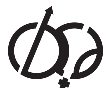



# *In vitro* production of *Bos taurus indicus* embryos with cysteamine

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## Abstract

Advanced reproductive techniques use three phases of bovine embryos in *in vitro* production (IVP), i.e. *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) for a variety of studies. This study assessed the effect of cysteamine, a thiol component, on the embryonic development during IVP. *Bos taurus indicus* cumulus-oocyte complexes (COC) from ovaries collected at an abattoir were randomly distributed in four groups: control-group (n = 544; without cysteamine), cysteamine in maturation (n = 543), cysteamine in fertilization (n = 540) and cysteamine in the culture medium (n = 557). All COC were matured for 24 h in TCM-199 + 0.01 IU r-hFSH/ml + 0.05 mg LH/ml + 10% fetal calf serum (FCS) at 38.5ºC in 5% $CO_2$ in humidified air. In the cysteamine-maturation group, the TCM-199 medium was supplemented with 150 µm cysteamine (CYS). The IVF (day 0 = fertilization day) was performed for 18-22 h in Fert-Talp medium + heparin + penicillamine, hypotaurine and epinephrine (PHE). The medium of the cysteamine-fertilization group was supplemented with 150 µm CYS. *Bos taurus indicus* frozen semen was selected by Percoll gradient, and incubated with the oocytes for 18 h. Presumed zygotes were cultured in 400 µl SOFaaci medium + 5% FCS. In the cysteamine-culture group the SOFaaci was supplemented with 150 µm CYS. Embryos were cultured at 5% CO2, 5% O2, 90% N2 and saturated humidity for 8 days. Cleavage rates were 86, 90, 88, and 91% respectively, for control, maturation, fertilization and culture groups. The blastocyst yield at day 7 was 29, 29, 38 and, 36% ($P < 0.05$) hatched blastocyst yield at day 9 was 21, 25, 27, and 29% ($P < 0.05$) in the control group and treatments, respectively. Results demonstrated that the addition of cysteamine to the fertilization or culture medium improved blastocyst production.

**Keywords:** antioxidants, *Bos taurus indicus,* bovine,cysteamine, IVP.

## Introduction

Reactive oxygen species (ROS) are free oxygen radicals that may result in oxidative injury. The superoxide anion ($O_2^0$-), hydroxyl radical ($OH^0$), hydrogen peroxide ($H_2O_2$) and peroxyl radical ($ROO^0$-) are among these short-lived molecules. Although the main source of intracellular ROS is oxidative phosphorylation in the mitochondria, external factors such as ultraviolet light and increased oxygen tension during culture increases the level of ROS. This additional oxidative stress alters the physiological functions in the embryo and negatively impacts development in mammals (Halliwell and Gutteridge, 1989).

Oxidative stress results in lipid peroxidation of membranes, amino acids, proteins, and nucleic acids; it also has the potential to cause cellular death, depending on the cell type, origin, and speed of oxidative stress production (Halliwell *et al*., 1992). As the production of free radicals normally occurs *in vivo*, cells develop a mechanism called "antioxidant defense system" to neutralize the reactive oxygen species and their effects (Halliwell and Gutteridge, 1989). Catalase and superoxide dismutase, as well as the “thiol” components act as metabolic lids to neutralize the reactive oxygen species (Del Corso *et al.,* 1994).

Cysteamine (CYS), a thiol component, acts within the embryo to increase glutathione synthesis, another potent antioxidant enzyme. Previous work demonstrated that the addition of cysteamine to bovine *in vitro* maturation medium could improve the embryonic development and quality (De Matos *et al*., 1995). However, the effect of such an antioxidant mechanism in other phases of *in vitro* production (IVP) has not been well identified.

The purpose of this study was to evaluate the effect of cysteamine on oocyte maturation (IVM), fertilization (IVF) and *in vitro* culture (IVC) of *Bos taurus indicus* embryos.

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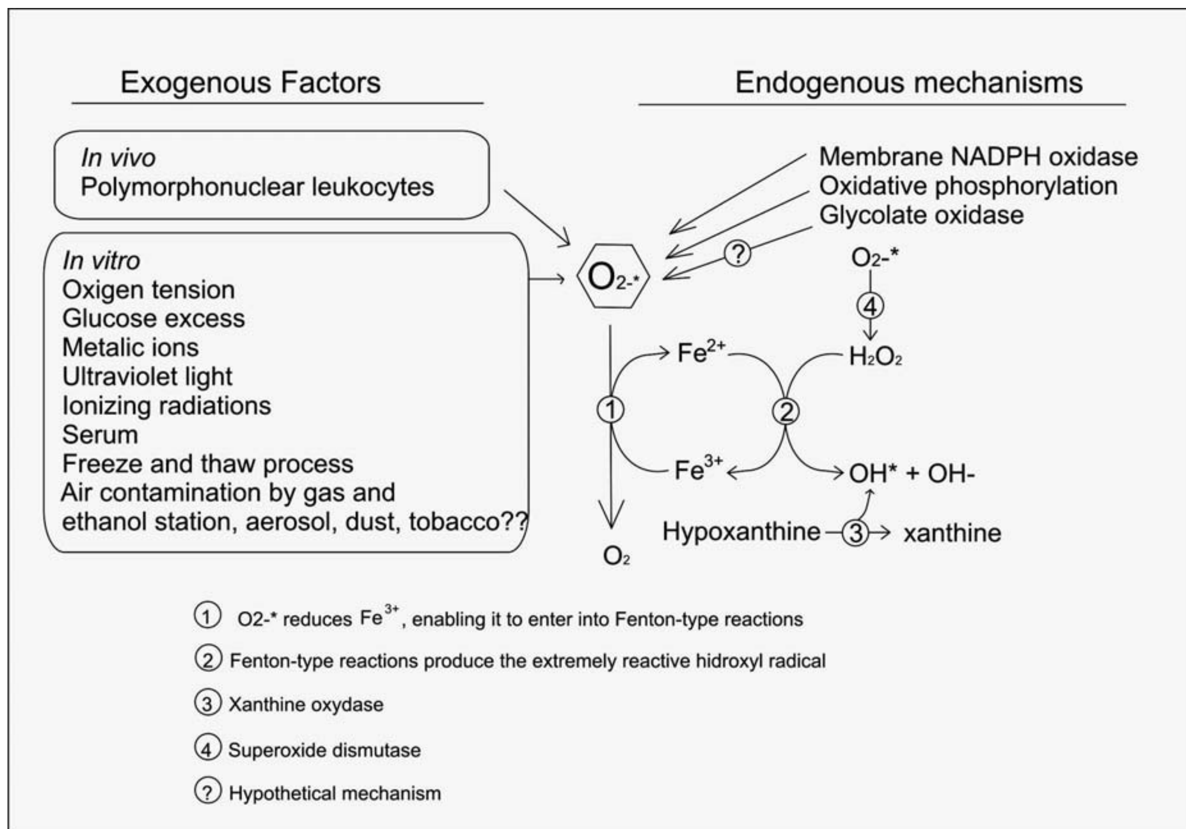



Figure 1. Schematic representation of the primary sources of reactive oxygen species in oocytes and embryos. Adapted from Guérin *et al*. (2001).

**Materials and Methods**

*Ovary collection and reagents*

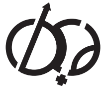
The ovaries from *Bos taurus indicus* cows obtained at an abattoir were transported for no more than 2 h in thermos bottles to the laboratory in physiologic solution (0.9% NaCl) with an initial temperature of 30ºC. Follicles with a diameter of 2 to 8 mm were identified and the follicular fluid was aspirated with a syringe and a 21 gauge needle. Immediately after aspiration, category I, II, and III (De Loos *et al*., 1989) cumulus-oocyte complexes (COC) were selected under a stereomicroscope. The COC were randomly distributed into four groups: control-group (without cysteamine; n = 544), cysteamine-maturation group (TCM-199 + CYS; n = 543), cysteamine-fertilization group (Fert-Talp + CYS; n = 540), and cysteamine-culture group (SOFaaci + CYS; n = 557). Unless otherwise stated, all reagents were purchased from Sigma (São Paulo, Brazil).

*In vitro maturation of oocytes*

Maturation was performed using 23 to 26 COC in 10 x 35 mm Petri dishes with 400 µl drops of modified TCM-199 medium containing Earle's salts (Gibco™, Ref.31-10-035, Invitrogen Corporation, USA) supplemented with 0.0225 mg/ml sodium pyruvate, 0.01 IU r-hFSH/ml, 0.05 mg/ml of sLH (Lutrophin-V, Bioniche Animal Health, Ontario, Canada) and 10% of fetal calf serum (FCS) for 24 h. Maturation was performed in an incubator at 38.5ºC with 5% $CO_2$ in air and saturated humidity. The COC from the cysteamine-maturation group were matured in TCM-199 maturation medium supplemented with 150 µm of cysteamine (Sigma® M9768).

*In vitro fertilization*

Fertilization was performed with frozen-thawed semen of a *Bos taurus indicus* bull, whose 0.25 ml straw was thawed for 10 sec at room temperature and subsequently for 20 sec in a 38.5ºC water-bath. The semen was placed in a Percoll gradient containing 1 ml of 45% Percoll over 1 ml 90% Percoll (Parrish *et al*., 1995) and centrifugated at 500 x g for 10 min. The pellet was reconstituted with 4 ml of Talp-Sperm and centrifuged at 500 x g for 10 min, and finally 200 µl of





the pellet was removed. A final concentration of 2 x $10^6$ 400 µl fertilization drop of Fert-Talp medium prepared with 0.03 mg/ml of BSA, 0.022 mg/ml of sodium pyruvate, PHE (penicillamine, hypotaurine and epinephrine) and 20 µg/ml of heparin, for 18 h in 5% $CO_2$ in air and saturated humidity at 38.5ºC. In the cysteamine-fertilization group 150 µm of cysteamine was added to the Fert-Talp medium.

*In vitro culture*

Presumed zygotes were denuded with a micropipette and washed five times in TCM-Hepes. Immediately, 20 to 25 zygotes were randomly distributed in 400 µl drops of modified Synthetic Oviduct Fluid (SOFaaci) medium according to Holm *et al.* (1999), with 5% FCS, 20 µl/ml of essential amino acids and 10 µl/ml of non-essential amino acids, under mineral oil in 10 x 35 mm Petri dishes. No feeding was performed during the 7 days of culture. The zygotes/embryos were maintained in an incubator with 5% $CO_2$, 5% $O_2$ and 95% $N_2$. Cysteamine (150 µm) was added to the culture medium of the cysteamine culture group.

*Embryo manipulation, development evaluation and statistical analysis*

Eleven replicates were performed to evaluate the cleavage, embryo production yield and the hatching rate. The cleavage rate was evaluated on day 2 of culture, considering the fertilization day as D0, and the uncleaved zygotes were removed. At day 7 the embryo spermatozoa/ml was incubated with 20 to 23 COC in production yield and the embryo quality were evaluated; as well at day 9, the hatching rate. Cleavage, embryonic production and hatching blastocyst formation were calculated based on the number of viable oocytes subjected to IVF. Embryo quality was evaluated using the International Embryo Transfer Society (Robertson and Nelson, 1998) recommended criteria. Oocytes and zygotes were exposed to ambient conditions when removed from the incubator for 20 to 60 min for processing. Cleavage and embryo development rates were analyzed by analysis of variance (ANOVA) and a value of 0.05 was used to define significant differences.

## Results

The cleavage rates of all groups were similar ($P > 0.05$); Fig. 2. Blastocyst yield at day 7 in cysteamine-maturation media (29.5%) was similar to the control group (29.0%), which was significantly lower than the cysteamine-fertilization and cysteamine-culture treatments (38.0 and 36.5% respectively; $P < 0.001$; Fig. 2). The blastocyst hatching yield at day 9 was significantly higher in the cysteamine-fertilization (27.2%; $P < 0.01$) and cysteamine-culture (29.4%; $P < 0.001$) than that of the control group (21.5%). The hatched blastocyst yield obtained with cysteamine-maturation was lower (24.9%; $P < 0.05$) than in cysteamine-culture (29.4%). However, no differences were observed between culture versus fertilization and maturation versus control treatments.

Embryo quality I and II at day 7 were similar ($P > 0.05$) in all treatments (Fig. 3).

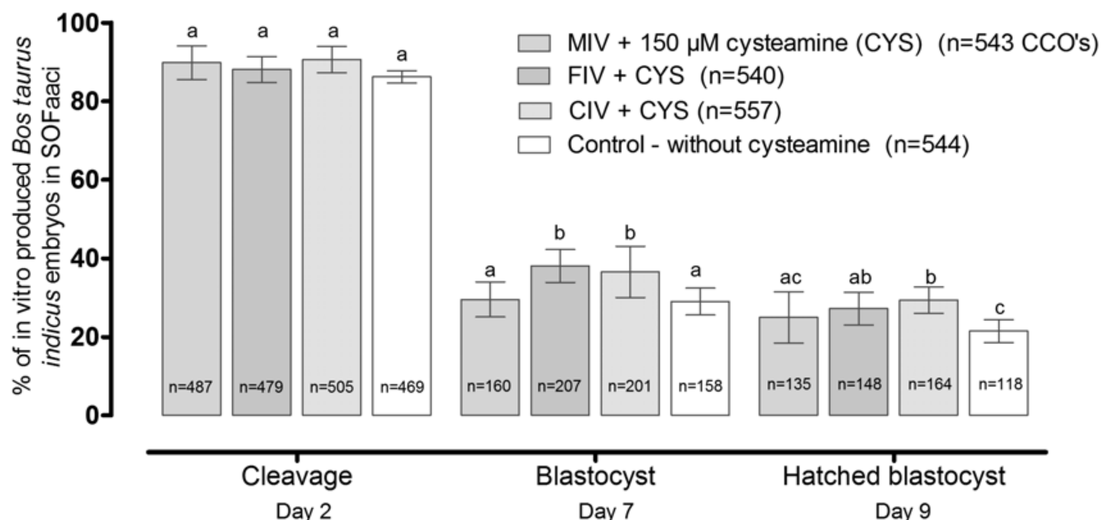



Figure 2. I*n vitro* production rate of *Bos taurus indicus* embryos in Synthetic Oviduct Fluid (SOFaaci) medium supplemented with 150 µm of cysteamine during maturation, fertilization or culture ($P < 0.05$).

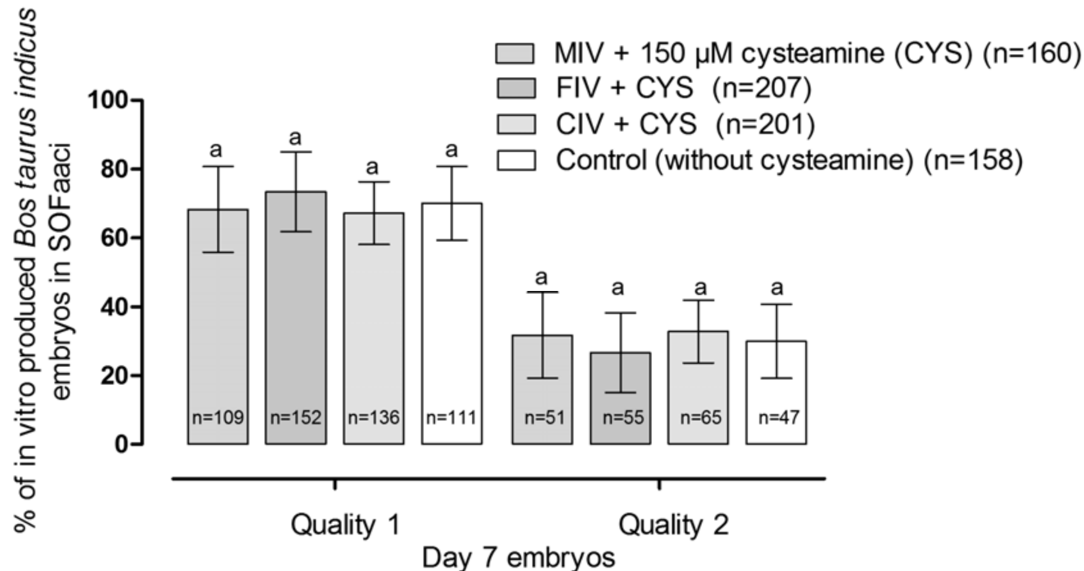



Figure 3. Quality of *Bos taurus indicus* embryos produced *in vitro* in Synthetic Oviduct fluid medium (SOFaaci) supplemented with 150 µm cysteamine ($P > 0.05$).

## Discussion

New knowledge of the embryonic metabolism and culture of bovine embryos is critical to improve efficiency of *in vitro* embryo production since the average blastocyst yield in Brazil is still lower than 50%.

In this research, the cleavage rates varied from 86 to 90% and were similar among the treatments and control group, which did not received cysteamine in any phase of the *in vitro* production. Similar rates were achieved in the study performed by Lonergan *et al*. (1999) using SOF medium, SOF + taurine, SOF + BSA + taurine or SOF + SFB + taurine in the culture of *in vitro* produced bovine zygotes under an atmosphere of 5% $O_2$ or 20% $O_2$.

During the 20 to 60 min manipulation procedures, oocytes, zygotes and embryos were exposed to the visible light and environmental oxygen (20%), which affects the embryonic development by producing free radicals (Goto *et al*., 1993; Nakayama *et al*., 1994). This time period may have been sufficient to allow the formation of ROS which could have detrimentally effected the subsequent embryological development.

Despite the negative effects of ROS, some studies suggest that they are beneficial under specific conditions. The study of Luvoni *et al*. (1996) highlights that 3 IU/ml of superoxide dismutase (SOD) and 1.0 mM of glutathione (GSH) reduced the bovine oocyte cleavage rate. However, a similar concentration of GSH was beneficial in the culture medium, increasing cleavage rates. In a later study, Blondin *et al*. (1997) concluded that the effect of ROS is dependent upon the reactive species, concentration and stage of *in vitro* production and that certain levels may be necessary for fertilization.

In this research, cysteamine added to maturation medium containing 10% of fetal calf serum did not significantly alter the cleavage (90 vs. 86%), blastocyst production (29 vs. 29%) or hatching rates (25 vs. 21%) compared to the control-group. Since the oocytes were maintained under low metabolic condition during maturation (Rieger *et al*., 1992) and the GSH is synthesized by the *Cumulus oophorus* cells (De Matos *et al*., 1997), it is possible that the cysteamine did not stimulate enough GSH production in 24 h to provide antioxidant protection.

In a series of three experiments, Ali *et al*. (2003) studied the development of bovine oocytes in antioxidant (cysteine, N-acetyl-cysteine, catalase, and superoxidase supplemented) supplemented *in vitro* maturation, fertilization and culture media. Compared to the control, the addition of cysteine (0.6 mM) to the IVM or IVC media increased the proportion of oocytes that developed into the morula and blastocyst stage. The results obtained in the IVM treated group differ from the present study where no improvement was noticed. This may be explained by the different antioxidants used: cysteine and cysteamine. These results indicate that the addition of an antioxidant to the IVC media is beneficial, and certain antioxidants at various concentrations added to the IVM could also improve blastocyst yield.

Ali *et al*. (2003) also reported that the addition of cysteine (0.6 mM), NAC (0.6 mM), catalase (127 U/ml), and SOD (100 U/ml) to the IVF media significantly reduced embryo development ($P < 0.05$). However, lower concentrations of the same antioxidants (0.1 mM,

0.1 mM and 5 U/ml, respectively) had no effect on the added to the fertilization medium resulted in the highest blastocyst formation at day 7 (38.0%) and an improved hatching rate at day 9. These results support the hypothesis that some amount of ROS might be necessary for *in vitro* fertilization and that antioxidant concentration above a currently undefined level is detrimental.

The benefits of adding antioxidants to the culture medium were described by Takahashi *et al*. (1993). Different cysteamine concentrations in TCM-199 + 10% of FCS *in vitro* culture without somatic cells resulted in a significant increase in the development of 6 to 8-cell embryos to the blastocyst stage. Additionally, the addition of cysteamine resulted in increased levels of GSH, providing further evidence for the use of antioxidants in the culture media.

Rosenkrans *et al*. (1993) achieved better embryonic development at day 7 (22 vs. 32%) in CR1 medium with amino acids (CR1aa) by reducing the concentration of $O_2$ from 20 to 5%. Lee *et al*. (1999) supplemented SOF with β-mercaptoetanol and the blastocyst production increased from 18 to 27%. In 2002, Fischer-Brown *et al*. reported the use of β-mercaptoethanol in their semi-defined SOFaa + BSA culture system with or without 10% FCS. In FCS-supplemented medium, embryos formed blastocoeles earlier and with 35% fewer cells than in unsupplemented medium. They demonstrated that the antioxidant increased hatching rate and day-6 development when FCS was added; however, the nature of this interaction was unclear. In our study, the SOF medium supplemented with 5% FCS, amino acids, citrate, inositol and cysteamine resulted in an embryo production of 36% (201/557) at day 7. This was probably due to the superiority of nutrients associated with the antioxidant action of the cysteamine. Although the percentage of emerged embryos was based on the whole number of maturated oocytes, the hatched blastocyst yield was lower than that reported in the literature.

According to Brum *et al*. (2004), 5, 10 and 20 COC fertilized in 1:5 and 1:10 oocyte/µl volume ratios had no effect on embryo production. Therefore, blastocyst production and the hatching rate at day 9 were neither influenced by the COC number, nor by the proportion of COC per medium volume used during fertilization. However, it is important to note that the eleven replicates in this study were performed using 20 to 25 COC/zygotes in 400 µl of maturation, fertilization or culture medium.

The cysteamine supplied to fertilization (38%) or culture media (36%) showed higher blastocyst yield at day 7 compared to maturation + CYS (29%) or control group (29%). These results may be due to the beneficial antioxidant mechanism of cysteamine in neutralizing the reactive oxygen species and their effects. Gordon (1993) emphasized that the accelerated embryo production. In our study, 150 µm of cysteamine development of *in vitro* produced bovine embryos causes increased susceptibility to oxidative stress. This suggests that cysteamine neutralizes the reactive oxygen species generated by the high metabolism of the embryos in early development.

Finally, the quality of *in vitro* produced embryos is not usually reported in the literature because the embryos are generally transferred to the recipient at day 7. In this study, the embryos were maintained until day 9 in order evaluate blastocyst hatching. Similar morphologic quality among the treatments and the control-group indicates that cysteamine does not influence the embryo production rates.

In conclusion, cysteamine increases the production of bovine blastocysts *in vitro* when added either to the fertilization or culture medium. However, the apparent embryonic quality is not influenced by cysteamine in any stage (maturation, fertilization or culture) of the *in vitro* production. Therefore, the addition of cysteamine may improve the IVP programs associated with commercial ovum pick-up.

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