

Vitamin C and Vitamin E supplementation reduce oxidative stress–induced embryo toxicity and improve the blastocyst development rate

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Objective: To evaluate the adverse effects of exogenously induced reactive oxygen species (ROS) on mouse embryo development by using the 12-phorbol 13-myristate acetate (PMA)–activated leukocyte model as a source of ROS, and to examine the protective effect of antioxidant supplementation (vitamin C and vitamin E).

Design: Prospective study.

Setting: Research laboratory.

Main Outcome Measure(s): Effects of ROS on the blastocyst development rate in the presence and absence of antioxidant supplementation.

Result(s): After incubation with the PMA-activated leukocyte supernatant, the median (25th, 75th percentile) rate of blastocyst development significantly decreased from 73% (60%, 80%) after 3 hours to 30% (20%, 40%) after 6 hours compared with control reactions (86% [80%, 100%]). Co-incubating the embryos with vitamin C (50 μ M) and the PMA-activated supernatant significantly increased the blastocyst development rate from 73% (60%, 80%) to 90% (80%, 91%) at 3 hours and from 30% (20%, 40%) to 91% (89%, 91%) at 6 hours—a level similar to that of control reactions. The blastocyst development rate increased after vitamin E supplementation (400 μ M) at 6 hours, but not significantly and not by as much as after vitamin C supplementation.

Conclusion(s): Exposure of mouse embryos to ROS for extended periods results in embryotoxicity. Vitamin C is more effective than vitamin E in reversing ROS-induced mouse embryo toxicity. (Fertil Steril® 2002;78:1272–7. ©2002 by American Society for Reproductive Medicine.)

Key Words: Reactive oxygen species, leukocytes, embryo, antioxidant, blastocyst development

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Reactive oxygen species (ROS) are produced during aerobic metabolism, even under basal conditions. In both oocytes and embryos, various metabolic pathways and enzymes can produce endogenous ROS (1–3). Reactive oxygen species may originate directly from the embryos or their surroundings. Several exogenous factors, such as oxygen consumption, metallic cations, visible light, amine oxidase, and spermatozoa, can increase the amount of ROS produced by embryos (4–7).

When ROS production overwhelms antioxidant capacity, oxidative stress occurs. Oxidative stress seems to damage embryos by causing peroxidation of membrane phospholipids and altering most types of cellular molecules,

such as lipids, proteins, and nucleic acids (8, 9). The consequences of such damage include mitochondrial alterations, embryo cell block, adenosine triphosphatase depletion, and apoptosis (programmed cell death) (10–12).

Oocytes and embryos seem to be protected against oxidative stress by oxygen scavengers that are present in follicular and oviductal fluids. In addition, several antioxidant enzymes guard against peroxidative damage (e.g., superoxide dismutase, catalase, and glutathione peroxidase) (13, 14).

However, when oocytes and embryos are removed from their natural environment for assisted reproduction techniques, this natural

defense system is lost. Therefore, the importance of protecting preimplantation embryos from oxidative stress *in vitro* is being increasingly recognized. To optimize *in vitro* embryo production, oxidative stress must be controlled during *in vitro* culture.

The objectives of this study were to evaluate the adverse effects of exogenously induced ROS on mouse embryo development *in vitro* by using a phorbol ester, 12-phorbol 13-myristate acetate (PMA)-activated leukocyte model as a source of ROS, and to examine the protective effect of antioxidant supplementation (vitamin C and vitamin E) in reducing ROS-induced embryotoxicity.

MATERIALS AND METHODS

Leukocyte Preparation and Activation

A purified population of polymorphonuclear granulocytes, hereafter referred to as "leukocytes," was isolated from freshly collected peripheral blood by centrifugation through a discontinuous Histopaque gradient (Sigma Diagnostics, St. Louis, MO). In brief, 3 mL of Histopaque-1119 (1.119 g/mL) was overlaid with 3 mL Histopaque-1077 (1.077 g/mL) in a 15-mL conical-bottomed centrifuge tube. A 5-mL sample of blood was pipetted onto this gradient and centrifuged at 700 *g* for 30 minutes. The leukocyte-rich population was removed from the high-density Histopaque layer and was washed three times (500 *g* for 10 minutes) with phosphate-buffered saline (pH, 7.4).

To stimulate ROS production, 100 nM of PMA was added to the leukocytes. A linear ROS response was determined by using a leukocyte concentration of 0.25 to 4×10^6 cells/mL. The PMA-activated leukocyte suspension was incubated at 37°C for 30 minutes in the dark. The leukocyte concentration was adjusted to a final concentration of 0.5×10^6 cells/mL by adding equilibrated human tubal fluid (HTF) (Irvine Scientific, Santa Ana, CA). The amount of ROS produced by the leukocytes alone (no PMA activation), the PMA-activated leukocytes, and the PMA-activated leukocyte-free supernatant was measured at 0, 3, and 6 hours.

Reactive Oxygen Species Measurement

Levels of extracellular and intracellular ROS (hydrogen peroxide, superoxide, and hydroxyl ions) were measured by using a chemiluminescence assay and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma Chemical Co., St. Louis, MO) as a probe (15). Measurements were made using a Berthold luminometer (Autolumat LB 953; Wallace, Inc., Gaithersburg, MD). A 400- μ L aliquot of unprocessed specimens was prepared in duplicate, along with the blank and control. Ten microliters of a 5-mmol solution of luminol prepared in dimethyl sulfoxide (Sigma Chemical Co.) was added to the 400 μ L of fresh culture media. Chemiluminescence was measured for 15 minutes in the integrated mode, and the results were expressed as $\log_{10}(\text{ROS} + 1) \times 10^6$ cpm.

Blastocyst Development Rate in a 2-Cell Mouse Embryo *In Vitro* Model

Human tubal fluid was equilibrated by overnight incubation at 5% CO₂ and 37°C. Frozen straws containing 2-cell mouse embryos (Embryotech Laboratories, Inc., Wilmington, MA) were exposed to room temperature for 2 minutes. Each straw was bisected between the lower heat seal and the column of medium. By using the stylet, the contents of the straw were flushed as a single drop into a sterile culture dish (Allegiance Health Care, Inc., McGaw Park, IL). Immediately thereafter, the embryos were divided into four groups: embryos and HTF (control group); embryos, HTF, and inactivated leukocytes; embryos, HTF, and PMA-activated leukocytes (0.5×10^6 cells/mL); and embryos, HTF, and PMA-activated leukocytes supernatant. These embryos were then incubated at 37°C in an atmosphere of 5% CO₂ for 3 hours and 6 hours.

After incubation, the embryos were transferred to freshly equilibrated HTF media, and the blastocyst development rate was calculated by determining the number of embryos that had reached the blastocyst development stage after 72 hours' incubation. Both late blastocysts (blastocoeles greater than half the volume of the embryo) and expanding blastocysts (blastocoeles that are fully expanded, with a thin zona pellucida) were included in the blastocyst development rate. The control group (embryos and HTF) demonstrated a blastocyst development rate >80%. For all subsequent studies, a blastocyst development rate <50% was considered embryotoxic.

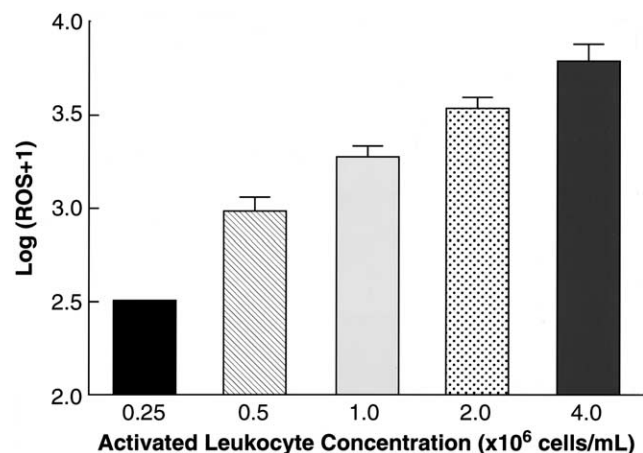
Supplementation of Culture Media With Antioxidants

To determine whether the toxic effects of the PMA-activated leukocyte supernatant were due to ROS formation and not to another secreted product, we assessed whether embryotoxicity (as reflected by a reduced blastocyst development rate) could be reversed by adding the antioxidants vitamin C and vitamin E to the culture media. If these substances improved the blastocyst development rate, ROS would be considered the reason for embryotoxicity.

Vitamin C (L-ascorbic acid; Sigma Diagnostics, St. Louis, MO) and vitamin E (water soluble α -trolox analogue; 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; Sigma-Aldrich, Milwaukee, WI) concentrations were standardized. The vitamin C concentration ranged from 0 to 400 μ M, and the vitamin E concentration ranged from 0 to 800 μ M. The optimum concentrations of vitamin C and vitamin E were added to the culture media and co-incubated with the embryos alone and with the embryos and the PMA-activated leukocyte supernatant for 3 hours and 6 hours. The embryos were then transferred to fresh HTF media and incubated for 72 hours to study changes in blastocyst development rate.

FIGURE 1

The linear response (mean \pm SD) of log (ROS + 1) values versus activated leukocyte concentrations. Reactive oxygen species (ROS) response was determined in triplicate by using activated leukocyte concentrations ranging from 0.25 to 4.0×10^6 cells/mL.



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Statistical Analysis

Continuous variables were compared between groups by using the Wilcoxon rank-sum test. Blastocyst development rates were compared by using logistic regression. Changes in the blastocyst development rate were compared by performing logistic regression for the blastocyst development rate at 6 hours and adjusting for blastocyst development rate at 3 hours as a covariate. Calculations were performed with SAS software version 8.1 (SAS Institute Inc., Cary, NC), and all tests were two-tailed. $P < .05$ was considered significant. Results are reported as the median (25th, 75th percentile), unless otherwise noted.

RESULTS

Reactive Oxygen Species Levels

In a preliminary study, we evaluated the concentration-dependent ROS response by using PMA-activated leukocytes at concentrations of 0.25 to 4.0×10^6 cells/mL. The mean (\pm SD) ROS level was 2.51 ± 0.006 at a leukocyte concentration of 0.25×10^6 cells/mL, 2.99 ± 0.07 at a concentration of 0.50×10^6 cells/mL, 3.27 ± 0.059 at 1.0×10^6 cells/mL, 3.53 ± 0.06 at 2.0×10^6 cells/mL, and 3.79 ± 0.10 at a leukocyte concentration of 4.0×10^6 cells/mL. Levels of ROS at these concentrations showed a linear response (Fig. 1). For all subsequent studies, a concentration of 0.5×10^6 cell/mL was used. Levels of ROS produced by the control reaction were similar (0.136 ± 0.07) at 3, 6, and 72 hours.

The median (25th, 75th percentile) level of ROS generation by the PMA-activated leukocytes was 2.94 (2.92, 3.01). The median ROS level in the PMA-activated leukocyte supernatant was 1.12 (1.07, 1.19) at 0 hours. The ROS levels significantly decreased after incubation with PMA-activated leukocyte supernatant for 3 hours (0.57 [0.34, 0.65]; $P < .001$) and 6 hours (0.53 [0.24, 0.43]; $P < .001$).

Effects of PMA-Activated Leukocyte Supernatant on Mouse Embryo Development

Similar mean ROS levels were produced by embryos incubated with HTF alone (0.14 ± 0.07 at 0 hours, 0.13 ± 0.11 at 3 hours, 0.12 ± 0.13 at 6 hours, and 0.14 ± 0.11 at 72 hours). When PMA-activated leukocytes were used, we observed leukocyte clumping and agglutination after 3 hours and 6 hours of incubation. This resulted in a cellular film that surrounded the mouse embryo, which made it difficult to transfer the embryos. Therefore, we decided to use the PMA-activated leukocyte supernatant.

After the embryos were incubated with PMA-activated leukocyte supernatant for 3 hours, the blastocyst development rate significantly decreased from 86% (80%, 100%) to 73% (60%, 80%) ($P < .02$). After 6 hours of incubation, the blastocyst development rate significantly decreased from 86% (80%, 100%) to 30% (20%, 40%) ($P < .0001$) (Table 1).

Effects of Vitamin C and E on Mouse Embryo Development

In the first set, we examined the embryotoxic effect of vitamin C at concentrations ranging from 25 to 400 μ mol. Vitamin C alone at concentrations of 50 and 100 μ mol did not result in embryotoxicity, and the blastocyst development rate was similar to that of the control reaction. However, at higher concentrations (200 and 400 μ mol), vitamin C resulted in embryotoxicity (Fig. 2A).

In the second set, we examined the concentration-dependent effect of vitamin E (100 to 800 μ mol) on mouse embryo development. Vitamin E alone at concentrations of 100, 200, and 400 μ mol did not have an embryotoxic effect, and the blastocyst development rates were similar to those of the control reaction. Higher concentrations of vitamin E (600 and 800 μ mol), however, resulted in embryotoxicity (Fig. 2B).

Co-incubation of Antioxidants With PMA-Activated Leukocyte Supernatant

Co-incubating the embryos with vitamin C at 50 μ M for 3 hours and 6 hours with the PMA-activated leukocyte supernatant significantly improved the blastocyst development rate. Incubation with vitamin C increased the blastocyst development rate from 73% (60%, 80%) (overall mean blastocyst development rate, 69%) to 90% (80%, 91%) (overall rate, 87%) at 3 hours and from 30% (20%, 40%) (overall rate, 30%) to

TABLE 1

Effect of reactive oxygen species–induced embryotoxicity after incubation with 12-phorbol 13-myristate acetate–activated leukocyte supernatant and reactive oxygen species scavenger effect of vitamin C and vitamin E.

Reaction	No. of embryos	No. of blastocysts	Blastocyst development rate (%) ^a
Control	51	45	86 (80, 100)
PMA-activated supernatant (3 h)	52	36	73 (60, 80)
PMA-activated supernatant (6 h)	40	12	30 (20, 40)
Vitamin C, 50 μ M (3 h)	31	27	90 (80, 91)
Vitamin C, 50 μ M (6 h)	31	28	91 (89, 91)
Vitamin E, 400 μ M (3 h)	33	22	72 (65, 77)
Vitamin E, 400 μ M (6 h)	33	17	51 (43, 61)

Note: PMA = 12-phorbol 13-myristate acetate.

^aValues are median (25% and 75% percentile).

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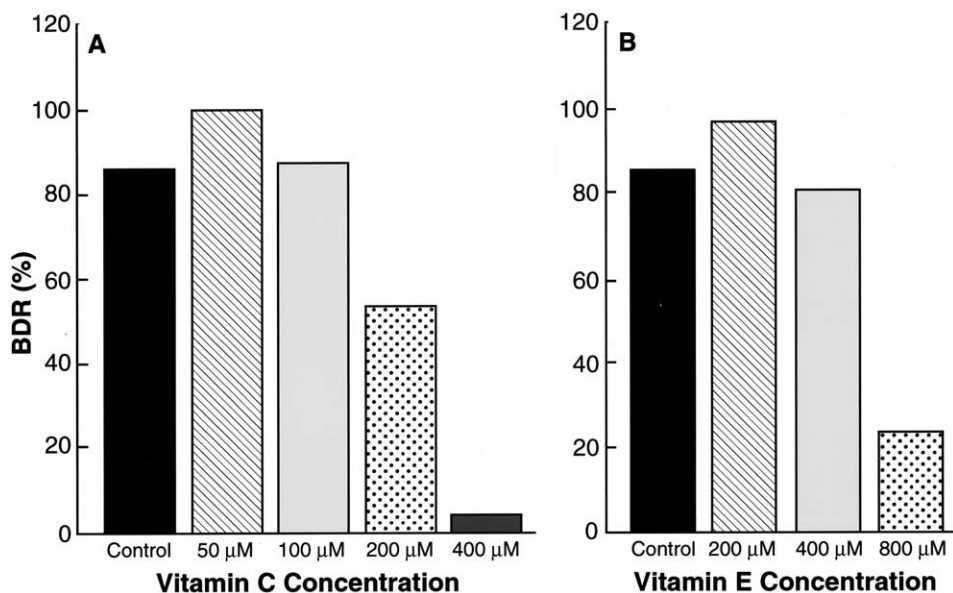
91% (80%, 91%) (overall rate, 87%) at 6 hours (Table 1, Fig. 3).

After 6 hours of incubation, vitamin C was more effective than vitamin E in reversing the embryotoxic effect of ROS generated by the PMA-activated leukocyte supernatant (overall mean blastocyst development rate, 90%), and the blastocyst development rate was similar to that of the control group (overall mean blastocyst development rate, 88%) (Table 1, Fig. 3). No significant difference was seen between embryos incubated with vitamin E (400 μ m)–

supplemented culture media for 3 hours in the presence of PMA-activated leukocyte supernatant (71.5% [65%, 76.5%]) and those incubated without vitamin E (73% [60%, 80%]). Embryos that were co-incubated with vitamin E for 6 hours with the PMA-activated leukocyte supernatant had a significantly improved blastocyst development rate, from 30% (20%, 40%) (overall mean blastocyst development rate, 30%) without vitamin E to 51% (43%, 61%) (overall rate of 52%) ($P < .04$) with 400 μ m of vitamin E. Vitamin E was less effective in reversing the embryotoxic effect of ROS (Table 1, Fig. 3).

FIGURE 2

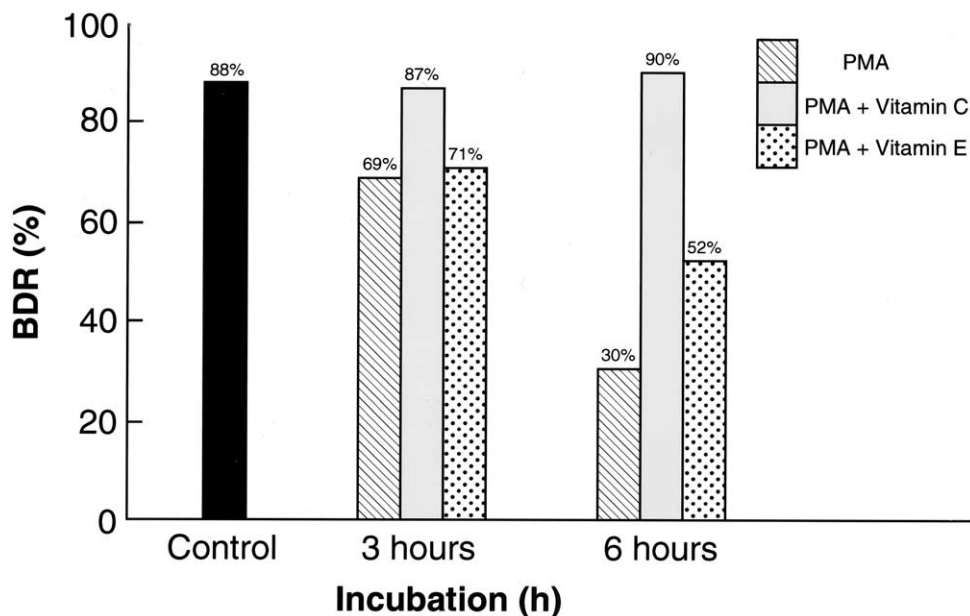
Effect of antioxidant supplementation on mouse embryo development, expressed as the blastocyst development rate (BDR). (A), Effect of vitamin C. The control group consisted of embryos cultured in human tubal fluid medium. (B), Effect of vitamin E.



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FIGURE 3

Comparison of ROS scavenging activity of the antioxidants vitamin C and vitamin E on development of mouse embryos, expressed as the mean overall blastocyst development rate (BDR). Control = embryos cultured in human tubal fluid medium only; PMA = embryos cultured in 12-phorbol 13-myristate acetate (PMA)-activated leukocyte supernatant; PMA + vitamin C = embryos cultured in PMA-activated leukocyte supernatant and vitamin C (50 μ M); PMA + vitamin E = embryos cultured in PMA-activated leukocyte supernatant and vitamin E (400 mg). Reactions were done for 3 hours and 6 hours. At 6 hours, vitamin C significantly reversed the embryotoxicity induced by PMA-activated leukocyte supernatant.



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DISCUSSION

Our study demonstrates that exposing mouse embryos to PMA-activated leukocyte supernatant for 3 hours and 6 hours produces ROS and results in oxidative stress, which in turn decreases the blastocyst development rate. When embryos were co-incubated for 72 hours, no embryo development occurred and the blastocyst development rate was 0%. Embryo arrest at the 2-cell stage was observed, and more than 90% of the embryos degenerated. This observation establishes that PMA-activated leukocyte supernatant results in a time-dependent embryotoxicity.

Production of ROS by embryos can be distinguished from other sources of ROS by three main characteristics. First, superoxide anion, hydrogen peroxide, and hydroxyl radical are produced, which are particularly important during in vitro culture. Second, production of hydrogen peroxide in vitro increases compared with in vivo-derived embryos. Finally, the amount of ROS varies according to the stage of development (1, 5).

Another potential mechanism responsible for ROS-induced embryo damage during in vitro fertilization could be related to the deleterious effect of ROS-producing spermatozoa during in vitro insemination of oocytes. In vitro incu-

bation of oocytes with a critical number of ROS-producing spermatozoa that remain outside the oocyte could lead to oxidative damage of the oocytes or pronucleate embryos (8). Deleterious effects of ROS during oocyte maturation may alter embryo development, and impaired embryo development may be associated with increased generation of ROS by the embryo (16).

When PMA-activated leukocytes were used, we observed leukocyte clumping and agglutination after 3 hours and 6 hours of incubation. This resulted in a cellular film that surrounded the mouse embryo, which made it difficult to transfer the embryos. Therefore, we decided to use the PMA-activated leukocyte supernatant.

Although PMA-activated leukocytes may release other secretory products, the embryotoxic effects were reduced or reversed by supplementation with antioxidants. Therefore, we concluded that the embryotoxic effects in the leukocyte-activated culture medium were largely due to ROS toxicity.

We chose the PMA-activated leukocyte supernatant model for ROS production because ROS is produced both by exogenous factors in the incubation media as well as endogenously by the leukocytes and embryos. Embryos may also have different sensitivities to ROS at different developmen-

tal stages (17). For example, 9- to 16-cell bovine embryos are reported to be more resistant to exogenous H₂O₂ than are zygotes and blastocysts. Different sensitivities could be attributed to variations in the defense mechanism thresholds. The PMA-stimulated peripheral blood leukocytes demonstrate the same characteristic pattern of ROS generation as seminal leukocytes (18).

Ascorbic acid (vitamin C) is an important water-soluble antioxidant that reduces sulfhydryls, scavenges free radicals, and protects against endogenous oxidative DNA damage (19). Vitamin C may become a pro-oxidant when free transition metals are present (1, 20). Addition of ascorbic acid to an embryo culture significantly affects embryo development. Vitamin E, the predominant lipid-soluble antioxidant in animal cells, protects cells from oxygen radicals both in vivo and in vitro. Significant amounts of vitamin E are present in the ovary and follicular fluid (15). Supplementation of culture media with vitamin E has been shown to increase the blastocyst development rate in vitro (21). Vitamin C supplementation in the culture media may be more beneficial than vitamin E supplementation.

Simultaneous supplementation with vitamin C (50 μM) and vitamin E (400 μM) in the HTF medium was more embryotoxic than incubation with each vitamin alone. Supplementation with both ascorbate and α-tocopherol in a sperm preparation medium has been shown to induce DNA damage and intensify the damage induced by hydrogen peroxide (22).

In conclusion, the presence of ROS in the culture media for extended periods (6 hours) results in mouse embryotoxicity. Hence, we recommend reducing incubation time and supplementing the culture media with vitamin C or vitamin E to help scavenge the excessive production of ROS in the culture media and improve embryo development.

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