium with cumulus cells under atmosphere of 5% CO_2 in air (high O_2) and embryos cultured in SOF medium under an atmosphere of 5% O_2 (low O_2)

Treatment	N	Cleavage (%)	Blastocyst (%)	
			D6	D7
High O_2	570	441 (77.4) a	125(21.9) a	254 (44.5) a
Low O_2	548	438 (79.9) a	205(37.4) b	258 (47.1) a

Different letters (a and b) in the same column differ statistically ($P < 0.05$) by chi-square test.

Table 3
Total (mean ± S.E.M.) cell number and cells at metaphase II of D7 embryos cultured in cultured in SOF medium with cumulus cells under atmosphere of 5% CO_2 in air (high O_2) and embryos cultured in SOF medium under an atmosphere of 5% O_2 (low O_2)

Treatment	*N*	Total cell number	*N*	Number of cells at metaphase
High O_2	44	175.6 ± 54.1 a	54	5.9 ± 3.1 a
Low O_2	50	160.6 ± 44.0 a	61	5.0 ± 2.8 a

Different letters (a and b) in the same column differ statistically ($P < 0.05$) by the analysis of variance.

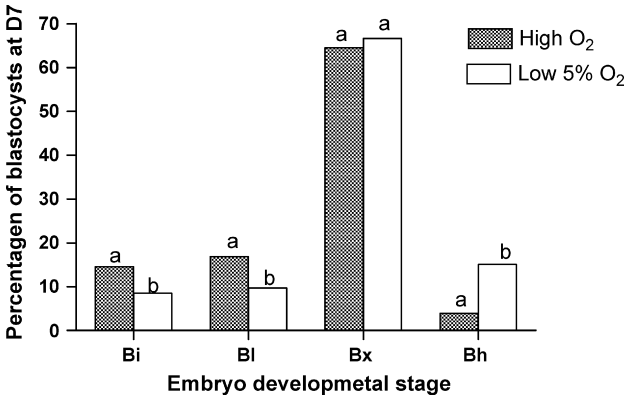

Fig. 1. Developmental stages at D7 of bovine embryos cultured in SOF medium with cumulus cells under atmosphere of 5% CO_2 in air (high O_2) and embryos cultured in SOF medium under an atmosphere of 5% O_2 (low O_2). Bi: initial blastocyst, Bl: blastocyst, Bx: expanded blastocyst, Bh: hatched blastocyst. Different letters (a and b) above bars indicate differences between treatments for each embryo developmental stage ($P < 0.05$).

Indeed when embryos were categorized in the various stage of development, the majority of the embryos on D7 were expanded blastocysts in both systems, and no difference ($P > 0.05$) in the percentage of embryos that reached such stage was observed between treatments (Fig. 1).

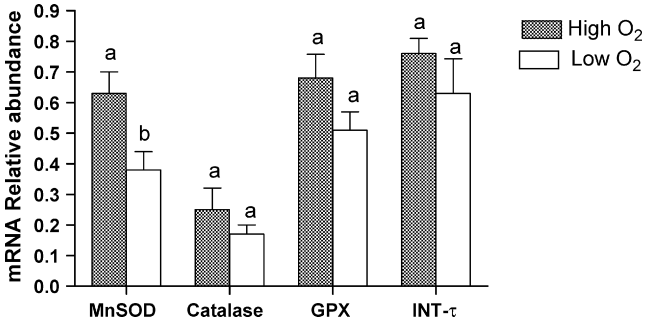

Fig. 2. Relative quantity of transcripts for the genes evaluated by semi-quantitative RT-PCR in D7 embryos culture in different system with greater and lesser O_2 tension. Different letters (a and b) over the bars, for each gene indicate differences ($P < 0.05$).

3.2. Gene expression

The relative abundance of each gene transcript studied is depicted in Fig. 2. Embryos cultured in the system using greater O_2 tension had a greater expression for Mn-SOD gene. Although the amount of transcript for GPX was similar for both treatments ($P > 0.05$), a tendency ($P = 0.0973$) for this gene to be expressed to a greater extent in embryos cultured in the greater O_2 tension system was noted. No change in transcript abundance between the two culture systems was observed for catalase and IFN-τ genes (Fig. 2).

4. Discussion

A suitable culture system which provides a greater *in vitro*-development of embryos with greater quality became an important concern for the commercial application of this technology. Oxidative stress during culture has been emphasized as one of the main factors responsible for the lesser production and poor quality of *in vitro*-produced embryos. It has been suggested that environmental factors such as exposure to light and greater O_2 tension, during culture, induce changes in embryo metabolism leading to an imbalance in the production and degradation of ROS (Goto et al., 1993; Kitagawa et al., 2004; Dalvit et al., 2005). This modification would cause oxidative stress that can alter important cellular functions including control of expression of several genes (Mouatassim et al., 1999), consequently compromising embryo quality and the results obtained with this technique.

Currently the majority of IVP laboratories culture *in vitro*-produced embryos under an atmosphere of 5% O_2. However, based in results from our laboratory, we hypothesized that the detrimental effects of greater O_2 tension during culture can be minimized if cumulus cells are used in co-culture systems. To test this hypothesis, we compared the culture system used in the present study with a system that uses the same medium but no co-culture and less O_2 tension (5%).

The data indicate that cleavage and blastocyst rates at D7 were similar for all treatments (Table 2). Similar results were reported by Khurana and Niemann (2000) and Booth et al. (2005), who found no difference in blastocyst rate when culture was performed under 5 or 20% of O_2. However, the majority of the studies report a deleterious effect of the greater O_2 tension, showing an increase in embryo development when culture is performed at 5% O_2 (Takahashi et al., 2000; Yuan et al., 2003; Kitagawa et al., 2004; Petersen et al., 2005). The authors suggested that a lesser O_2 tension would decrease ROS formation and consequently diminished oxidative stress leading to greater embryo development. Conversely, development to the blastocyst stage was unaffected when oxygen concentration was decreased to 2% during the post-compaction period (Harvey et al., 2004, 2006). However, in both previous studies the embryos were subjected to changes in O_2 tension after D5 of culture. Therefore, the embryos in these previous studies were subjected to 7% O_2 during the majority of the period of *in vitro* development, especially during the critical period of maternal zygotic transition. In these previous studies, the beneficial effect of the lesser oxygen conditions (2%) during the entire culture period on blastocyst rate could not be evaluated.

The similar results in the present study between culture systems with greater and lesser O_2 tension could be explained by the co-culture with cumulus cells that remained attached to the zygote after IVF. It is well established that somatic cells have a beneficial effect on the development of bovine embryos, mainly because of secretion of embryotrophic factors (Reed et al., 1996; Geshi et al., 1999; Holm et al., 1999). In addition, these cells also play a role in removing embryotoxic substances from medium by secreting neutralizing agents such as antioxidant and chelants of

heavy metals (Donnay et al., 1997). These cells also have an important role in the production of glutathione (GSH) (Luciano et al., 2005) by enhancing the stimulatory effect of cysteine and cystine (Luberda, 2005). Glutathione is the major non-protein sulphydryl compound present in mammal's cells that participates in protection against oxidative stress, therefore, the involvement of the cumulus cells in GSH production confirms the role of these cells as a scavenger of ROS.

The number of embryonic cells have been extensively used as an indicator of embryo quality (Khurana and Niemann, 2000; Lequarre et al., 2003; Pereira et al., 2005). In the present study, the total cell number of the *in vitro* produced embryos by the two systems was similar (Table 3), suggesting that the quality of the embryos produced also was not affected by the culture conditions. It is possible that the presence of the cumulus cells contributed to minimizing oxidative stress caused by the greater O_2 tension which could affect embryo quality.

The other approach we used to evaluate embryo quality was the expression of IFN-τ gene. The abundance of transcripts for this gene has been used as a marker for embryo quality (Rizos et al., 2003, 2004), because variation in expression was observed in *in vitro*-produced embryos (Wrenzycki et al., 1999; Rizos et al., 2003). In addition, differences in amounts of transcripts for the gene are observed when *in vitro*- and *in vivo*-cultured bovine embryos are compared (Lonergan et al., 2003b; Rizos et al., 2004; Wrenzycki et al., 2001). In the present study, no differences were observed in amount of transcript for this gene between embryos produced in the two systems, which confirms the similar embryonic quality.

Considering that embryo protection against ROS relies, in part, on the activity of antioxidant enzymes (Harvey et al., 1995) and that the gene expression for those enzymes can be induced by oxidative stress, we evaluated the expression of genes coding for Mn-SOD, GPX and catalase. Embryos cultured in the greater-O_2 tension system had a greater expression of the Mn-SOD gene. Although not statistically different, the amounts of transcripts for GPX ($P = 0.0973$) showed a tendency to be more abundant in those embryos. However, the expression of catalase gene was not different in embryos from the two culture systems assessed in the present study. Several antioxidant enzymes are involved in the protection of embryos against oxidative stress. Mn-SOD, which is located in the mitochondria, allows superoxide radicals to be scavenged. This enzyme in involved in the first enzymatic pathway that protects cells against toxic oxygen radicals and its by-product, hydrogen peroxide (H_2O_2), is then eliminated by catalase or GPX (Guérin et al., 2001). In contrast to catalase, which is effective only against H_2O_2, GPX has a greater spectrum of action because it also reduces lipid hydroperoxides (Limaye et al., 2003). This suggests that GPX has a pivotal role in cell antioxidant protection (Guérin et al., 2001). The activity of the GPX is more beneficial for oocytes than catalase activity (Remacle et al., 1992; Mouatassim et al., 1999).

Expression of the Mn-SOD gene *in vitro* embryo production can change according to culture conditions (Lequarre et al., 2001). In addition, the greater gene expression, as shown in the present study, has been associated with greater embryo quality (Rizos et al., 2002, 2003, 2004; Lonergan et al., 2003a). Indeed, greater abundance of the transcript for Mn-SOD but not for Cu/Zn-SOD, catalase or GPX was related to a reduction of number of apoptotic cells as well as reduction of atretic follicles in mouse ovaries, showing that it is related to cell quality (Tilly and Tilly, 1995).

Considering this information together, we can ascertain that in the present study embryo culture in greater oxygen tension system, probably resulted in greater ROS production than in the lesser O_2 culture system, which induced a greater expression of the Mn-SOD gene and a tendency for increased GPX gene expression by the embryos. However, the stress was not severe enough to cause a change in the main enzymatic antioxidant system because the amounts of catalase transcript were similar, with neither affecting blastocyst production nor embryo quality, as measured by cell

number and IFN-τ expression. The presence of cumulus cells during culture probably had a role in attenuating the ROS production due to the greater O_2 tension. The greater expression of the Mn-SOD gene indicated that these embryos had the ability and the plasticity for protection against the adverse culture conditions, without compromising developmental potential and quality.

5. Conclusion

Based on the results of the present study, we conclude that culture of bovine embryos can be performed either in SOF medium under greater O_2 tension in the presence of cumulus cells or in SOF medium under lesser O_2 tension, without affecting embryo production and quality.

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