

# Effect of pentoxifylline in reducing oxidative stress-induced embryotoxicity<sup>1</sup>

Xiaoyan Zhang,<sup>2</sup> Rakesh K. Sharma,<sup>2,3</sup> Ashok Agarwal,<sup>2</sup> and Tommaso Falcone<sup>2</sup>

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**Purpose:** To 1) evaluate the embryotoxic effects of hydrogen peroxide on mouse embryo development and 2) examine if pentoxifylline can reverse hydrogen peroxide induced embryotoxicity.

**Methods:** Prospective in vitro study examining the effects of varying concentrations of hydrogen peroxide and pentoxifylline on the blastocyst development rate alone as well as in combination.

**Results:** A dose-dependent decrease in % BDR was seen with increasing concentrations of H<sub>2</sub>O<sub>2</sub>. High concentrations of hydrogen peroxide (>60 μM) were embryotoxic. Pentoxifylline (500 μM) was able to reduce the embryotoxic effect of hydrogen peroxide. Percent blastocyst development rate increased from 44% in hydrogen peroxide alone to 85% in hydrogen peroxide and pentoxifylline coincubation.

**Conclusions:** Pentoxifylline may be beneficial in reducing hydrogen peroxide induced embryo damage and improve IVF outcome. Patients with endometriosis-associated infertility may benefit from the use of pentoxifylline without significantly affecting embryo development.

**KEY WORDS:** Embryotoxicity; hydrogen peroxide; pentoxifylline.

## INTRODUCTION

Reactive oxygen species (ROS) is involved in the modulation of an entire spectrum of physiological reproductive functions such as oocyte maturation, ovarian steroidogenesis, ovulation, implantation, formation of blastocyst, maintenance in pregnancy, corpus luteal function and even luteolysis (1,2). Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a strong oxidant and a major source of ROS. Oxidative stress occurs as

a result of an imbalance between the pro-oxidants and the ability of the antioxidants to scavenge the excess ROS production. Oxidative stress in female reproduction may be a major link in the infertility puzzle and in some reproductive diseases of women such as endometriosis (1,3).

High ROS levels are associated with endometriosis, unexplained infertility and poor in vitro fertilization (IVF) outcome. Peritoneal, follicular and hydrosalpingeal fluids represent important reproductive microenvironments (3–6). Evidence show increased oxidative stress in women with endometriosis (7). There may be multiple sources of ROS in an IVF setting including the oocytes, cumulus cell mass and spermatozoa used for insemination (8). Oxidative stress can damage oocytes and cause mitochondrial alterations, embryo block, adenosine triphosphatase depletion and apoptosis (9,10).

Pentoxifylline, a methylxanthine acts as a phosphodiesterase inhibitor. It inhibits phagocytosis and

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<sup>2</sup> Center for Advanced Research in Human Reproduction, Infertility and Sexual Function, Department of Obstetrics-Gynecology, and Glickman Urological Institute, Cleveland Clinic Foundation, Cleveland, Ohio.

<sup>3</sup> To whom correspondence should be addressed at Desk A19.1, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195, USA; e-mail: sharmar@ccf.org.

generation of toxic ROS and proteolytic enzymes. Pentoxifylline influences both the production of inflammatory mediators and the responsiveness of immunocompetent cells to inflammatory stimuli (11–14). The importance of protecting preimplantation embryos from oxidative stress is important and must be carefully monitored. The present study was designed to evaluate the embryotoxic effects of H<sub>2</sub>O<sub>2</sub> and pentoxifylline alone as well as in combination and examine if pentoxifylline could reverse H<sub>2</sub>O<sub>2</sub> induced embryotoxicity.

## MATERIALS AND METHODS

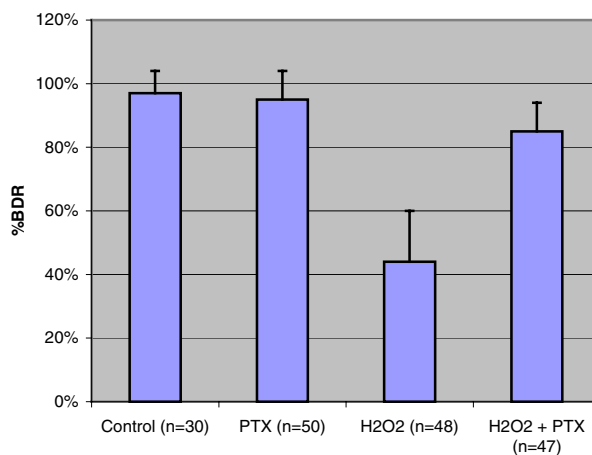
A culture system using cryopreserved two-cell mouse embryos (Embryotech laboratories, Inc., Wilmington, MA) was used to evaluate the effects of exogenous addition of hydrogen peroxide and pentoxifylline (Sigma Chemical Co., St. Louis, MO) alone as well in combination. In brief, frozen straws were thawed and only embryos with a normal morphological appearance were used. Incubation was carried out at 37°C under a humidified atmosphere of 5% CO<sub>2</sub> in air. Groups of 9–10 embryos were randomly added to the inner well of a petri dish containing 1 ml of human tubal fluid (HTF, Irvine Scientific, Santa Ana, CA) without oil overlay. Embryos in all groups were monitored daily at the same time in the morning using Normarski and bright field inverted optics. The number of embryos cleaving to morula and blastocysts (early, expanding, and expanded) were recorded. A blastocyst development rate (% BDR) of over 80% was considered satisfactory for internal control.

Hydrogen peroxide was diluted with HTF to give five working concentrations of 25, 50, 60, 75 and 100  $\mu$ M. A total of 326 embryos were divided into five groups and each concentration of H<sub>2</sub>O<sub>2</sub> was repeated at least three times. Similarly, a total of 239 embryos were used to examine the embryotoxicity of pentoxifylline. Pentoxifylline was dissolved in HTF to give five working concentrations of 250, 500, 1000, 2000 and 4000  $\mu$ M. Embryos cultured in HTF media ( $n = 72$ ) served as the control. Once the lowest dose of H<sub>2</sub>O<sub>2</sub> that was embryotoxic and the highest dose of pentoxifylline that was not embryotoxic was determined, we examined the beneficial effect of pentoxifylline in reversing H<sub>2</sub>O<sub>2</sub> induced embryotoxicity. A total of 175 embryos were cultured in three groups: H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) alone, pentoxifylline (500  $\mu$ M) alone and H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) + pentoxifylline (500  $\mu$ M).

Comparisons between groups were performed with Fisher's exact test. A  $P$ -value of  $<0.05$  was considered significant using the two-tailed test. Computations were performed with GraphPad In-Stat version 3.00 statistical software (GraphPad Software, Inc., San Diego, CA).

## RESULTS

A total of 740 embryos were used in the study to examine the embryotoxic effects of hydrogen peroxide and pentoxifylline. A dose-dependent decrease in % BDR was seen with increasing concentrations of H<sub>2</sub>O<sub>2</sub> compared with controls. A significant increase in embryotoxicity was seen at 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> compared with controls (% BDR: 69% versus 94%  $P < 0.01$ ). Higher concentrations were embryotoxic and a complete block was seen at the two-cell stages with 75  $\mu$ M of H<sub>2</sub>O<sub>2</sub>. Concentrations up to 500  $\mu$ M were not embryotoxic and. Compared with control, a significant decrease in % BDR (75% versus 96%  $P < 0.008$ ) was seen at 1000  $\mu$ M of pentoxifylline. Pentoxifylline at 500  $\mu$ M was able to reduce the embryotoxic effect of H<sub>2</sub>O<sub>2</sub>. A significant increase in % BDR was seen when embryos were incubated in the presence of pentoxifylline (Fig. 1). The % BDR increased from 44% in embryos cultured in the presence of H<sub>2</sub>O<sub>2</sub> (60  $\mu$ M) alone to 85% in culture medium containing H<sub>2</sub>O<sub>2</sub> and pentoxifylline ( $P = 0.0043$ ).



**Fig. 1.** Effect of pentoxifylline (PTX: 500  $\mu$ M) in combination with 60  $\mu$ M H<sub>2</sub>O<sub>2</sub> in reducing the embryotoxic effect of H<sub>2</sub>O<sub>2</sub>. Each point represents the mean of the readings in triplicate with 6–8 embryos in each group. The % BDR increased from 44% in H<sub>2</sub>O<sub>2</sub> alone to 85% in H<sub>2</sub>O<sub>2</sub> and pentoxifylline ( $P = 0.0043$ ).

## DISCUSSION

Oxidative stress has been proposed as a potential factor involved in the pathophysiology of endometriosis. Large amounts of ROS are released after chronic stimulation of peritoneal fluid macrophages in women with endometriosis. Oxidative stress-induced increase in autoantibody titers along with elevated levels of lysophosphatidyl choline have been demonstrated in the peritoneal fluid women with endometriosis (15).

During in vitro fertilization-embryo transfer (IVF-ET), the oxygen concentration is higher in in vitro culture environment compared with the in vivo conditions. Embryos and sperms may also generate ROS when they are cultured in vitro. Furthermore, when oocytes and embryos are removed from their natural environment for assisted reproduction techniques, their natural defense system is lost resulting in formation of ROS.

Reactive oxygen species in culture media affect fertilization, embryo development and clinical pregnancy rates. Elevated concentrations of ROS in day-1 culture media have been shown to be associated with lower pregnancy rates both with IVF and intracytoplasmic sperm injection (ICSI) (8). Our study demonstrates that the blastocyst development rate decreased significantly even at 60  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and higher concentrations caused a block at two-cell stage. Overproduction of ROS resulting from impaired intracellular milieu and disturbed metabolism is detrimental for the embryo (2).

Pentoxifylline is a 3',5'-nucleotidase phosphodiesterase inhibitor that protects cells from lipid peroxidation by ROS. It could be used as antioxidant in IVF. Furthermore, because of its potent immunomodulatory properties, pentoxifylline has been proposed as a treatment for endometriosis-associated infertility. Steinleitner and collaborators reported that pentoxifylline might fully reverse the infertility effects observed in rodent models of endometriosis (12,13). In our study, pentoxifylline at 500  $\mu\text{M}$  significantly reduced the embryotoxic effects of  $\text{H}_2\text{O}_2$ . This concentration of pentoxifylline is much higher than the peak plasma concentrations of pentoxifylline seen after intravenous infusion of 200 mg (about 10  $\mu\text{M}$ ) or 400 mg (about 4  $\mu\text{M}$ ) oral doses of capsule formulation (11). In this study, we did not examine the lower dose, it would be interesting to see if the lower doses of pentoxifylline are also equally effective in reducing the  $\text{H}_2\text{O}_2$  induced embryotoxicity. Our results indicate that pentoxifylline

may be beneficial in reducing  $\text{H}_2\text{O}_2$  induced embryo damage and improve IVF outcome. Patients with endometriosis-associated infertility may benefit from the use of pentoxifylline without significantly affecting the embryo development.

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