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In vitro production of bovine embryos using Sigma antioxidant supplement®, α-tocopherol and L-ascorbic acid

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Abstract

This study tested the effect of Sigma antioxidant supplement®, α-tocopherol (vitamin E) and L-ascorbic acid (vitamin C) in the culture medium of bovine embryos. In experiment 1, *in vitro* produced bovine zygotes were cultured in Human Tubal Fluid (HTF): Eagle's Basic Medium (BME) with: Group 1 – 50 µm vitamin C; Group 2 – 200 µm vitamin E; Group 3 – 25 µm vitamin C and 100 µm vitamin E; Group 4 – 1 µl/ml Sigma antioxidant supplement®; and the Control group – HTF:BME only. In experiment 2, embryos were cultured in high or low oxygen tension with HTF:BME + Sigma antioxidant supplement® or in HTF:BME alone (Control). The data were analyzed using ANOVA followed by Tukey's test. The results of experiment 1 showed a negative effect ($P < 0.05$) of vitamin E on blastocyst production in Group 2 (19.7 ± 0.1%). This effect was reduced in Group 3 by the addition of vitamin C (26.1 ± 0.2%). The use of vitamin C alone (34.9 ± 0.3%) or the Sigma antioxidant supplement® (33.3 ± 0.7%) did not increase ($P > 0.05$) the number of blastocysts produced compared with the control group (30.1 ± 0.5%). During experiment 2, there was no effect ($P > 0.05$) from the culture medium or the O_2 concentrations used, indicating that the reduction of the O_2 concentration did not improve blastocyst production.

Keywords: antioxidants, bovine embryos, *in vitro* embryo production, vitamin C, vitamin E.

Introduction

For the success of *in vitro* embryo production, several barriers need to be overcome. Optimizing the culture medium to produce an *in vitro* environment similar to the one obtained in the oviduct and uterus (Yuan *et al*., 2003) is an important step toward achieving this goal.

According to the literature, the oxygen concentration in the lumen of the female reproductive tract is between 3 and 7% (Fischer and Bavister, 1993). Therefore, embryos from mice (Umaoka *et al*., 1992), sheep (Thompson *et al*., 1990), cattle (Fujitani *et al*., 1997; Takahashi *et al*., 2000), and humans (Dumoulin *et al*., 1999) cultured *in vitro* under a low O_2 atmospheric concentration (5%) have shown higher developmental rates than those cultured under 20% O_2. This finding indicates that a high O_2 concentration during the *in vitro* culture reduced developmental ability, which may have resulted from the increased accumulation of reactive oxygen species (ROS) in the cytoplasm of developing embryos (Goto *et al*., 1993; Yang *et al*., 1998). The oxidative stress reduces embryo quality and viability because of the peroxidation of membrane lipids and the modification of important molecules, such as proteins and nucleic acids, which results in cellular death through apoptosis (Nasr-Esfahami *et al*., 1990; Van Soom *et al*., 2002).

ROS are formed in the intermediary steps of oxygen reduction during aerobic metabolism, even in basal conditions. The main radicals that originate are superoxide (O_2), hydrogen peroxide (H_2O_2) and hydroxyl (OH^-). Several exogenous factors, such as the presence of dead sperm cells after fertilization, the oxidation of proteins, and the presence of metallic ions, can increase the production of ROS by the embryo in the *in vitro* culture (Goto *et al*., 1993; Harvey *et al*., 2002). The embryo undergoes oxidative stress when the production of ROS is higher than the cells capacity to produce antioxidants (Droge, 2002). Therefore, the balance between ROS and the presence of antioxidants is a key factor in the success of embryonic development (de Lamirande *et al*., 1997).

During the *in vivo* culture, embryos can be protected from oxidative stress by antioxidants produced by the embryo in combination with the ones present in the follicular fluid and the oviductal fluid (Gardiner and Reed, 1995). However, during *in vitro* culture, the embryonic physiological antioxidants production is not enough to prevent oxidative stress (Ali *et al*., 2003), so exogenous antioxidant supplements may be necessary.

The antioxidants that can be added to culture medium can be divided into two large groups: ones with an enzymatic action, such as catalase, superoxide dismutase and glutathione peroxidase; and ones that are non-enzymatic or metabolic with low molecular weight, such as cysteine, β-mercaptoethanol, L-ascorbic acid (vitamin C) and α-tocopherol (vitamin E; Nordberg and Arnér, 2001). Moreover, several commercial solutions

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composed of a mixture of those components can be found Vitamin E is a fat-soluble antioxidant vitamin that suppresses the peroxidation of membrane lipids (Chow, 1991; Guerin *et al*., 2001). The peroxidation of fatty acids is known to inhibit the function of cells and might induce cell death (Spiteller, 2001). In bovine embryos, the addition of vitamin E to the medium improved the developmental competence up to the blastocyst stage. Olson and Seidel (2000) suggested that the supplementation of the culture medium with vitamin E increased bovine embryo development and blastocyst formation due to the inhibition of NADPH oxidase, protecting cell membranes.

Alternatively, ascorbic acid is a water-soluble vitamin that has been considered the most important antioxidant in extracellular fluids (Buettner, 1993; Rose and Bode, 1993). Vitamin C antioxidant effect protects DNA from exogenous oxidation (Fraga *et al*., 1991). In physiologic concentrations (0.3 µm), vitamin C can reduce embryo oxidative stress by inducing the synthesis of hypotaurine and taurine in the rabbit oviduct (Guerin *et al*., 1995, 2001). Adding vitamin C into the culture medium prevents apoptosis in rat and mouse follicles and also improves blastocyst production in mice (Tilly and Tilly, 1995; Eppig *et al*., 2000). Vitamin C acts synergistically with vitamin E under some conditions by regenerating tocopherol from tocopheroxyl radicals, the products of tocopherol and free radical interactions (Chow, 1991).

Considering these results, this experiment hypothesizes that the addition of Vitamin C, Vitamin E or the commercial antioxidant from Sigma® would have a beneficial effect on the number of bovine blastocysts produced in a concentration of 5% CO_2 in air atmosphere (~20% O_2). It also hypothesizes that, independent of the presence of antioxidants, blastocyst production would be higher in an atmosphere with 5% O_2.

Materials and Methods

The present research used 1,510 oocytes divided between 2 experiments. A total of 661 oocytes were used in experiment 1, and 981 oocytes were used in experiment 2. In both experiments, five replicates were performed. Unless otherwise specified, all drugs used were from Sigma (Sigma-Aldrich Corp., St. Louis, MO).

Oocyte collection

Bovine (*Bos taurus indicus*) ovaries were collected at a local slaughterhouse and transported to the laboratory in physiological saline supplemented with 100 U/ml penicillin G at approximately 30-35°C within 2 hours of collection. Cumulus-oocyte complexes (COCs) from follicles with 2-8 mm in diameter were aspirated using an 18-gauge needle attached to a 10 ml disposable syringe. Only oocytes with homogeneous cytoplasm and at least three layers of compact cumulus cells were used.

Oocyte maturation

The selected oocytes (25 - 30) were matured *in vitro* at 38.5°C in four-well dishes containing 400 µl of maturation medium covered with mineral oil. The maturation medium was composed of TCM-199 with Earle's salt and L-glutamine (Gibco 31.100; Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS), 2.2 mg/ml sodium pyruvate, 1 mg/ml 17β estradiol, 50 µg/ml hCG (Profasi®- 5.000 IU, Serono, Barueri, Brazil.), 1 µg/ml FSH (Foltropin-V®, Vetrepharm, Ontario, Canada), and 75 µg/ml gentamicin were added to the wells, and the dishes were placed in an air incubator containing a concentration of 5% CO_2 for 22 to 24 h. At the end of the maturation period, the oocytes were transferred to fertilization drops, and the maturation plate was kept in the incubator for further use during culture.

In vitro fertilization (IVF)

Oocytes were fertilized with a single semen batch from the same bull. Sperm cells were selected by swim up, and the final concentration was adjusted to $1x10^6$ spermatozoa/ml. Fertilization was performed within 18 h in a 90 µl drop of Human Tubal Fluid (HTF - Irvine Scientific, Santa Ana, USA) that was supplemented with 0.5% BSA, 2.2 mg/ml sodium pyruvate, 0.5 mg/ml caffeine and 75 µg/ml gentamicin.

Embryo culture

The presumptive zygotes were denuded and transferred to culture dishes. Basic culture medium (BCM) was composed of HTF:BME (Human Tubal Fluid:Eagle's Basic Medium - 1:1) supplemented with 0.6% BSA, 10% FCS, 0.01% myoinositol and 75 µg/ml gentamicin.

Experiment 1

The same four-well dishes used during maturation were double-washed with BCM before receiving the zygotes in groups of 20 to 25 per 400 µl of culture medium covered with mineral oil. Five experimental groups were tested:

Group 1: BCM + 50 µm of vitamin C (L-ascorbic acid);

Group 2: BCM + 200 µm of vitamin E (α-tocopherol);

Group 3: BCM + 25 µm vitamin C and 100 µm Vitamin E – association in order to maximize the antioxidant activity and a reduced vitamin concentration to avoid an eventual embryotoxic effect of the simultaneous supplementation (Donnelly *et al*., 1999; Wang *et al*., 2002);

Group 4: BCM + 1 µl/ml Sigma antioxidant supplement® (Sigma, A-1345);

Control group: only BCM.

Embryos were cultured for 7 days at 38.5°C with a concentration of 5% CO_2 in air (~20% of O_2) and 100% humidity.

Experiment 2

In experiment 2, the addition of Sigma antioxidant supplement® was tested under two different oxygen concentrations (5 and ~20%).

Group Air: BCM + 1 µl/ml Sigma antioxidant supplement® under an atmosphere of 5% CO_2 in air (~20% O_2);

Group 5% O_2: BCM + 1 µl/ml Sigma antioxidant supplement® under a 5% CO_2, 5% O_2 and 90% N_2;

Control group Air: BCM under an atmosphere of 5% CO_2 in air (~20% O_2);

Control group 5% O_2: BCM under a 5% CO_2, 5% O_2 and 90% N_2.

For 7 days, all of the embryos were kept in four-well dishes in groups of 20 to 25 per 400 µl of culture medium covered with mineral oil. The feeding (partial change in medium) and cleavage analysis were performed on Day 3, and blastocyst formation was checked on Day 7.

Statistical analyses

In both experiments, the percentage of cleavage and blastocyst formation was calculated from the total number of oocytes selected for maturation.

For statistical analysis, the percentage data were transformed using arcsine (arcsin $\sqrt{y/100}$ transformation) and analyzed by ANOVA followed by Tukey's test. The untransformed data are presented in Tables 1 and 2. In both experiments, 5% was used as the significant level ($P < 0.05$). All tests were performed with a GraphPad InStat 3.05 statistical software package (Graph Pad Software Inc., San Diego, CA).

Results

The results of experiment 1 showed that the utilization of Vitamin C, Vitamin E and Sigma antioxidant supplement® did not increase ($P > 0.05$) the cleavage rate of bovine oocytes in comparison with the control group (Table 1). However, the addition of vitamin E alone (Group 2) or in combination with vitamin C (Group 3) was detrimental ($P < 0.05$) to embryo development until the blastocyst stage in comparison to either the use of Vitamin C alone (Group 1) or the use of Sigma antioxidant supplement® (Group 4). Nevertheless, the results of Group 1 and 4 were not significantly different ($P > 0.05$) from the ones observed in the control group (Table 1).

In experiment 2, the results showed that, independent of the O_2 concentration in the atmosphere during culture, the addition of antioxidants did not improve ($P > 0.05$) the cleavage rate and blastocyst formation (Table 2).

Table 1. (Mean (± s.d.) cleavage and blastocyst formation obtained using different antioxidants in the culture media of bovine embryos. Experiment 1.

Group	Antioxidant	N° oocytes	% cleavage	% blastocyst
1	50 µm Vit. C	125	72.0 ± 0.9	34.9 ± 0.3[a]
2	200 µm Vit. E	131	60.4 ± 0.8	19.7 ± 0.1[c]
3	25 µm Vit. C + 100 µm Vit. E	134	72.9 ± 0.1	26.1 ± 0.2[b,c]
4	1 µl/ml Sigma antioxidant ®	134	74.8 ± 0.7	33.3 ± 0.7[a]
Control	-	139	73.7 ± 0.8	30.1 ± 0.5[a,b]

[a,b]Values with uncommon superscript differ $P < 0.05$. Cleavage was analyzed on Day 3 and blastocyst formation on Day 7 of culture.

Table 2. Mean (± s.d.) cleavage rate and blastocyst formation observed on Day 3 and Day 7 of culture in atmospheres with different O_2 concentrations. Experiment 2.

Group	Sigma antioxidant®	O_2 concentration	N° oocytes	% cleavage	% blastocyst
Air	1 µl/ml	~20%	234	74.8 ± 0.7	33.3 ± 0.7
5% O_2	1 µl/ml	5%	275	72.0 ± 0.9	35.2 ± 0.2
Control air[†]	-	~20%	220	66.8 ± 0.9	29.3 ± 0.5
Control 5%O_2[††]	-	5%	252	69.8 ± 0.2	29.0 ± 0.5

Values in the same column did not differ ($P > 0.05$). [†]Atmosphere: 5% CO_2 in air (~20% O_2). [††]Atmosphere: 5% CO_2, 5% O_2 and 90% N_2.

Discussion

Oocytes and embryos produce endogenous ROS by various enzymatic actions during the metabolic process (Riley and Behrman, 1991; Gardner and Lane, 2002; Harvey *et al*., 2002). ROS participate in various cell processes, especially in tissue remodeling, hormone signaling, steroidogenesis and germ cell function at a physiological level. However, when the critical level is overwhelmed, ROS may modify normal cell functions,

endanger cell survival, or both (Droge, 2002). Therefore, ROS must be inactivated continuously in order to maintain only the small amount necessary to maintain normal cell functions.

The present study was undertaken to evaluate the effect of antioxidant supplements included in the *in vitro* fertilized bovine embryo culture medium to improve embryo development and its quality. Two well-known antioxidants, vitamin E and vitamin C were tested alone or in combination, together with a commercial solution from Sigma.

The results of experiment 1 demonstrate that, although the antioxidants tested did not influence the percentage of cleavage embryos, the use of vitamin E significantly reduced the number of blastocysts produced in comparison with the medium containing vitamin C and Sigma antioxidant supplement® (Table 1). This result diverges from those obtained by Olson and Seidel (2000), who demonstrated that culture medium supplemented with 100 µm of vitamin E increased bovine blastocyst production. Also, Kitagawa *et al*. (2004) report that culture medium supplemented with 100 µm of vitamin E increased porcine embryo development and cell number. In contrast, the controversial results of Wang *et al*. (2002) report that 400 µM of α-tocopherol induced a dose-dependent decrease in mouse blastocyst development and blastocyst cell number. In the present study, a concentration of 200 µM of vitamin E was used, and while appropriate for mouse embryos, this concentration may have been too high for bovine embryos. In fact, when vitamin E was used in association with vitamin C, the dose used was 100 µM, and this concentration resulted in blastocyst formation rates similar to the ones obtained in the control group (Table 1). However, it is not clear if this improvement in embryo production resulted from the low concentration of vitamin E or from the addition of vitamin C. A synergic effect from the combination of vitamins cannot be ignored, since the functional interrelation between α-tocopherol and micronutrients, notably L-ascorbic acid, has long been recognized (Chow, 1991).

Debate about ascorbic acid embryotrophic effects surrounds its use as a supplement in *in vitro* maturation (IVM) and *in vitro* culture mediums. Tatemoto *et al.* (2001) used 250 µM L-ascorbic acids during the IVM of porcine oocytes and reported that it did not improve the developmental competence of porcine embryos after IVF. However, Wang *et al*. (2002) reported that adding vitamin C to an embryo culture medium significantly affected embryo development in a dose-dependent manner. The present study demonstrates that supplementing 50 µM of L-ascorbic acid in embryo culture medium as a single supplement improved embryo development in comparison to vitamin E alone (Table 1). Similar results were obtained when the Sigma antioxidant supplement was used. Although the numeric results obtained using those antioxidants were superior, the blastocyst formation rate was not different from the control group (Table 1). This finding may be a result of the culture system used. In experiment 1, the embryos were cultured in the presence of granulosa cells attached to the culture plate during maturation (Co-culture). It is well established that the presence of granulosa cells helps with the removal of toxic substances present in the culture medium (Fujitani *et al*., 1997), which reduces oxidative stress (Fatehi *et al*., 2005). Also, the presence of FCS and the high amino acid concentration in the BME solution may have a beneficial synergic effect on the co-culture with granulosa cells.

Another important point is the timing of the addition of antioxidants during culture. Hossein *et al*. (2007) reported that the embryotrophic effects of vitamin E and vitamin C on porcine embryos vary according to the timing of the supplements addition. The supplementation of culture medium with 100 µM of vitamin E within the first 48 h of the culture was beneficial for embryo development. However, when vitamin C was used, two supplementations of 100 µM (at 0 and 96 h of culture) were needed to produce the same effect. In experiment 1, all of the antioxidants were supplemented at 0 h of culture, and no medium change was performed. Even in that condition, a benefit from the addition of vitamin C and Sigma antioxidant supplement was observed, and this effect may be increased if the supplementation was performed twice during culture.

Although several authors suggest that a high O_2 tension during culture is detrimental to embryo development due to high oxidative stress (Goto *et al*., 1993; Kitagawa *et al*., 2004; Dalvit *et al*., 2005), the results of experiment 2 showed no benefit in the use of Sigma antioxidant supplement® independently of the O_2 concentration during culture. Moreover, no differences were observed in the blastocyst production when culture was performed under an atmosphere of 5% O_2 in comparison with ~20%.

Currently, most culture systems that produce bovine embryos *in vitro* use SOFaa in atmosphere with 5% O_2 (Hashimoto *et al*., 2000; Van Soom *et al*., 2002; Ali *et al*., 2003; Luciano *et al*., 2005). This system eliminated the necessity of co-culture, but it increased the time during which the embryos are exposed to a potential oxidative stress (Holm *et al*., 1999). In experiment 2, a similar system was used, but the medium was changed to a mixture of HTF and BME. It was expected that the absence of cumulus cells and the elevated O_2 concentration when embryos were cultured in 5% CO_2 in air (~20% O_2) would be detrimental to embryo development and that the addition of antioxidants would minimize this effect. However, the blastocyst production rate was similar among all of the groups (Table 2). Similar results were reported by Khurana and Niemann (2000) and by Correa *et al*. (2008), who found no differences in embryo

development when culture was performed at 5 or ~20% O_2. These results may be linked with a low ROS generation in the conditions of the present experiment, in association with the possible activation of embryonic defense mechanisms. Correa *et al*. (2008) studied the expression of several genes in bovine embryos exposed to different concentration of O_2 during culture and observed a higher expression of the gene Mn-SOD (Manganese-superoxide dismutase) together with a tendency toward an increase in the expression of the gene GPX (glutathione peroxidase) when high O_2 concentrations were used. Although this gene expression pattern was expected to happen in response to a high production of ROS, the oxidative stress was not high enough to affect blastocyst production. A similar situation may be happening in experiment 2. However, it was not possible to estimate the amount of oxidative stress during culture because the concentration of ROS in the system was not measured.

This great deal of variation in the results of antioxidant supplementation and O_2 atmospheric tension in the *in vitro* production of embryos present in this work and in the literature are in agreement with discrepancies regarding the requirements of reactive oxygen species or lipid peroxides in the IVF process. Reactive oxygen species and lipid peroxidation exert a detrimental effect during *in vitro* fertilization and early embryo development (Nasr-Esfahami *et al*., 1990; Spiteller, 2001; Droge, 2002; Van Soom *et al*., 2002; Ali *et al*., 2003). However, some authors observed beneficial effects due to ROS. In human, lipid peroxidation increased the ability of spermatozoa to bind to both homologous and heterologous zona pellucida (Aitken *et al*., 1989). In mouse spermatozoa, a mild peroxidative condition improved the fertilizing potential of spermatozoa by increasing their binding capacity to zona pellucida (Kodama *et al*., 1996). Human spermatozoa need superoxide anion generation for the development of hyperactivation and capacitation (de Lamirande and Gagnon, 1995). Hydrogen peroxide plays a major role during hamster sperm capacitation, possibly in membrane reorganization to produce acrosomal reaction (Bize *et al*., 1991). In bovine, the use of α-tocopherol and acid ascorbic impaired the *in vitro* fertilization process (Dalvit *et al*., 1998).

In conclusion, the results of both experiments indicated that vitamin C and the Sigma antioxidant supplement® did not affect bovine embryo production. However, the addition of vitamin E in the culture medium was deleterious to the embryo development. Moreover, the reduction of O_2 concentration did not improve the number of blastocysts obtained.

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