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Oxygen tension in IVM and IVF of bovine oocytes: effect on embryonic development and pregnancy rate

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Abstract

In order to evaluate the effect of O_2 tension on the *in vitro* maturation (IVM) and fertilization (IVF) process, 11 replicates of OPU/IVP were performed using 1092 viable oocytes obtained from 48 *Bos indicus* embryo donors of commercial herds. Cumulus-oocyte complexes (COCs) from each donor were homogeneously and randomly distributed across two treatments. The IVM was performed in TCM-199 with ovine FSH, ovine LH, porcine estradiol, human EGF, porcine insulin, and FCS. The IVF was performed in Fert-TALP medium with heparin and PHE using Percoll–gradient-selected spermatozoa from a *Bos indicus* bull. During IVM and IVF, the oocytes were incubated in 5% CO_2 in air (Group 20% O_2) or 5% CO_2, 5% O_2, and 90% N_2 (Group 5% O_2) for 18-24 h at 39ºC. The embryos were incubated in Synthetic Oviduct Fluid (SOF) with aminoacids and citrate at 39ºC for 6-8 days with 5% CO_2, 5% O_2, and 90% N_2. A number of COCs (n = 187) were evaluated until Day 9 when hatching rates were recorded. For the remaining embryos produced on Day 7 (n = 905), pregnancy rate was evaluated after the embryo transfer. There were no differences ($P < 0.05$) in the cleavage rate (69.6% and 70.4%), embryo development rate (37.3% and 38.4%), or Quality-I blastocyst percentage on Day 7 (30.8% and 30.4%) between the 5% O_2 and 20% O_2 groups, respectively. Considering the number of matured oocytes, the percentage of hatching blastocysts on Day 9 was greater in the 5% O_2 group compared to the 20% O_2 group (21.3% vs. 10.8%, respectively). The pregnancy rates were similar ($P = 0.15$) between 5% O_2 (25.8%; 34/132) and 20% O_2 (33.6%; 49/146) groups. The results of this study indicate the possibility of using a 5% oxygen tension during the entire IVP procedure for cattle.

Keywords: OPU, oxygen tension, *in vitro* maturation, *in vitro* fertilization, *Bos indicus.*

Introduction

The technology of *in vitro* production (IVP) of bovine embryos has been in constant development and has recently yielded more consistent results, thus making its commercial application a reality. However, pregnancy rates are still inferior to those of embryos obtained *in vivo* (Merton *et al.*, 2003). An important aspect to consider is protocol differences of IVP when working with oocytes obtained from a slaughterhouse compared to those obtained via ultrasound-guided transvaginal follicle aspiration (OPU). Differences in the methods of collection, selection of oocytes, including morphological quality and number of oocytes or embryos, hormonal status of the donor, and bulls used should be taken into account.

Embryonic development is affected by the quality of the aspirated oocytes and by the conditions of IVM and IVF. A deficient IVM, especially cytoplasmic maturation, is responsible for the low rates of blastocyst formation (Oyamada and Fukui, 2004). The gaseous atmosphere normally used in IVM is 5% CO_2 in air; the concentration of oxygen is not controlled but is close to the atmospheric level (20%). The concentration of oxygen in the reproductive tract of bovine females is approximately 5% (Mastroianni and Jones, 1965; Bavister, 1995). High levels of oxygen (20%) are toxic for different types of mammalian cells, including oocytes and spermatozoa, probably due to the formation of free radicals which cause severe cell damage by oxidation, enzyme inactivation, and DNA damage (Umaoka *et al.*, 1992). In *in vitro* culture (IVC) of bovine embryos, the tension of 5% O_2 results in better embryonic development rates as well as embryo quality (Gordon, 2003). Growth factors or antioxidants have been used during IVM and IVC in order to prevent the effects of the oxidative stress (Alli *et al.*, 2003; Oyamada and Fukui, 2004). Oxygen tension in follicular fluid decreases as the follicle grows (McNatty, 1978), suggesting that the low concentration of oxygen (5%) can be beneficial to oocyte maturation.

In mice, the atmosphere of 5% O_2 in the IVM has no deleterious effect on oocyte development (Eppig and Wigglesworth, 1995; Adam *et al.*, 2004). However in pigs, the quality of embryos produced with IVM oocytes at 5% O_2 was superior to those produced using 20% O_2 (Kikuchi *et al.*, 2002). De Matos *et al.* (1996) reported faster development until the blastocyst stage (6 days) with IVM oocytes at 5% O_2 and suggested a higher

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resistance of oocytes and embryos to cryopreservation produced in this system. The reduction to 5% O_2 during IVM resulted in slow maturation (Caiado *et al.*, 2003), whereas Azambuja *et al.* (1993) reported higher rates of morulae and blastocyst formation at 20% O_2 compared to 5% O_2. Hashimoto *et al.* (2000) concluded that IVM at 5% O_2 is beneficial to bovine oocytes. Kruip *et al.* (2000) reported the IVM at 20% O_2 is harmful to embryonic development, and Siqueira-Pyles *et al.* (2004) produced IVM embryos after oocyte transportation in 5% O_2 gasified tubes.

Unlike the 5% CO_2 in air atmosphere, O_2 tension is not usually controlled during IVF. There are a few studies regarding the effect of O_2 concentration on the success of IVF and the production of blastocysts in mice and humans (Dumoulin *et al.*, 1995). It has been shown that the reduction of O_2 during IVF was beneficial to cattle embryonic development (Lazzari *et al.*, 1998). The concentration of 5% O_2 was used successfully for bulls with low rates of fertility in conventional protocols (20% O_2; Galli *et al.*, 2001). Takahashi and Kanagawa (1998) did not detect any difference in fertilization rates; however, embryonic development was superior in the group in which IVF was conducted in a 5% O_2 atmosphere.

The goal of this study was to evaluate the influence of atmospheres of 5% O_2 (5% CO_2, 5% O_2, and 90% N_2) compared to 20% O_2 (5% CO_2 in air) during IVM and IVF of bovine oocytes obtained by OPU on the production and quality of embryos and pregnancy rates after embryo transfer.

Materials and Methods

Recovery of cumulus-oocyte complexes (COCs)

Fourteen, cycling Brahman heifers between 13 and 18 months old, kept on pasture (*Brachiaria decumbens*), and supplied daily with sorghum silage *ad libitum* served as oocyte donors during the period between June and September 2004 in Uberaba, MG, Brazil. The heifers underwent a minimum of 2 and a maximum of 4 OPU procedures at intervals of 15 days. A total of 48 sessions of OPU-IVP were carried out on 11 different days, and 1,092 viable COCs for IVM were collected. An ultrasound scanner Aloka SSD500 (Aloka Co. LTD, Mure 6-22-1, Mitaka, Tokyo 181-8622, Japan) equipped with a 5 MHz probe (UST Aloka 9111) connected to a transvaginal guide was used for OPU. Follicle aspiration was performed with an 18-gauge needle 55cm length (Vopal 1855-Cook Company-Queensland 4113, Australia) connected to an aspiration system (Cook Co. LTD-Vopal 1800) and vacuum pump (Cook Co. - VMAR 500) set to a flow of 15 ml of medium per minute. The COCs were kept in 45-ml tubes in PBS (Nutricell, Campinas, Brazil) with 1% fetal calf serum (FCS) and 5 UI heparin/ml. The content of OPU was cleaned in the same solution, filtered out, and after identification and classification under a stereomicroscope, Quality-I, -II, or -III COCs (De Loos *et al.*, 1989) were considered viable. Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Experimental design

The oocytes from each donor were equally distributed across two groups taking into account oocyte number and quality. In vitro maturation and the IVF were performed in 5% CO_2 in air (Group 20% O_2 = control) or in 5% CO_2, 5% O_2, and 90% N_2 (Group 5% O_2).

In vitro maturation

The maturation was performed with 10 to 20 COCs in 100-µl drops of TCM-199 to which 10% FCS, 5µg/ml ovine FSH, 50 µg/ml ovine LH, 1µg/ml porcine estradiol, 100 µg/ml hEGFr, 6.25 µg/ml swine insulin, 22 µg/ml pyruvate, and 1µg/ml gentamicin were added. The medium was covered in mineral oil in Petri dishes (35x15mm) and kept in an incubator for 24 h at 39°C in saturated humidity and a gaseous atmosphere of 5% CO_2 in air or 5% O_2 in air, as described above.

In vitro fertilization

After the period of IVM was over, the COCs were transferred to 100-µl drops of Fert-TALP medium (Parrish *et al*., 1986) to which 22 µg/ml pyruvate, 6 mg/ml BSA, 10 µg/ml heparin, 20 µM penicillamine, 10 µM hypotaurine, and 2 µM epinephrine were added. Frozen semen from two Bos indicus bulls was used for IVF. For this procedure, all COCs were exposed to spermatozoa from only one bull for fertilization within each replicate. The spermatozoa selected by Percoll gradients of 90, 60, and 30% were prepared at a final dose of 2 x 10^6 spermatozoa/ml. The day of IVF was considered Day 0. Spermatozoa and oocytes were incubated for 18 to 24 h at 39°C in saturated humidity and a gaseous atmosphere of 20% O_2 in air or 5% O_2 in air.

In vitro culture

Presumptive zygotes underwent successive aspirations to extract cumulus oophorus cells. These were immediately transferred to 100-µl drops of SOFaaci medium with 4 mg/ml of BSA (Holm *et al.*, 1999) under mineral oil in Petri dishes and stored for 6 to 8 days in incubator at 39°C in saturated humidity and a gaseous atmosphere of 5% CO_2, 5% O_2, and 90% N_2.

Embryo evaluation and transfer

The effect of oxygen tension during IVM and IVF was evaluated *in vivo* and *in vitro* on Days 2, 7, 9, and 45 after IVF (cleavage, blastocyst, expanded or hatched blastocyst, and pregnancy, respectively). A total of 1,092 oocytes were selected to be in vitro matured. Part of the oocytes (n = 187) had their development followed

until Day 9 for hatching evaluation. The remaining oocytes (n = 905) were evaluated for morphology on Day 7 (International Embryo Transfer Society - IETS, 1998). The blastocysts were loaded into 0.25-ml straws with TCM-Hepes medium and 10% FCS for transport in thermal boxes at room temperature (25-30°C) as far as the location of the transfer. Embryo transfer was performed within 8 h after embryo evaluation to recipients on Day 6 to Day 8 after estrus. Estrus synchronization was facilitated using prostaglandin-$F_{2\alpha}$ or progesterone + estradiol benzoate. Estrus was confirmed by observation of estrous behavior. Pregnancy diagnosis was carried out via rectal examination and confirmed by ultrasound 45 days after transfer. The pregnancy rate was assessed considering the number of transferred embryos as well as the total number of viable, mature COCs.

Statistical analyses

The percentages of cleaved embryos, blastocysts and hatched blastocysts related to the number of oocytes were analyzed with GLM procedure (SAS, 1998) after arcsine square root transformation. The effect of replicate was maintained in the model to evaluate the blastocyst rate. Due to the effect of treatment itself, the number of blastocysts present at Day 9 was not homogeneously distributed among replicates. Also, the number of transferred embryos from each replicate was not the same. Thus, Chi-square test was used to compare hatched/Day 9 blastocysts and pregnancy rates rather than a variance analysis. A probability of $P < 0.05$ was considered significant.

Results

The percentage of oocytes that cleaved and embryonic development on Day 7 of IVC were similar between both experimental groups. The morphological quality of Day 7 blastocysts as well as Quality-I blastocysts in the IVC was not influenced by the treatment (Table 1).

Table 1. Percentage of cleaved oocytes, blastocysts, and Quality-I blastocysts on Day 7 produced with cumulus-oocyte complexes of *Bos indicus* cows collected by OPU and matured and fertilized *in vitro* in a 5% or 20% O_2 atmosphere.

IVM and IVF atmosphere	Oocytes (n)	Cleavage (%)	Day 7 embryos	
			Blastocysts (%)	Quality 1 (%)
5% O_2	448	69.6	37.2	30.8
20% O_2	457	70.4	37.4	30.4

There was no difference between treatments ($P > 0.05$).

The number of blastocysts that hatched on Day 9 of culture, considering the number of oocytes (n = 187), was greater ($P < 0.05$) in the 5% O_2 group whereas Day 9 blastocyst hatching was not different between groups (Fig. 1). There was no difference in pregnancy rates (Fig. 2) between 5% and 20% O_2 atmospheres considering the number of matured COCs (n = 905) and transferred embryos (n = 278).

Results of the IVM and IVF in a 5% or 20% O_2 atmosphere were grouped by donor (Table 2) in order to verify a possible relationship between *in vitro* and *in vivo* performance.

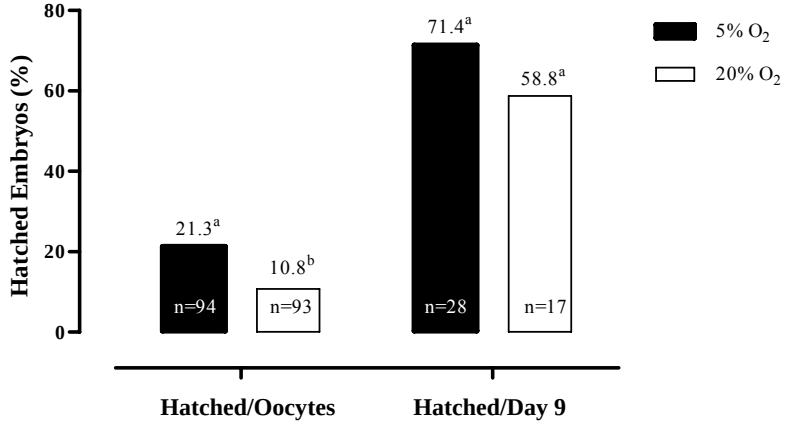

Figure 1. Bovine blastocysts hatched percentage from cumulus-oocyte complexes obtained from *Bos taurus indicus* cows collected by OPU and *in vitro* matured and fertilized in a 5% or 20% O_2 atmosphere. [a,b]Values differ significantly; $P < 0.05$.

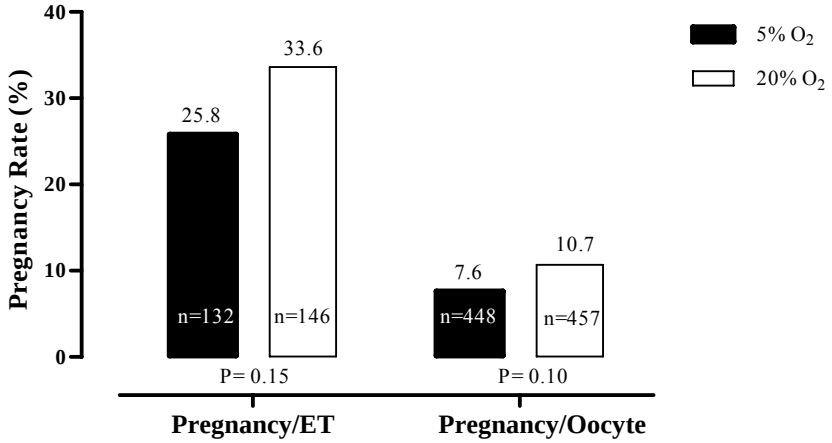

Figure 2. Pregnancy rate at 45 days after embryo transfer of bovine blastocysts obtained from *Bos taurus indicus cows* collected by OPU/IVM and submitted to IVF in a 5% or 20% O_2 atmosphere. ET = embryo transfer.

Table 2. Oocyte cleavage, blastocyst development, and Quality-I blastocyst percentages of Day 7 embryos produced with cumulus-oocyte complexes (COCs) obtained from 10 *Bos indicus* cows and collected by OPU and matured and fertilized *in vitro* in a 5% or 20% O_2 atmosphere.

Donor	Replicates (n)	COC (n)	Cleavage (%)	Day 7 blastocyst (%)	Day 7 Quality-I blastocyst (%)	Pregnancy rate (n/n;%)
A	4	111	95 (85.6)	60 (54.1)	54 (48.6)	16/56 (28.6)
B	4	116	106 (91.4)	63 (54.3)	53 (45.7)	19/52 (36.5)
C	2	92	74 (80.4)	50 (54.3)	39 (42.4)	13/28 (46.4)
D	2	51	38 (74.5)	27 (52.9)	20 (39.2)	9/20 (45.0)
E	3	159	94 (59.1)	31 (19.5)	23 (14.5)	2/11 (18.2)
F	3	48	39 (81.3)	22 (45.8)	9 (18.8)	3/17 (17.6)
G	2	58	32 (55.2)	21 (36.2)	17 (29.3)	8/18 (44.4)
H	2	123	51 (41.5)	31 (25.2)	22 (17.9)	7/22 (31.8)
I	3	92	82 (89.1)	34 (37.0)	22 (23.9)	4/23 (17.4)
J	2	48	37 (77.1)	22 (45.8)	15 (31.3)	2/16 (12.5)

Discussion

It is known that the O_2 tension (5%) in the female reproductive tract is lower than that of the atmospheric air (20%). Similar O_2 concentrations to that of atmospheric air can cause damage to mammalian cells, including oocytes, spermatozoa, and embryos (Mastroianni and Jones, 1965; Umaoka *et al.*, 1992). During IVM, no routine control of O_2 tension is carried out, and it remains around 20%. Although IVM is a very important stage to subsequent embryo development, few studies have been done that examined the effect of O_2 tension reduction during IVM to levels close to those found in the bovine reproductive tract. In this study, the reduction of oxygen tension during IVM and IVF from 20% to 5% did not impair embryonic development, and the oocyte cleavage and blastocyst development on Day 7 were similar between experimental groups. Azambuja *et al.* (1993) and Caiado *et al.* (2003) reported that 20% O_2 was beneficial for IVM, whereas other groups (Hashimoto *et al.*, 2000; Kruip *et al.*, 2000; Siqueira-Pyles *et al.*, 2004) had successful results with a low (5%) concentration of O_2.

The oocytes obtained via OPU usually have few cumulus-oophorus cell layers, and this can compromise maturation and embryo development (Gordon, 2003). In cattle, these cells are important due to their contribution to male pronucleus formation and subsequent oocyte development (Chian *et al.*, 1994). To verify this, Konishi *et al.* (1996) demonstrated the beneficial effects of granulosa cells in IVM medium on the quality of aspirated oocytes with 4 or less layers of cells. In research programs, the IVP protocols are performed with oocytes selected according to quality, either I or II, matured and fertilized in groups of 20 to 40 oocytes/drop and in a 20% oxygen atmosphere. Those oocytes have a large amount of cells from the cumulus oophorus, which can reduce the concentration

of O_2 (Konishi *et al.*, 1996). Our study was carried out with Quality I, II, and III oocytes obtained by OPU and matured in groups of 10 to 20 oocytes/drop. With OPU oocytes, the reduced amount of cells from the cumulus-oocyte complexes and a lower density of oocytes per volume of medium may result in different oxygen concentrations when compared to the conditions usually provided to oocytes from slaughterhouse cows. The 5% oxygen tension might be beneficial to IVM and IVF of OPU oocytes similar to what occurs in conventional IVP with Quality I and II oocytes, which have a greater number of layers of cumulus-oophorus cells and are usually kept in a high (20%) oxygen tension.

When Pinyopummintr and Bavister (1995) evaluated the effect of the atmospheric conditions on IVM and IVF in cattle, maturation with 20% O_2 resulted in a high rate of oocytes in Metaphase II compared to 5 and 10% O_2. When the effect of O_2 (5, 10, and 20%) was studied only during IVF, fertilization occurred appropriately and was higher (71%) in the 20% O_2 atmosphere. Oyamada and Fukui (2004) concluded that the addition of glucose was beneficial during IVM in low concentration of oxygen. In this study, glucose was not used in the IVM medium, but we realize that the concentration of 10% of FCS used in the IVM medium might have added glucose, which could have a positive effect on maturation in low O_2 tension. However, high concentrations of glucose could be the source of free radicals (Iwata *et al.*, 1998). In this study, the EGF added to the IVM medium might have contributed to the embryonic development of the IVM/IVF 5% O_2 group. The addition of growth factors such as EGF or antioxidants such as cysteamine, despite not improving nuclear maturation of bovine oocytes, has increased the fertilization rates and embryonic development of IVM, IVF, and IVC oocytes individually maintained in 5% CO_2, 5% O_2, and 90% N_2 (Oyamada and Fukui, 2004).

There was a higher hatching percentage of blastocysts produced with oocytes of IVM and IVF at 5% O_2 (21.3% vs. 10.8%). On the other hand, this difference was not noticed with Day 9 blastocyst hatching rates nor the pregnancy rate after transfer to the recipients, thus indicating no difference in the embryo quality produced in an atmosphere with two different oxygen concentrations. The data collected via *in vitro* evaluation of embryos, such as blastocyst hatching and blastocyst cell number (Gordon, 2003) might have limited application, since there are differences between the *in vitro* and *in vivo* (pregnancy rate) evaluations. In pigs, the quality of blastocysts from IVM oocytes at 5% O_2 was superior to IVM oocytes at 20% O_2 (Kikuchi *et al.*, 2002). The low O_2 tension might be responsible for cytoplasmatic maturation, improving embryonic development by reducing oxidative stress (Kikuchi *et al.*, 2002; Adam *et al.*, 2004), as well as by producing substances involved in stimulating oocyte maturation, such as GSH (Oyamada and Fukui, 2004). In this study, the high percentage of blastocysts that hatched compared to the number of IVM oocytes indicates that embryo development was faster in the IVM and IVF oocytes group at 5% O_2, which suggests that low oxygen tension during maturation and fertilization might have stimulated embryonic development. Although pregnancy and embryonic development rates were not significantly increased, the 5% O_2 tension could be used in IVP programs to improve damage resistance in cryopreserved IVM oocytes and IVP embryos (De Matos *et al.*, 1996), since it is known that embryos which develop faster are also more resistant to freezing.

The reduction of oxygen tension during IVF has not interfered with fertilization rate, but it resulted in superior embryonic development compared to the 20% O_2 group (Takahashi and Kanagawa, 1998). In addition, Lazzari *et al.* (1998) showed the benefits of IVF in 5% O_2 with IVF of bovine oocytes. Galli *et al.* (2001) produced blastocysts by performing IVF in 5% O_2 with spermatozoa of bulls from which it was not possible to produce embryos in a 20% O_2 atmosphere. In this study, fertilization performed in 5% O_2, similar to the process that occurs *in vivo*, did not influence oocyte cleavage and embryonic development. Due to the fact that IVF has not been evaluated separately, but together with IVM in 5% O_2, it remains to be determined whether the oxygen tension affects the maturation and fertilization processes in the same way.

Four donors (A, B, C, and D) achieved the best results *in vitro* yielding high Day 7 blastocyst development rates and Quality-I blastocyst percentage. Three of the donors also had pregnancy rates above 35% (B, C, and D). From the other six donors (E, F, G, H, I, and J), whose percentage of embryo development on Day 7 was less than 50%, only Donor G had a pregnancy rate greater than 35%. A low number of embryos were transferred; therefore, a definitive conclusion cannot be drawn. However, these results highlight the importance of transferring blastocysts obtained from donors with high-quality *in vitro* developed embryos so that pregnancy rate can be maximized.

The results of this study demonstrated the viability of the IVP technique (IVM, IVF, and IVC) when performed in 5% O_2 concentration. Therefore, the use of only one incubator in all stages of IVP is feasible, as well as the possibility of oocyte transportation from the collection site to the IVP laboratory in gasified tubes (Siqueira-Pyles *et al.*, 2004). Other aspects are yet to be investigated, such as oocyte density per medium volume, the use of different energetic sources, and the viability of these IVM oocytes and embryos after cryopreservation. These results have shown the potential of IVM and IVF performed in a 5% oxygen tension given the fact that there was no damage to blastocyst production or to embryo quality and pregnancy rates. Therefore, the use of 5% O_2 throughout the OPU/IVP procedure is a viable alternative to the traditional method.

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