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Brief Note

Reactive oxygen species in bovine embryo *in vitro* production

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ABSTRACT: Oxidative modifications of cell components due to the action of reactive oxygen species (ROS) is one of the most potentially damaging processes for proper cell function. However, in the last few years it has been observed that ROS participate in physiological processes. The aim of this work was to determine ROS generation during *in vitro* production of bovine embryos. Cumulus-oocyte complexes were recovered by aspiration of antral follicles from ovaries obtained from slaughtered cows and cultured in medium 199 for 22 h at 39ºC in 5% CO_2: 95% humidified air. *In vitro* fertilization was carried out in IVF-mSOF with frozen-thawed semen in the same culture conditions and embryo *in vitro* culture in IVC-mSOF at 90% N_2: 5% CO_2: 5% O_2. ROS was determined in denuded oocytes and embryos at successive stages of development by the 2´,7´-dichlorodihydrofluorescein diacetate fluorescent assay. ROS production was not modified during oocyte maturation. However, a gradual increase in ROS production was observed up to the late morula stage during embryo *in vitro* culture ($P<0.05$). In expanded blastocysts, ROS level decreased to reach values similar to the corresponding in oocytes. In the bovine species, the variation in ROS level during the complete process of embryo *in vitro* production was determined for the first time.

Introduction

The concept of oxidative stress has been widely used in biological sciences to describe an enhanced state of oxidants or a lack of antioxidants in cells, a situation in which the concentration of reactive oxygen species (ROS) increases above its biological values (Gonzales Flecha *et al*., 1993; Sikka, 2001). The oxidative modification of cell components due to the action of ROS is one of the most potentially damaging processes for proper cell function that may lead to cell death by necrosis or apoptosis (Sarafian and Bredesen,1994; Yang *et al*., 1998).

Embryo *in vitro* culture affords higher oxygen concentrations than *in vivo* environments, resulting in increased ROS production (Luvoni *et al.*, 1996). In the bovine species, the procedure for producing embryos *in vitro* is still unsatisfactory, ranging 30-40% of blastocyst rate at day seven. ROS generation has been implicated as a major cause of low percentages of bovine embryo *in vitro* production. ROS has been suggested to

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participate in meiotic arrest in oocytes (Downs and Mastropolo,1994; Nakamura *et al*., 2002), embryonic block and cell death (Hashimoto *et al.*, 2000). However, during the last years it has been observed that physiological concentrations of ROS participate in normal cell processes as major factors in growth and development regulation (Hancock *et al.*, 2001).

Therefore, in the bovine species the role of ROS as regards *in vitro* maturation, fertilization and embryo development, remains controversial. Although the effect of antioxidants in these processes has been described, there are no studies that document whether during *in vitro* maturation and embryo development conditions there are variations in ROS production attributable to oocytes and embryos. Accordingly, the aim of this work was to determine the production of ROS in one of the most widely systems used for bovine *in vitro* production.

Materials and Methods

Ovaries were obtained from slaughtered cows. Cumulus-oocyte complexes (COCs) were recovered by aspiration of antral follicles and only good quality oocytes completely surrounded by compact and thick cumulus were used. COCs were matured in medium 199 supplemented with 10% steer serum and 50 mg/l gentamicine sulfate at 39ºC during 22 h in 5% CO_2 with humidified air. Meiotic maturation was evaluated using 40% of the samples as described Cetica *et al.* (1999).

In vitro fertilization was carried out using frozen semen, thawed at 37ºC in mSOF medium (Takahashi and First,1992) with 10 mmol/l theophylline, centrifuged at 500g twice for 5 min and resuspended in the fertilization medium to a final concentration of $2x10^6$ motile spermatozoa/ml. Fertilization was carried out in IVF-mSOF medium (mSOF supplemented with 10 iu/ml heparin and 5 mg/ml BSA) under mineral oil at 39ºC, 5% CO_2 and 100% humidity during 20 h. Zygotes were denuded by pipetting and placed in IVC-mSOF (mSOF supplemented with 30 µl/ml of MEM solution of amino acids, 10 µl/ml of MEM solution of non-essential amino acids, 2 mmol/l L-glutamine, 6 mg/ml BSA and 5% FBS) under mineral oil at 90% N_2: 5% CO_2: 5% O_2 and 100% humidity during 24 h. *In vitro* fertilization percentage was determined by evaluating the number of embryos divided into 2 or more blastomeres.

Embryo *in vitro* culture was carried out in IVC-mSOF medium under mineral oil at 90% N_2: 5% CO_2: 5% O_2 and 100% humidity. Blastocyst percentage was determined at the seventh day of insemination. Only grade 1 (excellent) and 2 (good) embryos at the expected stage of development for each age were used (Kennedy *et al.*, 1983).

To measure the production of ROS, immature and matured oocytes were completely denuded by vortex agitation in physiological saline solution for 1 min and then washed to eliminate cumulus cells. Samples of 20 denuded oocytes or embryos at different stages of development were incubated in 40 mmol/l Tris-HCl buffer pH 7.0 at 37°C for 30 min in the presence of 5 µmol/l

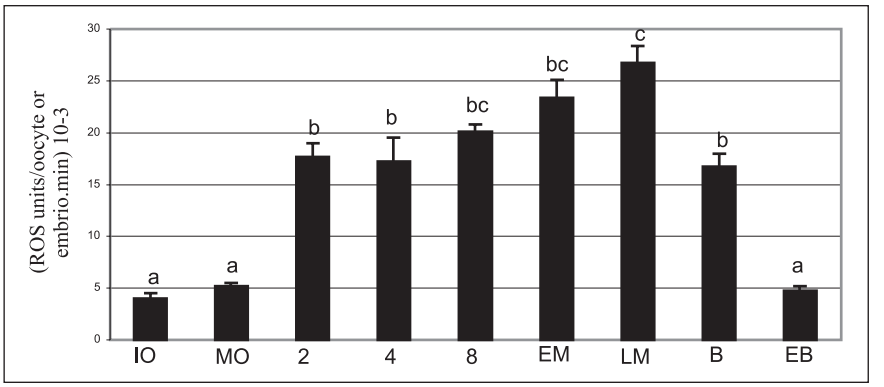

FIGURE 1. ROS levels determined by spectrofluorometry using 2´,7´-dichlorodihydrofluorescein diacetate assay during bovine oocyte maturation and embryo development *in vitro.*
IO, immature oocyte; MO, matured oocyte; 2, 2 cell embryo; 4, 4 cell embryo; 8, 8 cell embryo; EM, early morula; LM, late morula; B, blastocyst; EB, expanded blastocyst. Values are expressed as (means ± SEM) 10^{-3} of 7 replicates for each stage. [a, b, c] bars with different superscripts are significantly different (P<0.05).

2´,7´-dichlorodihydrofluorescein diacetate (DCHFDA), washed, sonicated at 50 W for 1 min and centrifuged at 4ºC and 10,000g for 20 min. Fluorescence was monitored in the supernatant using a spectrofluorometer at 488-nm excitation and at 525-nm emission (LeBel *et al.*, 1992; Cetica *et al.*, 2001). Corrections for autofluorescence were made by inclusion of parallel blanks in each experiment.

Data were expressed as Arbitrary ROS Units/denuded oocyte or embryo/min (means ± SEM) x 10^{-3} of seven replicates for each cellular stage. Statistical comparisons were made by ANOVA.

Results

In our system of bovine embryo *in vitro* production, the percentages of meiotic maturation, *in vitro* fertilization and blastocysts obtained at day 7 were 85%, 77% and 38%, respectively.

ROS determination by spectrofluorometry using DCHFDA showed that their generation remained constant during bovine oocyte *in vitro* maturation due to ROS level was not significantly different between immature and matured denuded oocytes (Fig. 1).

A significant increase in ROS level in 2 cell embryo was detected with respect to the oocyte ($P<0,05$) and then, during embryo *in vitro* culture there was a gradual enhancement starting from the 2-cell embryo up to the late morula stage ($P<0.05$) (Fig. 1).

In the blastocyst, ROS values decreased until they reached levels similar to those of oocytes in the expanded blastocyst stage ($P<0.05$) (Fig. 1).

Discussion

It has been shown that DCFHDA probe is oxidized by hydrogen peroxide, its derived oxidants, other peroxides and indirectly by the superoxide anion when generating hydrogen peroxide, thus providing an useful test to evaluate ROS production (LeBel *et al.*, 1992).

The production of ROS in denuded bovine oocytes from immature and *in vitro* matured COCs was unaltered by maturation, indicating that culture conditions employed were not responsible for oxidative stress in the female gamete. The maintenance of ROS production levels during maturation could be due partly to the action of the antioxidant system described in bovine COCs (Cetica *et al.*, 2001; Dalvit *et al*., 2005). This steady state concentration of ROS could be necessary for the maturation process, as suggested by Blondin *et al.* (1997), who documented that a certain production of ROS during bovine oocyte *in vitro* maturation is required to increase blastocyst percentage.

The significant enhace in ROS generation observed in 2 cell embryo with respect to the oocyte would be related to diverse factors. It could be due to modifications in embryo *in vitro* culture conditions with regard to those of maturation, including changes in culture medium, the corresponding gas mixture used and the total separation of cumulus cells starting from the zygote stage, or the increase of metabolic activity due to embryo cleavage.

The gradual increase in ROS levels from the 2-cell embryo up to the late morula stage could depend on the metabolic change undergone by the embryo during its development. Thompson *et al.* (1996a) reported a sustained increase in oxygen, glucose and pyruvate uptake during embryo *in vitro* development. This enhancement in oxidative metabolism of the embryo could be linked to the detected increase in ROS level.

The decrease of ROS values detected in blastocysts may be related to the onset of cellular differentiation. This onset would modify oxygen metabolism and/or the acquisition of autonomy by the embryonic genome to induce the synthesis of the antioxidant system that controls ROS production. It has been demonstrated that oxygen uptake diminishes in expanded blastocysts due to they have lower physiological ATP demand (Thompson *et al.*, 1996b) because glycolysis begins to contribute to ATP production (Thompson *et al.*, 1996a, 2000). Alternatively, the embryonic genome may acquire the autonomy to induce its own antioxidant system to control ROS production. Harvey *et al.* (1995) reported that in the bovine species certain antioxidant enzyme transcripts increase or appear in the blastocyst stage.

In conclusion, the present work is the first in determining the level of ROS production during the entire process of bovine embryo *in vitro* production. On the basis of our results, it is suggested that oocyte maturation conditions are not responsible for oxidative stress. However, metabolic changes during embryo development would lead to a gradual increase in ROS production, to peak in the late morula stage, then dropping abruptly in the expanded blastocyst stage. Given such data, in future work it would be of interest to control ROS levels by focusing on the successive stages of embryo *in vitro* culture and to determine whether they lead to cell deterioration due to oxidative stress or whether they are involved in the normal process of embryo development.

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