

# Preimplantation embryotoxicity after mouse embryo exposition to reactive oxygen species

ELISA CEBRAL<sup>1,2</sup>, ISABEL CARRASCO<sup>1</sup>, DAVID VANTMAN<sup>1</sup> AND ROSITA SMITH<sup>1</sup>.

1. Instituto de Investigaciones Materno-Infantil, Hospital Clínico Materno-Infantil San Borja-Arriarán, Facultad de Medicina, Universidad de Chile. Santa Rosa 1234, Santiago, Chile.
2. Laboratorio de Biología del Desarrollo, Instituto de Fisiología, Biología Molecular y Neurociencias (IFIBYNE-CONICET), Pabellón II, 4to. Piso, Ciudad Universitaria, Capital Federal, Buenos Aires, Argentina. Centro de Estudios Farmacológicos y Botánicos (CEFYO-CONICET). Paraguay 2155, piso 16. Capital Federal, Buenos Aires, Argentina.

**Key words:** reactive oxygen species, preimplantation embryogenesis, toxicity, mouse.

**ABSTRACT:** Exposure of either gametes or embryos to conditions and/or factors that generate oxidative stress has been associated with impaired early embryogenesis. The effects of reactive oxygen species (ROS) on mouse preimplantation development, depending of the ROS-concentration and time of exposition, were studied. Two-cell embryos were incubated with 5, 10, 25 and 50  $\mu\text{M}$  of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) for 30 and 60 minutes of exposition and allowed to develop for 72 h to study the quality of development. The incubation with 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 30 or 60 minutes, strongly inhibited the 2-cell embryo development as compared to the control ( $p < 0.001$ ). Twenty-five  $\mu\text{M}$   $\text{H}_2\text{O}_2$  produced inhibition of blastocyst formation ( $p < 0.001$ ) and 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  significantly decreased the percentages of expanded and hatched blastocysts, which resulted morphologically altered ( $p < 0.05$  and  $p < 0.01$ , respectively). The higher  $\text{H}_2\text{O}_2$  concentrations were able to elicit necrotic morphology in the 2-cell arrested embryos, while 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  induced moderate damage with the arrested embryos partially fragmented. In conclusion, important causes for defective preimplantation development and for early embryo losses may be due to oxidative stress because early mouse embryos exposed to ROS for short times arrested at the first cellular cycle (2-cell) and/or impaired embryo differentiation and morphogenesis, being these effects ROS-concentration-dependent.

## Introduction

Oxidative stress occurs as the result of an imbalance between the pro-oxidants and the ability of the antioxidants to scavenge the excess reactive oxygen species (ROS) production.

It is well known that ROS such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), the superoxide anion ( $\text{O}_2^-$ ) and the hydroxyl

radicals ( $\text{OH}$ ), are generated in several pathological conditions. It was suggested that ROS play an essential role in several reproductive dysfunctions. Evidence has been presented for the detrimental effects of ROS on male reproductive function (Aitken *et al.*, 1993). High seminal ROS have been related to poor semen quality, loss of sperm function, impaired fertilization and lower pregnancy rates after IVF (Aitken, 1994; Zorn *et al.*, 2003), because lipid peroxidation, even at a level that does not affect motility, may decrease the fertilizing potential spermatozoa (Aitken *et al.*, 1989). These studies suggest that ROS can be produced by living spermatozoa after incubation in aerobic conditions (Iwasaki and Gagnon, 1992).

On the other hand, ROS are involved in the modulation of a wide spectrum of physiological female re-

Address correspondence to: Dra. Elisa Cebal. IFIBYNE-CONICET / DBBE-FCEN. Lab. 15-21, 4to Piso, Pab.II. Ciudad Universitaria, (CP 1428) Capital Federal, Buenos Aires, ARGENTINA.

Phone: (+54-11) 4576 3300. E-mail: ecebral@hotmail.com

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productive functions such as oocyte maturation, ovarian steroidogenesis, ovulation, implantation, blastocyst formation, pregnant maintenance, corpus luteal function and even luteolysis (Guerin *et al.*, 2001; Sharma and Agarwald, 2004). ROS can mediate some female fertility disorders, such as endometriosis (Sharma and Agarwald, 2004; Bedaiwy *et al.*, 2002) and unexplained infertility. Lipid peroxidation has also been shown to decrease sperm-oocyte interaction, as measured by a decreased penetration of zona-free hamster oocytes by human spermatozoa (Aitken *et al.*, 1989).

Male and female reproductive disorders induced by oxidative stress have been reported; however little is known about the effects of ROS on zygotes and/or early embryo development. Oxidative stress may be responsible for the embryotoxicity in hydrosalpinx pathology (Attaran *et al.*, 2000). Reactive oxygen species in culture media may affect fertilization, embryo development and clinical pregnancy rates. In both IVF and intracytoplasmic sperm injection (ICSI) programs, elevated concentrations of ROS in culture media at day 1 have been shown to be associated with lower pregnancy rates (Bedaiwy *et al.*, 2004). In this regard, in human *in vitro* fertilization-embryo transfer programs, it was suggested that low numbers of good quality embryos developed were due to high oxygen concentration of the *in vitro* culture system (Goyanes *et al.*, 1990; Nars-Esfahani *et al.*, 1990; Goto *et al.*, 1992). Moreover, it was shown a direct relationship between high hydrogen peroxide ( $H_2O_2$ ) concentration and elevated fragmentation grade or apoptosis in mouse embryos (Yang *et al.*, 1998).

We hypothesized that a brief exposure of 2-cell embryos to low levels of ROS, that can derived from sperm suspensions or from the *in vitro* culture conditions, induces abnormal development and embryo arrest with necrotic or apoptotic features depending on the exposure conditions. This study was conducted to ascertain the effects of different  $H_2O_2$  levels on the quality and cleavage of embryos and to know the thresholds in concentration and exposure time needed to disrupt the embryo development.

## Materials and Methods

### Materials

Pregnant mare's serum gonadotrophin (PMSG), human chorionic gonadotrophin (hCG), Human Tubal Fluid (HTF) medium, bovine serum albumin (BSA),

mineral oil, hydrogen peroxide ( $H_2O_2$ ) and catalase were all obtained from Sigma Sigma Chemical Co., St Louis, MO, USA.

### Animals preparations

CF-1 mice, outbred in the laboratory of the Instituto de Investigaciones Materno-Infantil, were fed *ad libitum* with a commercial mouse chow diet, and were maintained under automatically controlled temperature (25°C) and 14 h light/10 h dark. Females were prepubertal 30-days-old and males were adult 75 days old.

Females were superovulated with PMSG 5 IU (i.p.) at 12:00 h and hCG 5 IU 48 h apart. At the moment of hCG injection, one female was paired overnight with a previously isolated fertile male. In the morning of the next day, successful mating was confirmed by presence of the vaginal plug.

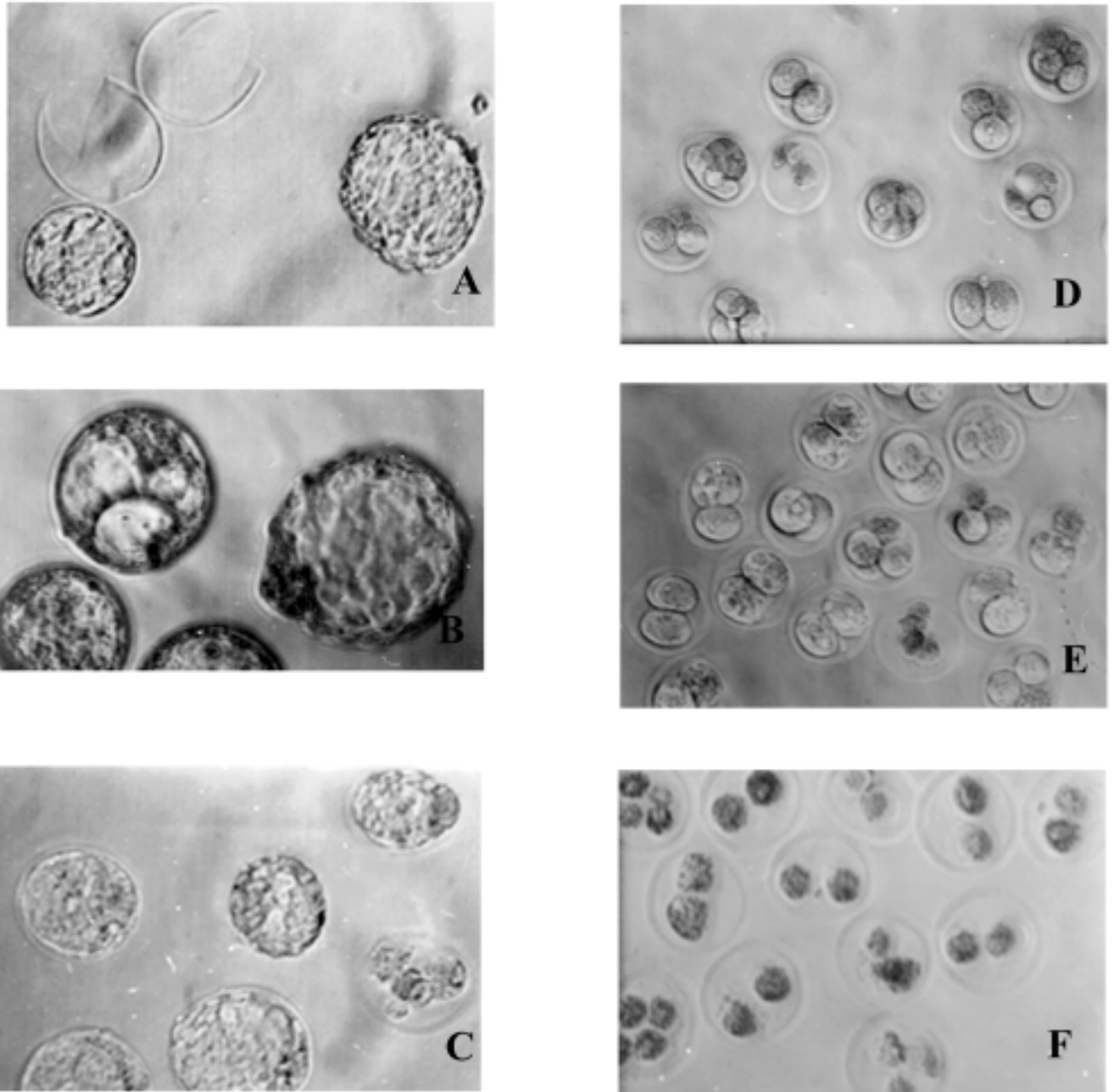
### Embryo collection

Mated females were sacrificed 46 h post-hCG and the 2-cell embryos were recovered from the oviducts by flushing with HTF medium supplemented with 0.4 mg/ml BSA. Immediately the embryos were pooled into drops of HTF-BSA overlaid with mineral oil, that was previously equilibrated in a humidified tissue culture incubator. The fragmented or dead eggs were discarded.

### Oxidative stress experiments

The 2-cells embryos were placed in groups of 10 embryos per 50  $\mu$ l drop of HTF-BSA medium overlaid with mineral oil. The induction of the oxidative stress on embryos was performed by addition different concentrations of hydrogen peroxide ( $H_2O_2$ ) and 30 and 60 minutes of incubation in 5%  $CO_2$  in air, at 37°C. Following the period of incubation, the two-cell embryos were placed in drops of the same medium added by catalase 1800 U/ml for 10 min, washed and cultured in 50  $\mu$ l drops of HTF + BSA 0.4%, overlaid with mineral oil for 72 h.

The following groups were performed: *HTF-control group*: 30 and 60 min of incubation in HTF-BSA medium and 10 min of incubation in HTF-medium; *Catalase-control group*: 30-60 min of incubation in HTF-BSA medium and 10 min of incubation in HTF-catalase medium;  *$H_2O_2$ -treated groups*: 30-60 min of incubation in 50, 25, 10 and 5  $\mu$ M of  $H_2O_2$ -HTF and 10 min of incubation in HTF-catalase medium.



**FIGURE 1. Preimplantation in-vitro development at 72 h of culture following  $H_2O_2$  exposition.**

**1.A.** Control groups. Differentiation to expanded and hatched blastocysts after 30 or 60 minutes of incubation (X 20). **1.B.** Morphologically abnormal expanded blastocyst (abnormal cavitation) after 30 or 60 minutes of incubation (\*).(X 40). **1.C.** Embryos exposed to  $5 \mu M H_2O_2$ . Early and expanded blastocysts after 30 or 60 minutes of incubation (X 20). **1.D.** Embryos exposed to  $10 \mu M H_2O_2$ . Embryos arrested at 2 and 4 cells stages after 30 or 60 minutes of incubation (X 20). **1.E.** Embryos exposed to  $25 \mu M H_2O_2$ . Embryos arrested at 2 cells stages and type I and II of embryo quality after 30 minutes of incubation (X 20). **1.F.** Embryos exposed to  $50 \mu M H_2O_2$ . Embryos arrested at 2 cells stages and type III of embryo quality after 30 minutes of incubation (X 20).

### Evaluation of embryo development

The *in-vitro* development was evaluated at 72 h of the culture under phase-contrast microscopy. The developed embryo stages were photographed using a Nikon inverted microscope.

The intact, fragmented and/or lysed 2-cell embryos that did not continued developing was recorded as “arrested embryos”. The following types of embryo arrest were considered: Type I: fully lysed, necrotic and/or fragmented embryos. Type II: embryos with partially lysed/fragmented blastomeres. Type III: embryos with some lysed/fragmented blastomeres and/or cytoplasmic vesicles.

The *embryo differentiation* was examined by quantification of non-compacted, compacted morulae, early blastocysts (with initial blastocele), expanded blastocysts, hatching blastocysts (zona-escaping blastocysts), hatched blastocysts (extruded or zona-free embryos).

The *embryo morphology* was scored in the developed blastocyst. Smaller blastocyst than the controls, with no intact blastomeres (necrotic or lysed), abnormal cavitation (two or more blastoceles) and/or vesicles in blastomeres were considered as morphologic abnormal embryo.

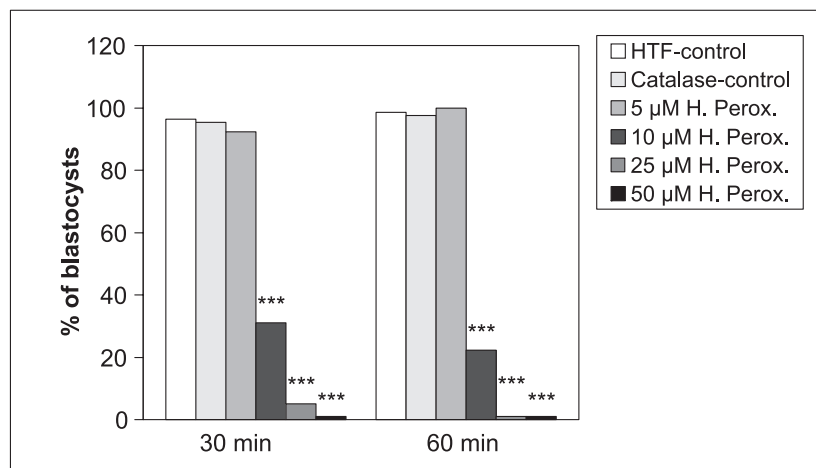
The *embryo-growth* and *the cleavage rate* was assessed by counting the cell number per embryo according to the air-drying technique of Tarkowski (1966). Briefly, the embryos were swollen in drops of 0.9% sodium citrate for 8-10 min, transferred to cleaned slides and fixed for 30 s by dropping methanol: acetic acid (3:1 v/v) over the embryos. The cells were stained with Giemsa solution (2:1, v/v, in PBS) for 10 min. The cytoplasm was dispersed leaving only chromatin behind. The nuclei were examined under light microscopy.

### Statistical analysis

The *in vitro* development was calculated as the number of embryos developed at 72 h over the total number of 2-cell treated embryos (N). The quality of embryo arrest was calculated as the number of 2-4 cell embryos and/or morulae with any of the three types of detention quality, over the total number of 2-cell treated embryos. The morphologically abnormal embryo percentages were calculated as the number of abnormal blastocyst over the total number of 2-cell embryos. The differences in these percentage comparisons were analyzed by Chi-square test. The numbers of embryos (N) analyzed for 30 and 60 min were: HTF-control N= 111, Cat-control N= 99 and 81, 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  N= 83 and 75, 25  $\mu\text{M}$   $\text{H}_2\text{O}_2$  N= 102 and 86, 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  N= 96 and 76, 5  $\mu\text{M}$   $\text{H}_2\text{O}_2$  N= 92 and 81, respectively. The embryo differentiation and the number of cells per embryo were calculated as means and SEM, with n= 5-8 experimental replicates or n = number of analyzed embryos, respectively. One-way ANOVA and Dunnett test compared them. A p value <0.05 was considered significant.

### Results

Embryos were studied after 30-60 min treatment with  $\text{H}_2\text{O}_2$  followed by 72 h incubation in fresh medium. For both exposition times, the quality and types of embryos resulted similar. The HTF and catalase-control groups had almost all embryos in the morphologically normal blastocyst stage (Fig. 1A). Abnormal blastocysts were those that presented fragmentation, lysis in the trophoectoderm or in the inner cell mass and/or with cavitation anomalies (Fig. 1B). Figure 1C shows nor-



**FIGURE 2. Blastocyst development**

Embryo development after exposition to different concentrations of hydrogen peroxide (H. Perox.) for 30 and 60 min was evaluated at 72 h of culture under a contrast microscope. The percentages (%) of blastocysts were recorded. \*\*\*: p<0.001 versus control groups, Chi-squared test.

mal blastocysts after incubation with 5  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and almost any 2-cells embryo arrest. Exposition to moderate  $\text{H}_2\text{O}_2$  concentrations (10 and 25  $\mu\text{M}$ ) produced embryo arrest type I, II and III; the 2 cells embryos appeared intact or partially fragmented and/or lysed (Fig. 1D and 1E, respectively). However, 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  arrested the 2 cells embryos and all of them were fully lysed and swollen (Fig. 1F).

The percentage of embryos developed at 72 h of culture following exposure of 2-cell embryos for 30 and

60 min to different concentrations of  $\text{H}_2\text{O}_2$  is shown in Fig. 2. Percentages of blastocysts were significantly reduced after treatment with 10  $\mu\text{M}$   $\text{H}_2\text{O}_2$  ( $p < 0.001$ ) and there was almost any blastocyst with 25 and 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$ , either after 30 or 60 minutes of incubation as compared to control groups ( $p < 0.001$ ). The analysis of the quality the arrest embryos after 30 minutes of incubation (Table 1) showed that 25 and 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$  increased detented 2 cells embryos. While the highest concentration produced 100% of fully lysed 2 cells embryos (Type I), 25

TABLE 1

Quality embryo arrest after 30 minutes of incubation

	Controls	5 $\mu\text{M}$ $\text{H}_2\text{O}_2$	10 $\mu\text{M}$ $\text{H}_2\text{O}_2$	25 $\mu\text{M}$ $\text{H}_2\text{O}_2$	50 $\mu\text{M}$ $\text{H}_2\text{O}_2$
<b>Stages of arrested embryos</b>	4-cells Morulae Blastocysts	2-cells Morulae	2-cells Morulae	2-cells	2-cells
<b>Arrest Type I</b>	1 (0.9 %)	1 (0.01 %)	6 (6.2 %)	31 (30.3 %) **	83 (100 %) ***
<b>Arrest Type II</b>	1 (0.9 %)	3 (3.2 %)	40 (41.6 %) ***	48 (47 %) ***	0
<b>Arrest Type III</b>	1 (0.9 %)	1 (0.01 %)	20 (20.8 %) ***	17 (16.6 %) ***	0
<b>N</b>	111	92	96	102	83

At 72 h of culture and after 30 minutes of exposition with different  $\text{H}_2\text{O}_2$  concentrations, the quality of the arrested 2 cells embryos was evaluated and classified as Type I, II and III by observation of the relative percentage of fragmented or lysed cells and/or presence of cytoplasm vesicles, as described in materials and methods. The percentages of different embryo stages and the types of arrest were recorded. \*\*\*:  $p < 0.001$ , \*\*:  $p < 0.01$ , Chi Squared test, N = total number of embryos analyzed.

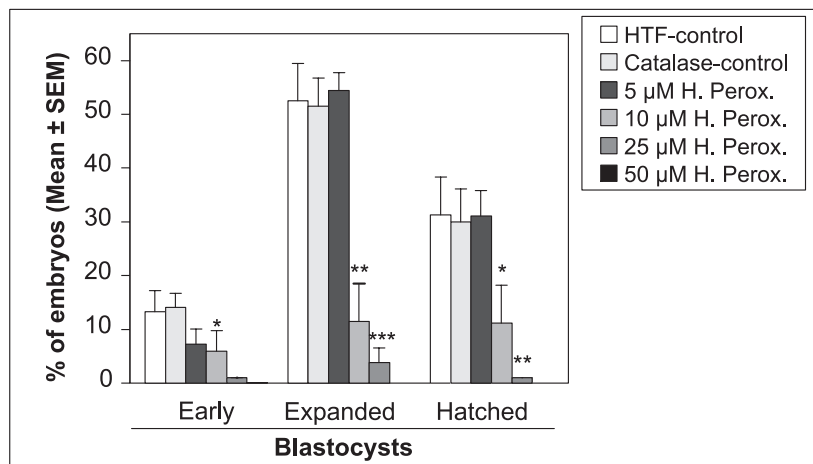


FIGURE 3. Embryo differentiation

The grades of embryo differentiation were analyzed at 72 h of culture following exposure to different hydrogen peroxide (H.Perox.) concentrations for 30 min. The blastocyst types were quantified as: early (with initial cavity), expanded (with expanded cavity) and hatched blastocyst (extruded from the pellucida zona). Values are expressed as the mean  $\pm$  S.E.M, n= 5-8 replicates. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ , versus control groups, ANOVA, Dunnett test.

$\mu\text{M H}_2\text{O}_2$  increased percentages of Types I, II and III of arrested embryos as compared to the control ( $p < 0.01$  and  $p < 0.001$ , respectively). Ten ( $10 \mu\text{M H}_2\text{O}_2$ ) produced significantly increased percentages of Type II and III arrest ( $p < 0.001$ ) and low percentage of Type I, while  $5 \mu\text{M H}_2\text{O}_2$  did not elicit almost any type of embryo arrest. These results suggest a differential effect and mechanism of embryo arrest depending on the  $\text{H}_2\text{O}_2$  concentration used for a short period of exposure.

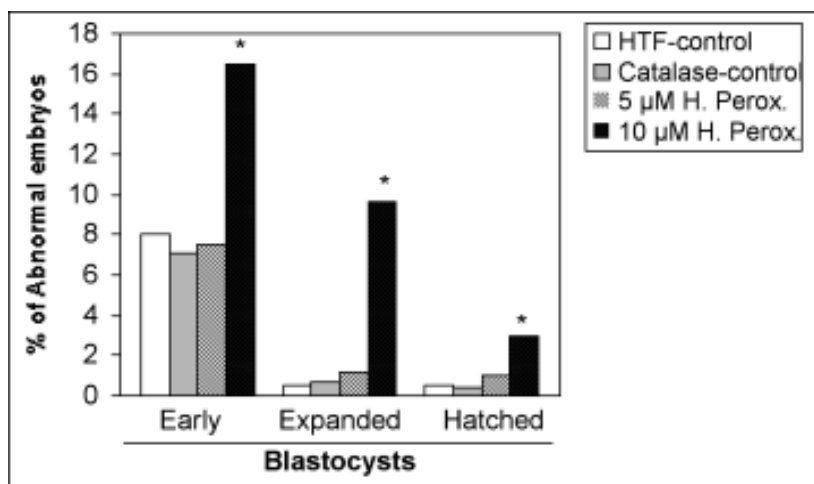
The analysis of embryo differentiation grade is shown in Figure 3. After 30 minutes of incubation, the percentage of expanded and hatching/hatched blastocysts were very significantly reduced with 10 and  $25 \mu\text{M H}_2\text{O}_2$  as compared to the control groups ( $p < 0.01$  and  $p < 0.001$ , respectively). However, similar percentage of early blastocyst was found with  $10 \mu\text{M H}_2\text{O}_2$  as compared to controls, although  $25 \mu\text{M H}_2\text{O}_2$  did significantly reduce the quantity of the type of blastocysts

( $p < 0.05$ ). Treatment with  $5 \mu\text{M H}_2\text{O}_2$  did not affect any of the embryo developmental stage studied.

Embryo morphology was studied after 30 min of incubation with  $\text{H}_2\text{O}_2$ , and is shown in Figure 4. Although  $10 \mu\text{M H}_2\text{O}_2$  exposition produced some blastocyst, there was a significantly increased percentage of morphologically abnormal blastocyst ( $p < 0.05$ ). Embryos were not affected with  $5 \mu\text{M H}_2\text{O}_2$  as compared to the control groups. The number of cells per embryo, however, was unaltered with either 5 or  $10 \mu\text{M H}_2\text{O}_2$  as compared to the control groups (Fig. 5).

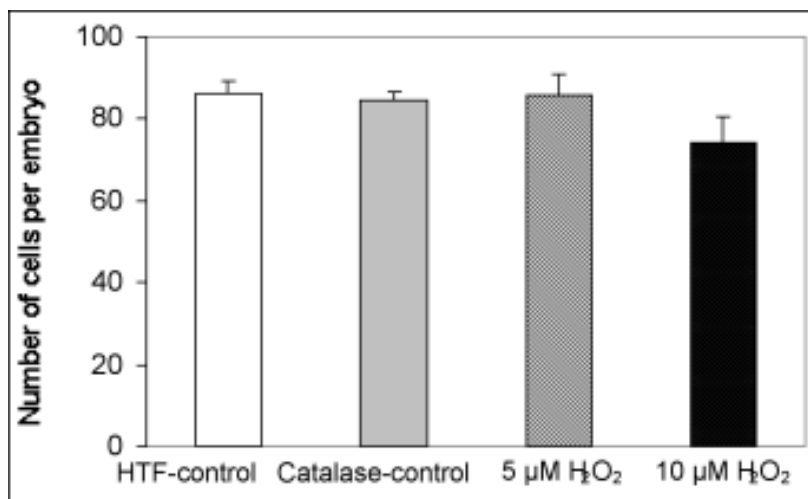
## Discussion

Oxidative stress, defined as an elevation in the steady-state levels of ROS that exceed the body's antioxidant defenses (Agarwal *et al.*, 2003), has been impli-



**FIGURE 4. Embryo morphology**

The quality of embryos developed at 72 h of culture after 30 minutes of incubation with hydrogen peroxide (H.Perox.) was recorded as the number of abnormal blastocysts (initial, expanded or hatched) over the total number of 2 cells embryos. \*:  $p < 0.05$  vs controls, Chi-squared test.



**FIGURE 5. Cleavage rate and embryo growth**

Following 30 minutes of incubation with different hydrogen peroxide (H.Perox.) concentration, the growth of embryos and their cleavage was analyzed by counting the number of cells per embryo. The results are expressed as the average number of cells, per embryo  $\pm$  S.E.M. Results were analyzed by Dunnett test.

cated in a number of different reproductive pathologies such as endometriosis, folliculogenesis, abnormal oocyte maturation, hydrosalpingeal fluid, necrozoospermia and asthenozoospermia (Goto *et al.*, 1993; Guerin *et al.*, 2001). The importance of the excessive production of ROS in the aetiopathogenesis of male and female infertility has been subject of a wide and intense analysis in the last years. The role of oxidative stress in the pathogenesis of early pregnancy losses, however, has not been completely documented.

The present results shows that early preimplantation embryogenesis is severely affected after short time exposition to ROS and that there were thresholds for H<sub>2</sub>O<sub>2</sub> concentration and time of exposition needed to induce embryo arrest or dismorphogenesis. Both, 30 and 60 minutes of exposition to H<sub>2</sub>O<sub>2</sub> negatively affected embryo development, resulting almost any blastocyst with 25 and 50 µM H<sub>2</sub>O<sub>2</sub> after 60 minutes of exposition and 10 µM H<sub>2</sub>O<sub>2</sub> incubation for 30 or 60 minutes produced very low blastocyst percentages. Embryo differentiation, morphology and embryo growth (number of cells per embryo) was, therefore, studied after H<sub>2</sub>O<sub>2</sub> exposition to 30 minutes.

While others authors reported that H<sub>2</sub>O<sub>2</sub> concentrations higher than 60 µM were embryotoxic (Zhang, 2005), we found that the minimal deleterious concentration of H<sub>2</sub>O<sub>2</sub> was 10 µM after a exposition for 30 minutes because it was sufficient to block 70% of 2 cells embryos for further development. This concentration produced a partial developmental inhibition since some embryos could reach the morula and expanded blastocyst stage; unlikely this later stage resulted morphologically abnormal. Higher concentrations of H<sub>2</sub>O<sub>2</sub> had strong inhibitory effects on preimplantation development since all 2 cells embryos were arrested and had fully lysed blastomeres at 72 h of culture. These results suggested a differential deleterious effect of H<sub>2</sub>O<sub>2</sub> depending on its levels. It is well known that the oxidative stress is involved in cell damage produced by *in vitro* culture. Some authors reported that the embryo detention at G2/M phase coincides with a rise in the level of ROS and that cleavage rates *in vitro* of mouse embryos can be increased by addition of ROS scavengers to the culture medium (Nasr-Esfahani and Johnson, 1991; Kuribayashi and Gagnon, 1996). Also, it was thought that low concentrations of H<sub>2</sub>O<sub>2</sub> derived from the sperm suspension after an *in vitro* fertilization can reduce the embryo development to blastocyst because ROS can be latent up to 3 to 5 days later (Aitken *et al.*, 1989). ROS can be produced either from intracellular sources, such as gametes or from extracellular ones, from environ-

mental factors. For instance, in the Assisted Reproductive Technology (ART), ROS can be generated in media during the preparation of semen, may increase within embryos or can be produced during culture *in vitro* (Goto *et al.*, 1993). Whether the increase of ROS from embryos is due to intrinsic changes in embryo metabolism or the result of a higher pO<sub>2</sub> or other environmental factors remains to be elucidated. Indeed, zygotes exposed to maternal heat stress had reduced embryo development by increasing intracellular oxidative stress (Orsi and Leese, 2001). Thus, ROS have an important role in the pathogenesis of the embryo arrest, in abnormal preimplantation embryo development and in early pregnancy losses.

Early embryos are more sensitive to the exposition time of an oxidative factor than somatic cells. Therefore, while concentrations of 500 µM to 5 mM of H<sub>2</sub>O<sub>2</sub> for 15-30 minutes affect the somatic cellular morphology producing oxidative effects such as rearrangements of the microfilaments and membrane blebbing prior to the cell death (Ozawa *et al.*, 2002), very short-term exposition to ROS may be sufficient to induce detention and abnormal preimplantation embryos. In the present work, 10 µM H<sub>2</sub>O<sub>2</sub> for 30 minutes resulted sufficient to produce an adverse effect on embryo development. Currently, incubation at shorter insemination times during IVF are preferred because defective spermatozoa can release ROS and produce subsequent oxidative damage (Quinn *et al.*, 1998). Reduction in the sperm-oocyte incubation time and lower sperm concentrations is recommended to minimize the development of oxidative stress.

It is known that apoptosis is a normal feature in preimplantation embryo development that plays an active role in the developing embryo through the removal of genetically or morphologically abnormal cells (Levy *et al.*, 2001). However, apoptosis rate may be affected by environmental factors including culture conditions and the composition of media (Brison, 2000). Studies have demonstrated that developmental toxicants, involved in pregnancy loss and gross structural anomalies, induce excessive apoptosis shortly after treatment (Toder *et al.*, 2002). Increased ROS levels can affect cell membranes, DNA, and mitochondria; these effects appear to be mediated, at least in part, by deregulation of the apoptotic cascade (Yang *et al.*, 1998). Extensive evidence suggest that high levels of H<sub>2</sub>O<sub>2</sub> induce cell death by apoptosis and/or necrosis in a variety of somatic cell systems depending on the concentration (Forrest *et al.*, 1994; Tada-Oikawa *et al.*, 1999; Halliwell and Aruoma, 1991; Wiese *et al.*, 1995). Mouse and hu-

man zygotes exposed to high  $H_2O_2$  levels displayed cell cycle arrest and cellular changes with apoptotic feature (Yang *et al.*, 1998; Liu *et al.*, 2000). Although it was shown that  $H_2O_2$ -treated zygotes shrank, they did not show the typical biochemical hallmarks of apoptosis during the first 48 h after treatment and only after 72 h of development arrest they exhibited nuclear DNA fragmentation (Orsi and Leese, 2001; Liu *et al.*, 2000; Harmon *et al.*, 1998). We suggest that  $H_2O_2$  inhibited and arrested the embryo division and then the embryo underwent or not to fragmentation in a similar manner to the apoptotic process, resulting at 72 h of culture in intact arrested 2 cells embryos or partially fragmented ones.

On the other hand, ROS can elicit apoptosis or necrosis depending on their concentration. The highest levels studied, 50  $\mu M$   $H_2O_2$ , could trigger a severe membrane damage by intensive oxidation, leading to immediately fully lyses, swollen and/or shrinkage blastomeres. Since 2 cells embryos found at 72 h of culture were morphologically fully lysed, we suggest that this high  $H_2O_2$  concentration had a necrotic effect. If the initial cell damage are moderate, it could be elicited an apoptotic response and therefore fragmented embryos appeared. The concentration of 25  $\mu M$   $H_2O_2$  produced necrotic and some fragmented 2 cells embryos. The fact that the arrested 2 cells embryos and morulae exposed to 10  $\mu M$   $H_2O_2$  were partially lysed, fragmented and/or had cytoplasm vesicles, suggest that this concentration produced a moderate damage at the membrane level, and that might produce embryo arrest and death by apoptosis rather than by necrosis. Therefore, 10  $\mu M$   $H_2O_2$  partially affected the 2 cells embryos allowing some of them undergo for further development, some embryos were arrested, and appeared fragmented and some developed to morphologically abnormal blastocysts. The fact that these blastocysts developed at 72 h did not show a reduced cell number, the timing of cell division appeared not to be affected by  $H_2O_2$ , suggesting that an apoptotic process could play a role in eliminating abnormal or defective embryos early in the first cell cycle similarly to other processes described (Jurisicova *et al.*, 1998).

It is known that oxygen concentrations used during *in vitro* culture not only can influence the embryo development and the cell number, but also gene expression. In this regard, compacting bovine embryo cultured under high oxygen tensions responded by altering the gene expression (Harvey *et al.*, 2004). Chromosome-type aberrations were found in metaphase cells 24 h after 10 min of  $10^{-5}$ - $10^{-3}$  M concentration- $H_2O_2$  (Oya *et al.*, 1986). Also, embryos of poor quality with various

degrees of fragmentation were found to have a higher incidence of cytogenetic abnormalities than embryos of good morphology (Pellestor *et al.*, 1994). We think that a similar process could be elicited by  $H_2O_2$  on the embryonic nuclei and that abnormal morphology of blastocysts may be associated with gene and/or chromosomal alterations. All these questions make the ROS exposition an important cause of congenital abnormalities, impaired gestation, early pregnancy losses and/or reproductive disorders that are commonly associated to unexplained infertility.

Finally, oxidative stress plays a significant role in the outcome of Assisted Reproductive Technology (ART). Many factors originated mainly from the external environment where the gametes and/or embryos are manipulated, can contribute to increase ROS during these procedures. For these important reasons, utmost cares should be taken to avoid inducing excessive ROS production. Efficient technical and quality control measures should be adopted for sperm preparation, laboratory environment should be optimized and proper culture media be selected in relation with ROS content and production. Additional studies are still needed to evaluate the use of antioxidants in an ART setting.

Although natural or *in-vivo* gamete damage induced by ROS is difficult to demonstrate (Agarwald *et al.*, 2003) because ROS levels are not normally measured (Goto *et al.*, 1993), experimental procedures as the present contribute to demonstrate an association between early ROS exposition, oxidative stress and impaired embryo development. Our results show that exposure of early embryos to low levels of  $H_2O_2$  for very short times produced deleterious effects on further embryo differentiation and that  $H_2O_2$  induced cell cycle arrest with morphological characteristics similar to apoptosis or necrosis. High levels of  $H_2O_2$  may induce necrosis while mild exposition may induce apoptosis. In absence of a severe effect, ROS exposition produced abnormal embryo morphogenesis at later preimplantation developmental stages. Future studies will allow elucidate the exact molecular mechanisms by which ROS induce oxidative stress, cellular damage and embryo arrest during early preimplantation development, and help to ascertain antioxidative therapies for treatment of infertility and /or early pregnancy losses.

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