5-1995

Effects of Antioxidants on Development of In Vitro Fertilized Bovine Embryos

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EFFECTS OF ANTIOXIDANTS ON DEVELOPMENT OF IN VITRO FERTILIZED BOVINE EMBRYOS

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Animal Science
(Reproductive Biology)

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

1995

ABSTRACT

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Free radicals are short-lived molecules that can cause decreased embryonic development in vitro. Antioxidants are molecules that block free radical formation or guard against their harmful effects. Many studies have linked exposure of media to light and culturing of embryos in high (20%) oxygen concentrations to free radical production. Some of the antioxidants used in culture media are superoxide dismutase (SOD), catalase, zinc (II), ethylenedinitrilo tetraacetic acid (EDTA), mannitol, vitamin E, dimethyl sulfide, and taurine. Most research involving antioxidants and embryonic development has been conducted on non-farm animals, particularly mouse and rabbit. Studies have shown that antioxidants in vitro culture improved embryo development to the blastocyst stage.

In this study, we evaluated the effects of SOD and catalase on bovine embryo development. Four concentrations

of SOD (0, 1500, 3000, 6000 IU/ml) and catalase (0, 75, 100, 125 µg/ml) and combinations of the two antioxidants were evaluated through maturation, fertilization, and culture. SOD and catalase were first reconstituted in water and then diluted to their final concentrations. Oocytes were matured in M-199 plus 0.5 µg/ml LH, 5 µg/ml FSH, and 10% FBS at 39 °C in 5% CO_2 for 24 hours. They were then placed in fertilization-TALP with heparin and 1 X 10^6/ml sperm. Embryos were cultured in CR2 medium supplemented with alanine, glycine, and 3 mg/ml of fatty-acid free bovine serum albumin in modular incubators with 5% CO_2, 5% O_2, and 90% N_2. Embryo development was evaluated on day 8. Three replicates with approximately 50 embryos per treatment were used to evaluate the effects of SOD and catalase. The control had better embryo development than all treatments. The treatment that was most similar to the control was treatment 2, which consisted of no SOD and 75 µg/ml catalase.

Based on these observations, levels of both SOD and catalase were lowered to 0, 100, 250, and 500 IU/ml and 0, 10, 25, and 50 µg/ml, respectively. Although these levels appeared to improve embryo development, there were no statistical differences. Based on the culture system and media currently used along with the precautions against light and oxygen concentration, we did not find any

beneficial effects of supplementing medium with SOD or catalase.

(51 pages)

ACKNOWLEDGMENTS

I would like to express my deep appreciation to Dr. Thomas D. Bunch, professor of animal, dairy and veterinary sciences, for his guidance and assistance throughout my graduate program at Utah State University. I would also like to thank the other members who served on my thesis committee: Dr. Kenneth L. White, associate professor of animal, dairy and veterinary sciences, and Dr. LeGrande C. Ellis, professor of biology.

In addition, I would like to extend appreciation to the following graduate students and laboratory technicians: Caiping Yue, Vicki Farrar, Charoensri Thonabulsombat, and William Reed for their time, assistance, and suggestions. I also wish to thank Eddie Sullivan for his patience as he taught me IVF.

I also wish to thank E.A. Miller Inc. for their generous donation of bovine ovaries. Without their contribution, work in this area would be next to impossible.

A special thanks to my dear family for the love, support, and encouragement as I pursued my master's degree.

I would also like to acknowledge the Experiment Station and the Utah Center of Excellence for their assistance in funding this project.

Bret L. Anderson

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INTRODUCTION

Factors that affect mammalian embryo development have been a topic of extensive research for a number of years. One area that continues to be of interest is creating an in vitro environment for embryo production that closely mimics conditions of the female reproductive tract. Numerous studies have been conducted to determine why embryos do not develop as readily in vitro as they do in vivo. The fact that mammalian embryos produced in vitro develop slower or less efficiently has been well documented in several species (Wright and Bondioli, 1981; Fisher, 1987). Embryos often stop their development at what is referred to as the "block." In most species the block occurs when the genetic system shifts from maternal to genomic DNA synthesis. Many researchers have speculated on the cause of the block. Most recently, there has been evidence reported in the literature that free radicals may be a contributing factor to the developmental retardation of embryos (Li et al., 1993).

Since free radicals are common in most in vitro cell culture systems, researchers have begun to either remove or minimize their effects. One of the first areas to be investigated was to alter the amount of oxygen concentration to which embryos are exposed. Most embryo culture systems are maintained under a gas atmosphere of 5% CO_2 and 95% humidified air. Some researchers have cultured embryos under an atmosphere of 5-8% O_2, which is within the range of O_2 concentrations within the mammalian oviduct. This is

considerably lower than the 20% O_2 that is in humidified air (Li et al., 1993). By lowering the O_2, there was an increase in the percentage of embryos that developed to the blastocyst stage. Beneficial affects of lowering O_2 concentrations could be detected as low as 1% O_2.

It has been established that the concentration of O_2 in in vitro culture systems plays a significant role in the production of harmful reactive oxygen species (Noda et al., 1991; Li et al., 1993; Nakayama et al., 1994). Studies have also shown that the incorporation of free radical scavengers into culture systems alters the adverse effects of reactive oxygen species (Noda et al., 1991; Li et al., 1993; Natsuyama et al., 1993a). One study performed in bovine (Luvoni et al., 1994) found that incorporation of superoxide dismutase (SOD) into embryo culture media exposed to atmospheric oxygen did increase the number of embryos developing to the blastocyst stage. However, if embryos were cultured with SOD under low oxygen concentration, the increase in embryo development was not nearly as great. Catalase and SOD, which are both free radical scavengers, have been shown to be beneficial to the in vitro development of mouse embryos (Nonogaki et al., 1991; Nonogaki et al., 1992; Natsuyama et al., 1993a; Chun et al., 1994; Luvoni et al., 1994). In these studies, they reported that blastulation rate increased with the use of radical scavengers.

free radical production. Another way that is probably equally damaging to embryos is exposure of media and(or) embryos to fluorescent light. An unpublished study performed in our laboratory showed that prolonged exposure of bovine embryos to fluorescent light decreased the percentage that developed to the blastocyst stage. Another study done on the hamster also verified the detrimental effects on embryo development (Nakayama et al., 1994). The detrimental effects on development have been linked to free radical production in prolonged light exposure and high oxygen concentration cultures. These two free-radical-causing factors should be of concern to anyone dealing with in vitro embryo production.

Our laboratory expends considerable effort studying factors that affect preimplantation embryo development in livestock species. The types of studies range from evaluating factors affecting oocyte maturation and in vitro fertilization, to the development of a better culture medium. This study was designed to evaluate the effects of catalase, SOD, and a reduced oxygen concentration in all phases (maturation of the oocyte, fertilization, and culture) of in vitro bovine embryo production.

LITERATURE REVIEW

A "free radical" is a molecule with one or more unpaired electrons (Gutteridge and Halliwell, 1988; Kehrer, 1993). Free radicals exist in free form, which makes them highly unstable. In their free form, they interact with various tissue components, causing metabolic and genetic dysfunction. Free radicals exist in various forms, consisting of both organic and inorganic molecules. While these molecules are very reactive and sometimes destructive, they are very necessary for the normal activity of many biological processes (Dianzani, 1992). Studies on free radicals date back to the mid 1950's. Their destructive and(or) beneficial effects on in vivo and in vitro systems continue to be actively pursued. The scope of this study will focus on the effects of oxygen free radicals in a bovine in vitro embryo culture system.

There are three types of highly reactive oxygen free radicals: superoxide radical, hydrogen peroxide, and the hydroxyl radical. These molecules have single unpaired electrons and therefore are classified as free radicals (Marklund, 1985).

Many studies have been conducted to find out where and how free radicals are formed. Free radicals are commonly formed within cells by the leakage of electrons to oxygen during electron transport. More specifically, this happens during oxidation of mitochondrial cytochrome P450, in the

endoplasmic reticulum, or by activity of other oxidase systems (Halliwell and Gutteridge, 1989; Gille and Joenje, 1991; Johnson and Nasr-Esfahano, 1994). A study that quantified free radicals by electron-spin-resonance signals in rat liver mitochondria found them to be very abundant. Not only were free radicals abundant in the liver samples, but the levels of radicals would also change by either starving the animals (increasing radical production) or by chemically inhibiting respiration (decreasing radical production) (Slater, 1972). Free radicals are also formed throughout normal cellular metabolism. They also result from the metabolism of some drugs and xenobiotics (Bendich, 1993).

Free radicals form in in vitro cultures. Exposure of cultures to light, both fluorescent and UV, have been shown to have detrimental effects. Living organisms absorb light by either endogenous or exogenous photosensitizers, which in the presence of oxygen cause oxidation, which leads to many chemical and biological effects (Blum, 1964). Unpublished studies in our laboratory have indicated that exposure of embryos to fluorescent light for as little as 15 min tended to cause developmental disruption.

The superoxide anion is produced through the reduction of molecular oxygen by the addition of a single electron (Fee and Valentine, 1977). Controlling the production of the superoxide radical may minimize potential adverse

effects in tissue culture systems. As was pointed out in a study done by Warren et al. (1987), there are quite a number of different ways that radicals can be produced. One of the common methods to produce oxygen radicals is by changing oxygen concentrations. Normally, $O_2 + 4e + 4H^+$ produces $2H_2O$. Variations in the normal pathway of production of $2H_2O$ creates highly reactive oxygen species. The addition of an electron to O_2 produces superoxide. The addition of a second electron and hydrogen atom to the superoxide anion results in hydrogen peroxide. The combination of two superoxide anions forms hydrogen peroxide. Hydrogen peroxide combined with another electron and a hydrogen atom results in the production of hydroxyl radicals (Ellis, 1990a). Another common means of hydroxyl radical production occurs during the Fenton reaction. In this reaction, ferrous iron reacts with hydrogen peroxide to produce ferric iron and hydroxyl radicals. As has been indicated, the processes that form oxygen free radicals are numerous.

Due to the configuration of free radicals, they are highly reactive and may cause cell injury. Injury is any deviation from normal metabolic activity, whether determined histopathologically or by chemical analysis (Slater, 1972). Whenever cellular defense mechanisms are unable to keep up with an assault, such as in times of disease, cellular dysfunction occurs (Kaul et al., 1993). An example is free radical buildup in tissues during organ transplant

procedures. Transplants are very susceptible to radical buildup due to long periods of ischemia. One study showed that after long-term ischemia, reoxygenation of the myocardium resulted in production of high levels of superoxide anions. The mechanism(s) of superoxide production may be by xanthine oxidase activity, neutrophil activation, and(or) arachidonate cascade activation. Superoxide accumulation may inhibit enzyme activation and lipid peroxidation in the sarcolemma, which then could lead to intracellular calcium buildup and excitation-contraction uncoupling (Keith, 1993). Another study showed that injury to myocardial cells may result from a disruption of the membranes, altering their integrity and increasing their fluidity and permeability (Ferrari et al., 1991). Transplants of liver, kidney, and lung have also been shown to produce free radicals during periods of ischemia. Presence of free radicals was determined by increased quantities of antioxidants (Paller and Jacob, 1994; Gao et al., 1995; Katz et al., 1995).

Lack of oxygen in tissues followed by reperfusion has been linked to perinatal brain damage and breast cancer. The perinatal brain is less susceptible than at any other time throughout development, but periods of hypoxia still lead to brain tissue injury. It is thought that the same deleterious mechanisms in heart transplants play a role in damaging the perinatal brain (Kjellmer, 1991).

Under conditions that cause localized hypoxia, ATP is broken down to the superoxide radical. This reaction occurs over several steps involving a host of intermediates. The products of superoxide radicals have been shown to be carcinogenic in breast tissue. Chemical carcinogens in the breast lead to fibroblast proliferation, hyperplasia of endothelium, cellular atypia, and then cancer. Murrell (1991) continued by suggesting that radicals tend to be formed in nonlactating breasts. Patients who have high levels of prolactin and low levels of oxytocin are most susceptible.

Free radicals have also been linked to diseases of the central nervous system and aging. Cadet and Kahler (1994) believe that oxygen free radicals play a role in some neuropsychiatric and movement disorders such as schizophrenia and neuroleptic-induced tardive dyskinesia. The presence of oxyradicals interferes with the normal metabolism of catecholamines. They have shown that treatment of tardive dyskinesia with antioxidants helps to alleviate some of the movement problems associated with the disease.

Researchers (Pacifici and Davies, 1991) have theorized on the role of free radicals and the process of aging. Their studies have shown an accumulation of oxidatively damaged cellular components, which increases in age. Their study proposes a free radical theory of aging. The theory states that the activities of oxidant repair enzymes

decrease and therefore contribute to the progressive accumulation of oxidant damage with aging. The theory continues by indicating that the ability of the organism to respond to the oxidative stresses may decline over years, thus predisposing older cells and organisms to oxidant damage.

Free radical production in vivo is primarily the result of oxygen concentration. The amount of oxygen in tissues in the intact animal, however, is much more difficult to determine than in a more simple in vitro culture system. The balance between too much oxygen and too little is called the "oxygen paradox." The oxygen paradox simply states that oxygen is necessary for higher life forms, but it can also be toxic to the same organism under certain conditions. As stated by Hooper (1989, p. 181), "We can't live without it, but it is hard to live with it."

Free radicals also cause adverse affects in plant cells (Yuan and Zhang, 1992). In barley cells, Yuan found that the frequency of sister-chromatid exchange (SCE) and sister-chromatid differentiation (SCD) could be controlled with oxidants. The amount of SCE increased when plants cells were exposed to oxidant compounds, which caused production of free radicals. Exposure to sulfhydryl compounds blocked any adverse effects and inhibited SCE. Yuan further reported that the amount of free radicals in root tips is directly correlated with the amount of SCE in root tip

cells. Free oxygen species have also been shown to damage DNA in human cell lines (Baker and He, 1991). Barker and He showed that in a human adenocarcinoma cell line, amounts of various hydroperoxide species were discovered to cause breaks in the DNA. Different reactive species caused breaks in single strands while others affected both strands of the DNA. In most cases, the affected cell lines had the inherent capability to repair the breaks caused by free radicals. However, some breaks were repaired only 80% of the time. Unchecked breaks lead to decreased cell growth and eventually death.

As indicated, free radicals damage chromosomes, which leads to altered cell growth. Dumitrescu (1992) speculated on how free radicals regulate normal cell proliferation. He suggested that oxygen-derived free radicals play a role in initiating and promoting neoplastic transformations in cultured cell lines and that transformations cause the activation of specific oncogenes. Thus, the presence of free radicals in some cell lines causes differentiation into unique cell types that behave differently from the original cell line.

Another way that free radicals affect cell viability is by interfering with cellular processes that are necessary for cell growth and proliferation (Vincent et al., 1991). Free radicals may interfere with the cell cycle. One group (Michel et al., 1992) observed that cells exposed to free

radicals arrest at G2 of the cell cycle. Hydrogen peroxide caused a variation in the amount of mRNA produced by c-myc and c-Ha-ras. Lower levels of mRNA were produced when hydrogen peroxide levels rose. These gene products have been shown to be essential in the G1 phase of the cell cycle (Vincent et al., 1991; Michel et al., 1992). Genes encoding for antioxidants have also been found in mouse and bovine embryos as well as bovine oviductal cultures (Harvey et al., 1995). In that study, evidence of antioxidant production was found in embryos, particularly viable embryos. Therefore, viable embryos have a means of protection against the assaults of free oxygen species.

In lieu of what is known about the effects of free radicals on biological systems, similar effects may come into play in in vitro embryo culture. Papers dealing with some of the factors that contribute to the "block" in embryo development are available (Noda et al., 1991; Legge and Sellens, 1991). The block usually occurs after a few cell divisions. In the mouse it is at the 2-cell stage (Natsuyama et al., 1993b). In cattle and sheep it is at the 8- to 16-cell stage (Kopecny and Niemann, 1993; Gardner et al., 1994). As already discussed, the higher the oxygen concentration the higher the production of free radicals. Until recently, researchers grew embryos in 5% CO_2 and atmospheric oxygen. However, with the discovery that embryos develop poorly when exposed to high concentrations

of oxygen, levels were lowered from those of standard protocols for in vitro culture. Many found that as the concentration of oxygen to which the embryos were exposed decreased, more embryos passed through the block (Goto et al., 1992; Umaoka et al., 1992).

Very little has been published regarding the physical effects of free radicals on embryo development. As indicated earlier, there are a number of factors that can lead to free radical production. By altering factors that contribute to free radical formation, there has been an improvement in the in vitro production of embryos. One example of regulating the oxygen concentration that affects the production of radicals has already been cited. Another example of potential effects is exposure of embryos to fluorescent light. Studies in our laboratory have shown that embryo viability decreases as exposure time to fluorescent light increases. Others have also found beneficial effects of regulating the amount of light (Nakayama et al., 1994). The presence of phenol red (a commonly used Ph indicator) in media plays a role in trapping light particles, which may lead to the production of free radicals. Zieger et al. (1991) found that free radicals increased in hepes buffered media exposed to fluorescent light from a flow hood. The increase in the amount of free radicals was significantly different from the media and cells held under dark conditions.

Tests are available to measure the presence of free radicals in culture media, but the numbers of embryos required for testing make the test impractical. The best approach is to assume that free radicals are present in in vitro culture systems and that by adding antioxidants it will reduce free radicals and their detrimental effects .

The role of the antioxidant is to inhibit oxidation. As was previously discussed, free radicals are the product of oxidation reactions. There are a number of different types of antioxidants. Some of the more commonly used antioxidants in media are catalase, ascorbate peroxidase, glutathione peroxidase, superoxide dismutase, and many others (Ellis, 1990b). Not all antioxidants work in the same manner. Each antioxidant targets a particular reaction product by either preventing its production or quickly changing it to a less destructive form.

Antioxidants have been shown to have positive effects on both in vitro and in vivo systems. Recent television advertisements have drawn attention to the beneficial affects of antioxidants. One advertisement claimed that using antioxidants, in this case a vitamin, would remove free radicals from around the brain and thus reduce headaches. Research that actually shows positive effects of dietary uses of antioxidants is scarce, probably due to the difficulty of proving that antioxidants were solely responsible for the outcome observed. This is why most

studies on the effects of antioxidants have been conducted in in vitro systems.

Some reports indicate that antioxidants work better when two or more types are used concomitantly in culture (Parshad et al., 1977). It has been shown, for instance, that the addition of SOD and catalase to an ascites tumor cell culture seemed to enhance better cell growth. Other tissues prefer either high or low levels of one or more antioxidants in order to thrive. Therefore, combinations of antioxidants that work for one type of tissue culture may not be beneficial for other types. Even the concentrations of antioxidants must be determined for specific cell types in order to obtain beneficial effects from their addition (Ellis, 1991).

The effects of SOD and catalase on the survival of pancreatic b-cells in culture were studied by Asayama et al. (1986). They showed that the presence of free radicals caused damage to the islet cells. The damage, however, could be inhibited when SOD and catalase were added to the cultures. SOD and catalase are two antioxidants that occur naturally and have thus been incorporated into many tissue culture systems. In another study by Reiter (1993), the effectiveness of melatonin (natural antioxidant) was compared to that of mannitol and glutathione. The study showed that melatonin was a more powerful free radical scavenger than either mannitol or glutathione. In fact,

melatonin protected susceptible molecules and prevented DNA degradation. It is thought that melatonin directly binds to specific regions of the nucleus, thus providing protection to DNA. Reiter further indicated that melatonin is a better antioxidant due to its twofold ability to protect. First, melatonin breaks down hydrogen peroxide to water and, secondly, in the event that hydroxyl radicals are formed, melatonin scavenges and removes them.

Vitamins are classified as antioxidants and are purported to have protective properties against reactive oxygen species (Zimmerman and Keys, 1991). In their study conducted on bovine rod outer segments, the addition of vitamin E and dithiothreitol proved to be beneficial in inhibiting ill effects of radicals on membrane phospholipids. The addition of these compounds to cultures assisted in the removal of peroxidized fatty acids, thus providing protection against oxidative damage. Vitamin E supplementation has also been shown to guard against chromosomal aberrations in Chinese hamster cells in culture (Sugiyama et al., 1991). Vitamin E was thought to protect against clastogenic and mutagenic affects of chromate compounds in culture.

The beneficial effects of antioxidants in tissue culture, as previously cited, have been well documented in the literature. The references cited herein represent a small portion of the work that has been conducted on free

radical scavengers. The beneficial supplementation of antioxidants to certain types of tissue culture media has lead to the incorporation of antioxidants into embryo culture media. For a number of years scientists have conducted experiments to improve the reproductive efficiency of animals. A major thrust has focused on the production of media that more closely resemble fluid that bathes the embryo in the female reproductive tract. To date, studies on the effects of antioxidants in embryo culture have been limited predominantly to the mouse, rabbit, and cattle. The majority of work has been done in the mouse.

In the mouse system, there is strong evidence that antioxidants increase the efficiency of embryo production in vitro (Ericksson and Borg, 1991; Nonogaki et al., 1991; Umaoka et al.,1992; Natsuyama et al., 1993a, 1993b; Chun et al., 1994). Part of this mouse research also focused on the reduction of oxidative stresses on developing embryos. In these studies, SOD, ethylenedinitrilo tetraacetic acid (EDTA), thioredoxin, apotransferrin, catalase, and glutathione peroxidase were tested for their efficacy in improving in vitro embryo development. Each free radical scavenger was found to exhibit some protection, which resulted in increases in blastulation, provided some growth-promoting factors, and decreased embryonic dysmorphogenesis.

Other studies on the mouse have used SOD and catalase to overcome the 2-cell block (Goto et al., 1992; Nonogaki et

al., 1992). Goto and Nonogaki further hypothesized that free oxygen radicals play a role in the termination of development at the 2-cell stage. A series of tests was conducted by two different research groups (Noda et al., 1991; Legge and Sellens, 1991). These studies concluded that the addition of SOD had a positive role in assisting embryos through the 2-cell block. Catalase was not as effective as superoxide dismutase.

There has been less research on the effects of antioxidants on rabbit embryos than on the mouse. Li et al. (1993) and Lindenau and Fischer (1994) reported there were no adverse or toxic effects on embryo development when adding SOD, catalase, or taurine to culture media. They also concluded that SOD and taurine, at the concentrations used in their studies, had a positive effect on embryo development. Catalase had neither a positive nor a negative effect.

There have been very few reports on the effects of antioxidants in media used for cattle embryo culture. One study, however, by Luvoni et al. (1994) showed that the addition of SOD at a low concentration in oocyte maturation media and a high concentration in the embryo culture media outperformed treatments without antioxidants. Luvoni et al. concluded that further work should be done to evaluate the effectiveness of antioxidants in culture of bovine embryos.

Throughout the course of this review, I have cited

methods for the detection of free radicals as well as oxidative stress. Methods still need to be developed to study the quantity as well as the cellular distribution of free radicals to fully understand their role in health and disease. Past studies do, however, show positive advantages to using antioxidants in tissue and embryo culture systems.

MATERIALS AND METHODS

Superoxide dismutase (SOD) (Sigma Chemical Co., St Louis, MO, catalog number S-5395) and Catalase (Sigma, catalog number S-40) were added either singly or in combination to culture medium. Both antioxidants were reconstituted in ultrapure sterile water at concentrations of 1mg/200µl for SOD and 1 mg/ml for catalase. Antioxidants were either freshly made before use, or frozen-stored at -20°C and thawed only once just before use.

Four different levels of each antioxidant were used in each phase of the study. In the first phase the levels of SOD were 0, 1500, 3000, and 6000 IU/ml, and for catalase 0, 75, 100, and 125 µg/ml. All combinations of SOD and catalase at these four levels were used. The concentrations for the first treatments are as follows (SOD/Catalase): T1 0/0, T2 0/75, T3 0/100, T4 0/125, T5 1500/0, T6 3000/0, T7 6000/0, T8 1500/75, T9 1500/100, T10 1500/125, T11 3000/75, T12 3000/100, T13 3000/125, T14 6000/75, T15 6000/100, and T16 6000/125. There were two replicates for each treatment level. In the second phase, the levels of SOD were 0, 100, 250, and 500 IU/ml, and for catalase 0, 10, 25, and 50 µg/ml. All combinations of SOD and catalase at these four levels were used and consisted of the following (SOD/Catalase): T1 0/0, T2 0/10, T3 0/25, T4 0/50, T5 100/0, T6 250/0, T7 500/0, T8 100/10, T9 100/25, T10 100/50, T11 250/10, T12 250/25, T13 250/50, T14 500/10, T15 500/25, and

T16 500/50. There were three replicates for each treatment level.

Stock medium was prepared at a 2x concentration for the in vitro maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC) processes. The medium was prepared separately for each treatment by mixing the 2x stock solution with the appropriate volume of the antioxidant solution and then adjusting with ultrapure sterile water to a 1x concentration.

Ovaries were collected from a local abattoir within 30 min after death of the animal. Ovaries were removed from the reproductive tracts and placed into a thermos bottle containing 0.9% saline and transported to the laboratory. The ovaries were washed with sterile 0.9% saline three times in order to remove any blood or debris from the ovaries.

Oocytes were then aspirated using a vacuum pump (150 mm Hg) attached to a 50-ml centrifuge tube (Fisher Scientific, Santa Clara, CA). Follicles ranging in size from 1 to 7 mm were aspirated using an 18-gauge needle. The aspirate was collected in the 50-ml centrifuge tube. When the level of the follicular fluid in a tube reached about 40 ml, the tube was replaced. The centrifuge tube was set aside for approximately 10 min, which allowed for the oocytes to settle to the bottom of the tube.

The selection of good quality oocytes was started as soon as the oocytes settled in the centrifuge tube. The

oocytes were located and examined in 100x15mm Falcon grid dishes (Falcon, Lincoln Park, NJ). A Hepes-buffered washing solution was poured into the search dish with just enough volume to cover the bottom of the dish. The pellet of cells at the bottom of the 50-ml tube was aspirated with a glass pasteur pipette. The fluid containing the pellet was then dispersed throughout the search dish. Oocytes with evenly granulated cytoplasm and with more than three to four layers of compact cumulus cells were removed from the search dish and placed into a drop of sterile washing medium (T.L. Hepes with 0.3 g BSA fraction V, 1% Pen/Strep, filter sterilized). Groups of 50 oocytes were then washed by transferring them through a series of four drops of the sterile washing medium. With each transfer, cellular debris was left behind. After the oocytes were washed, they were randomly assigned to 1 of the 16 treatment groups for maturation.

The medium used for oocyte maturation was an M-199/Earles salt stock solution (Hyclone Laboratories Inc., Logan, UT) supplemented with 10% fetal bovine serum (FBS) (HyClone), 0.5% bovine follicle stimulating hormone (bFSH) (Nobl Laboratories Inc., Sioux Center, IA), 0.5% bovine luteinizing hormone (bLH) (Nobl), 1% penicillin/streptomycin (pen/strep) (Sigma), and various concentrations of antioxidants as described. Maturation medium was prepared approximately 12 h before it was used to allow sufficient time to equilibrate in the incubator. The final volume of

the maturation medium in each treatment was 250 µl. Maturation was carried out in 4-well Nunc dishes (Nunc, Denmark) in 5% CO_2 at 39°C. Oil was not layered on top of the medium during maturation.

After 24 h in the maturation media, the oocytes were fertilized. Oocytes from each group were washed separately in the sterile washing media. This was necessary to remove glucose from the surface of the oocytes. Failure to wash glucose off the oocytes before exposure to sperm resulted in delayed fertilization. After the oocytes were washed, they were returned to a 4-well Nunc dish (Nunc) containing fertilization talp (Bavister and Yanagimachi, 1977) as well as the specific concentration of antioxidant for a given treatment. Care was given to ensure that each group of oocytes remained in the correct treatment.

The fertilization talp (fert-talp) (Bavister and Yanagimachi, 1977), also a 2x stock solution, was prepared as follows. Five milliliters of fert-talp is placed into a clean 15-ml Falcon tube (Falcon) and 60 mg of fatty acid-free bovine serum albumin (BSA) (Sigma) was added. Next, 50 µl of a 25-Mm stock solution of pyruvate (Sigma) was added, followed by 50 µl of pen/strep (100x) (Sigma). The medium was prepared 4 to 6 h before the expected time of usage so that the pH had time to equilibrate to 7.4 in the incubator. The methods used for the in vitro fertilization were similar to those reported by Parrish and others (1986).

Sperm was prepared for fertilization by first thawing a straw of characterized semen in a 37°C water bath for 1 min. Characterization of the semen had been done previously. Characterization of semen is done by adding various known concentrations of Heparin to fert-talp in order to maximize the number of oocytes fertilized but minimize the amount of polyspermy. Once the appropriate concentration of Heparin has been identified, the semen is said to be characterized. The semen used for this experiment was taken from a single collection of one bull. This was done to minimize the any variation between straws of semen. The semen was layered on top of a Percoll separation gradient (90% on the bottom and 45% on the top), and placed into a centrifuge at 700 xG for 30 min. During the Percoll separation, motile sperm pelleted at the bottom of the 15-ml Falcon (Falcon) centrifuge tube.

After 30 min of centrifugation, the Percoll gradients were aspirated to the 200-µl mark on the tube, thus leaving the sperm pellet at the bottom of the tube. The sperm pellet was gently resuspended with 180 µl of sperm talp. A portion (5 µl) of the resuspended sperm was transferred into a 500-µl snap-top microfuge tube containing 95 µl water. The water killed the sperm and the concentration of the sperm was then calculated using a hemocytometer. Upon calculating a final sperm concentration of 1 million sperm per ml, 20 µl of the sperm and sperm T.L. suspension were

added to the 445 µl of fert-talp containing the washed oocytes. Heparin was then added at a concentration of 0.025 mg/ml. Heparin is added to assist in the capacitation of the sperm. Oocytes were exposed to the sperm for 18 to 20 h.

After 18 h of fertilization, the embryos were moved to CR2 medium (Rosenkrans and First, 1991). This was accomplished by removing each group of embryos from the fertilization dish and placing them into a 15-ml Falcon (Falcon) tube containing about 1 ml of sterile washing media. The embryos were then vortexed on a Baxter S/P vortex mixer at a setting of 8 for 2 min 15 s. The embryos were then rinsed from the 15-ml tube and placed into another drop of sterile washing media. The CR2 used for IVC was also a 2x concentration. CR2 was supplemented with 3 mg/ml of BSA fraction V (Sigma), 1% glycine (1 M stock solution) and 1% alanine (100 mM stock solution). CR2 was prepared about 12 h in advance so that it had time to equilibrate in the incubator. Drops of CR2 (30 µl) were placed in a dish and covered with silicon oil (Dimethylpolysiloxane) (Sigma), and allowed to equilibrate. Groups of 10 embryos were transferred from the washing medium into each 30 µl drop of CR2. Each treatment was placed into the incubator until all 16 treatments were completed. By placing each treatment into culture individually, it increased the time required for all treatments, but it prevented mixing treatments by mistake.

When all treatments were transferred into culture, the 16 dishes were placed in a modular incubator and charged with a gas mixture consisting of 5% CO_2, 5% O_2, and 90% N. Gas was passed through the dish for 3 min to ensure that all other gas present was replaced. The modular incubator was then sealed and incubated at 39°C for 7 d. After 7 d in culture, dishes were removed from the modular incubator and the embryos evaluated for stage of development. The data were then analyzed by ANOVA.

RESULTS AND DISCUSSION

The results of this study did not support the hypothesis that the incorporation of antioxidants into embryo culture media during in vitro processes increases the percentage of embryos developing to the blastocyst stage. The results that refute the hypothesis will be presented in two sections: the high concentrations and then the lower concentrations of antioxidants.

The development of embryos exposed to high concentrations of antioxidants are shown in Table 1. Concentrations of SOD ranged from 1500 to 6000 ug/ml of medium and catalase ranged from 75 to 125 ug/ml. The percentage of 2-cell development among treatments ranged from 54 to 73. The percentage of embryos developing to morulae and blastocysts ranged from 5 to 27 and 1 to 20, respectively. Analysis of variance of the data was based upon the variables: run, SOD, catalase, interaction between run and SOD, interaction between run and catalase, interaction between SOD and catalase, and the interaction between run, SOD, and catalase. Table 2 shows the results from the ANOVA with three significant values. The run was found to be significant. This could be explained by the fact that all of the oocytes that were collected for a given run were collected on the same day. Therefore, it is possible to have variation between runs. This variation in runs could also be accounted for by the fact that the

Table 1. Embryo development at high antioxidant concentrations

SOD[a]	CAT[b]	# of oocytes[c]	% 2-cell	% Morula	% Blastocyst
0	0	102	63	27	20
0	75	102	70	23	19
0	100	86	73	17	13
0	125	101	61	9	7
1500	0	89	70	16	7
3000	0	86	64	17	13
6000	0	87	54	14	3
1500	75	84	67	14	6
1500	100	81	68	17	6
1500	125	86	72	17	8
3000	75	75	61	11	3
3000	100	94	69	14	6
3000	125	88	69	7	2
6000	75	94	71	13	3
6000	100	93	67	9	1
6000	125	93	66	5	1

[a]SOD concentration is shown as IU/ml.

[b]Catalase is shown as µg/ml.

[c]# of oocytes indicates the number of oocytes that were fertilized. The % 2-cell, morula, and blastocyst are all based on the # of oocytes fertilized.

Table 2. ANOVA table on the results of embryos cultured in high antioxidant concentrations

Source	DF	MS	F Value
Run	1	104.82	31.20**
SOD	3	16.67	4.96**
Run & SOD	3	8.25	2.46
Cat	3	3.76	1.12
Run & Cat	3	16.93	5.04**
Cat & SOD	9	4.65	1.39
Run & Cat & SOD	9	4.16	1.24

** Effect significant at $P < 0.01$

Run*Cat interaction was also significant. Superoxide dismutase in the culture media had a negative effect. As the level of SOD increased, fewer embryos developed to the blastocyst stage.

Table 3 is a comparison of all combinations of SOD (0, 1500, 3000, 6000 ug/ml), with significance based upon the average number of embryo divisions per SOD concentration. As the concentration of SOD increased, the average number of divisions per embryo decreased. Upon comparing levels 1 and 2 with level 4, there are significant differences in embryo development with $P = 0.0005$ and $P = 0.0026$, respectively. The P-value for the comparison of level 2 with level 3 was close to significant $P = 0.0570$.

Table 3. Comparison of all combinations of the four levels testing significance and the average number of embryo divisions based on SOD concentration

SOD Concentration (IU/ml)	Division #
0	2.086[a]
1500	2.042[a]
3000	1.826[ab]
6000	1.624[b]

[a,b] means with different superscripts differ significantly $P < 0.01$

Based upon the results from high level antioxidant effects, concentrations of SOD and catalase were reduced and a low-level experiment was conducted. Initial inspection of data from various low-level antioxidant combinations showed that treatments that resulted in poor embryo development at the high concentrations now seemed to be beneficial. Table 4 is a summary of the developmental results for the lower concentrations. SOD concentrations ranged from 100 to 500 ug/ml of medium and catalase ranged from 10 to 50 ug/ml. The percentage of 2-cell development among treatments ranged from 62 to 80. The percentage of embryos developing to morulae and blastocysts ranged from 12 to 23 and 7 to 28, respectively. Ten of the treatments produced blastocyst percentages that were equal to or greater than the control. This was not the case for the higher concentrations of

Table 4. Embryo development at reduced antioxidant concentrations

SOD[a]	CAT[b]	# of Oocytes[c]	% 2-Cell	% Morula	% Blastocyst
0	0	76	67	16	17
0	10	82	74	17	22
0	25	82	74	12	7
0	50	73	67	14	15
100	0	74	62	20	23
250	0	71	68	17	21
500	0	77	71	23	19
100	10	71	68	21	28
100	25	72	69	18	18
100	50	78	73	23	26
250	10	75	72	12	12
250	25	70	80	20	26
250	50	72	68	13	15
500	10	76	59	16	21
500	25	70	61	9	7
500	50	68	68	19	24

[a]SOD concentration is shown as IU/ml.

[b]Catalase is shown as µg/ml.

[c]# of oocytes indicates the number of oocytes that were fertilized. The % 2-cell, morula, and blastocyst are all based on the # of oocytes fertilized.

antioxidants. Even with what appeared to be greater embryo development within some of the lower level antioxidant treatments, there were no significant differences based on ANOVA.

Table 5 is the ANOVA table and P-values. Only the run variable was significant. Run had a P-value of 0.0001, which is highly significant. The other significant value was the interaction between run, SOD, and catalase (P = 0.0107). The significance of these two values is explained by variation in the three groups of oocytes which were used in the study.

Table 5. ANOVA table on the results of embryos cultured in low antioxidant concentrations

Source	DF	MS	F Value
Run	2	39.92	9.83**
SOD	3	2.72	0.67
Run & SOD	6	3.39	0.83
Cat	3	1.67	0.41
Run & Cat	6	3.14	0.77
Cat & SOD	9	4.96	1.22
Run & Cat & SOD	18	7.86	1.94*

* Effect significant at P < 0.05

** Effect significant at P < 0.01

In both high and low antioxidant concentrations the run was significant. The variation is primarily due to the variability in oocyte quality between collection periods, which is a common problem in trying to maintain quality control for in vitro fertilization systems. Variability in oocyte collections is difficult to control since ovaries are coming from slaughter animals whose nutrition, hormonal, and reproductive status is unknown. Furthermore, cows coming into the slaughterhouse have oftentimes been on exogenous hormones. The only way to alleviate some of the variation is to routinely followed a rigid protocol for collecting and handling the ovaries. Therefore, variation between collection periods, which has shown up as a significant difference in this study, is most likely a composite cow effect within the group slaughter on a given day.

The results of this study do not corroborate the studies conducted in the mouse (Ericksson and Borg, 1991; Nonogaki et al., 1991; Goto et al., 1992; Nonogaki et al., 1992; Umaoka et al.,1992; Natsuyama et al., 1993a, 1993b; Chun et al., 1994) and the rabbit (Li et al., 1993; Lindenau and Fischer, 1994). Other work on the effects of antioxidants in the bovine have shown similar results to what we found in this study (Luvoni et al., 1994). George Seidel, Jr. (1995-personal communication) at Colorado State University indicated that his research group tested the

effects of antioxidants in bovine embryo culture media and observed no beneficial effects.

The types of media used for embryo culture often account for differences in embryo development among species. In this study, bovine embryos were cultured in CR2, which is a simple-defined medium. Luvoni et al. (1994) cultured embryos in a complex medium that was necessary for co-culture and reported beneficial effects in supplementing media with antioxidants. In this case the beneficial effects experienced by the use of antioxidants could be associated with free radical production by the monolayers on which the embryos were growing. If somatic cell monolayers are responsible for radical production, then antioxidants would be an important factor in their removal.

It is also possible that different media trap light differently. Therefore, more free radicals would be produced in light-trapping media. An example is phenol red trapping light in culture media. The CR2 medium used in this study contained no phenol red. If in the previously cited studies phenol red was used in the media, then a greater amount of fluorescent light would have been trapped. Antioxidants would therefore have had a positive effect on embryo development by tying up free radicals. This could possibly explain why the treatments in those studies out-perform the controls.

Another possibility for the differences between the

results of this study as compared to the other cited reports may be the difference in the culture systems in which the embryos were grown. Noda et al. (1991) and Li et al. (1993) cultured in 5% CO^2 in humidified air. In this study embryos were cultured in a humidified atmosphere of 5% CO^2, 5% O^2, and 90% N^2. Higher O^2 concentrations can cause an increased production of free radicals, which negatively affect embryonic development. Antioxidants do not freely cross embryo membranes without some type of carrier. Therefore, without incorporation of a carrier with the antioxidants, the positive effects seen by using antioxidants in embryo culture are probably due to the elimination of radicals produced by excessive oxygen concentration or exposure to fluorescent light. If caution is given to both oxygen concentration and exposure to light, I do not believe that it is necessary to add antioxidants.

Another possibility may be that in this study, high levels of SOD in the culture had a detrimental effect on embryo development. However, Luvoni et al. (1994) reported some beneficial effects using SOD in a bovine embryo culture system. The difference between this study and the study of Luvoni et al. (1994) is the difference in the embryo culture environment. In this study extreme effort was made to reduce the exposure of embryos to factors shown to cause free radical development. Superoxide dismutase and catalase were not necessary to scavenger free radicals and therefore

did not have a positive effect. One study showed that addition of exogenous SOD caused variable results (Johnson and Nasr-Esfahani, 1994). Those researchers further stated that it is still unclear how exogenous SOD could remove intracellular superoxides that do not readily cross membranes. Free radicals are, however, important for electron transport. One possible explanation to the negative response in this study may have been the low levels of free radicals present during embryo culture. The increased concentrations of antioxidants in the high-level treatment groups could have removed the beneficial effects of free radicals. High levels of antioxidants may have interfered with the production of free radicals, which in turn could limit the production of ATP. Without sufficient amounts of ATP, essential life processes would be altered and the embryo would then die.

Finally, some have shown that viable embryos produce antioxidants. It is then conceivable to hypothesize that if caution was given to factors causing radical formation, that the embryos could be more viable. If they were more viable, they were probably producing antioxidants and the additional antioxidants may have had a toxic or negative effect on development.

Whether antioxidants have a positive or negative effect will depend on the embryo culture system used. High concentrations of O_2 or light-trapping substances in the

media may require the use of antioxidants to improve embryo development. On the contrary, if O_2 tension is lowered and light-trapping compounds such as phenol red are removed from the media, then adding antioxidants may have a negative effect on embryo growth and development.

CONCLUSION

Even though previous studies showed that there were beneficial effects to the use of antioxidants in embryo production, my results do not corroborate this in the bovine model. I believe that free radicals play a role in developmental obstruction of embryos. I am also confident that certain types of media, laboratory procedures, and culture systems are conducive to free radical production. Our system of IVF embryo production pays special attention to the presence of phenol red in the media, exposure of embryos to direct fluorescent light, and culturing of embryos in reduced O_2 concentration. Each of these factors has been cited previously as a factor contributing to radical production. It is my belief that if the above mentioned factors are not closely monitored, free radicals may be produced in concentrations that could depress embryo development. By controlling the factors that cause radical production, it is possible to successfully culture embryos without antioxidants. However, if those factors are not controlled, antioxidants may need to be added in order to achieve desired embryo development. Therefore, at this time, the use of SOD and catalase in maturation, fertilization, and culture will not be incorporated into our laboratory standard operating procedures.

In the future, I hope that studies will continue to be done in order to elucidate the role of free oxygen

radicals in embryo production. Understanding the positive role (if one exists) of free radicals could perhaps shed some light on controlling the concentration of radicals that embryos are exposed to. Then, free radicals could be allowed in culture at concentrations necessary to carry out cellular processes, and any concentrations above that could be removed by antioxidants before cellular damage occurs.

REFERENCES

Asayama, K., N.W. Kody, and I.M. Burr. 1986. Effect of vitamin E deficiency and selenium deficiency on insulin secretory reserve and free radical scavenging systems in islets decreased of islet manganosuperoxide dismutase. J. Lab. Clin. Med. 107:459.

Baker, M.A., and S.Q. He. 1991. Elaboration of cellular DNA breaks by hydroperoxides. Free Radic. Biol. Med. 1(6):563.

Bavister, B. D., and R. Yanagimachi. 1977. The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa in vitro. Biol. Reprod. 16:228.

Bendich, A. 1993. Physiological role of antioxidants in the immune system. J. Dairy Sci. 76(9):2789.

Blum, H.F. 1964. Photodynamic Action and Disease Caused by Light. Hafner, New York.

Cadet, J.L., and L.A. Kahler. 1994. Free radical mechanisms in schizophrenia and tardive dyskinesia. Neurosci. Biobehav. Rev. 18(4):457.

Chun, Y.S., J.H. Kim, H.T. Lee, and K.S.Chung. 1994. Effect of superoxide dismutase on the development of preimplantation mouse embryos. Theriogenology 41(2): 511.

Dianzani, M.U. 1992. Free radicals in physiology and pathology. Boll. Soc. Ital. Biol. Sper. 68(8-9):491.

Dumitrescu, C. 1992. The role of free radicals in normal and pathologic cell proliferation. Rom. J. Endocrinol. 30(1-2):45.

Ellis, L.C. 1990a. Free radicals in tissue culture: Part I, What are free radicals. Art Sci. 9(2):1.

Ellis, L.C. 1990b. Free radicals in tissue culture: Part III. Antioxidant systems and antioxidants. Art Sci. 9(4):1.

Ellis, L.C. 1991. Free radicals in tissue culture: Part IV. Effects on cells in culture. Art Sci. 10(1):1.

Ericksson, U.J., and L.A. Borg. 1991. Protection by free oxygen radical scavenging enzymes against glucose-induced embryonic malformations in vitro. Diabetologia. 34(5):325.

Fee, J.A., and J.S. Valentine. 1977. Chemical and physical properties of superoxide. In: A.M. Michelson, J.M. McCord, and I. Fridovich (Ed.) Superoxide and Superoxide Dismutase. p. 78. Academic Press, New York.

Ferrari, R., C. Ceconi, S. Curello, A. Cargnoni, O. Alfieri, A. Pardini, P. Marzollo, and O. Visioli. 1991. Oxygen free radicals and myocardial damage: Protective role of thiol-containing agents. Am. J. Med. 91(3C):95S.

Fisher, B. 1987. Developmental retardation in cultured preimplantation rabbit embryos. J. Reprod. Fertil. 79:115.

Gao, W., H.D. Connor, J.J. Lemasters, R.P. Mason, and R.G. Thurman. 1995. Primary nonfunction of fatty livers produced by alcohol is associated with a new antioxidant-insensitive free radical species. Transplant. 59(5):674.

Gardner, D.K., M. Lane, A. Spitzer, and P.A. Batt. 1994. Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in the absence of serum and somatic cells: Amino acids, vitamins, and culturing embryos in groups stimulate development. Biol. Reprod. 50(2):390.

Gille, J.J.P., and H. Joenje. 1991. Biological significance of oxygen toxicity: an introduction. In: C. Vigo-Pelfrey (Ed.) Membrane Lipid Oxidation. p. 1. CRC Press, Boca Raton, FL.

Goto, Y., Y.Noda, K. Narimoto, Y. Umaoka, and T. Mori. 1992. Oxidative stress on mouse embryo development in vitro. Free Radic. Biol. Med. 13(1):47.

Gutteridge, J.M.C., and B. Halliwell. 1988. The antioxidant proteins of extracellular fluids. In: C.K. Chow (Ed.) Cellular Antioxidant Defense Mechanisms, Vol. II. p. 1. CRC Press, Boca Raton, FL.

Halliwell, B., and J.M.C.Gutteridge. 1989. Free Radicals in Biology and Medicine, 2nd ed. New York: Oxford University Press, New York.

Harvey, M.B., M.Y. Arcellana-Panlilio, X. Zhang, G.A. Schultz, and A.J. Watson. 1995. Expression of genes encoding antioxidant enzymes in preimplantation mouse and cow embryos and primary oviduct cultures employed for embryo coculture. Biol. Reprod. 53:532.

Hooper, C. 1989. Free Radicals: Research on biochemical bad boys comes of age. J. NIH Res. 1:181.

Johnson, M.H., and M.H. Nasr-Esfahano. 1994. Radical solutions and culture problems: Could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos in vitro? Bioessays 16:31.

Katz, A., K.T. Oldham, K.S. Guice, and A.G. Coran. 1995. Oxidized glutathione as a marker of ischemia reperfusion associated with single lung transplantation. J. Am. Coll. Surg. 180(1):25.

Kaul, N., N. Siveski-Iliskovic, M. Hill, J. Slezak, and P.K. Singal. 1993. Free radicals and the heart. J. Pharmacol. Toxicol. Methods. 30(2):55.

Kehrer, J.P. 1993. Free radicals as mediators of tissue injury and disease. Crit. Rev. Toxicol. 23(1):21.

Keith, F. 1993. Oxygen free radicals in cardiac transplantion. J. Card. Surg. 8(2 supplement):245.

Kjellmer, I. 1991. Mechanisms of perinatal brain damage. Ann. Med. 23(6):675.

Kopecny, V., and H. Niemann. 1993. Formation of nuclear microarchitecture in the preimplantation bovine embryo at the onset of transcription: Implications for biotechnology. Theriogenology 39(1):109.

Legge, M., and M.H. Sellens. 1991. Free radical scavengers ameliorate the 2-cell block in mouse embryo culture. Hum. Reprod. 6(6):867.

Li, J., R.H. Foote, and M. Simkin. 1993. Development of rabbit zygotes cultured in protein-free medium with catalase, taurine, or superoxide dismutase. Biol. Reprod. 49(1):33.

Lindenau, A., and B. Fischer. 1994. Effect of oxygen concentration in the incubator's gas phase on the development of cultured preimplantation rabbit embryos. Theriogenology 41(4):889.

Luvoni, G.C., L. Keskintepe, J. Rzucidlo, A. Kenimer, and B.G. Brackett. 1994. Superoxide dismutase (SOD) nd glutathione (GSH) affect fertilization and development of bovine oocytes. Biol. Reprod. 50(Supplement 1):189.

Marklund, S.L. 1985. Oxygen toxicity and protective systems. Clin. Toxicol. 23(4-6):289.

Michel, C., F. Vincent, C. Duval, M.C. Poelman, and M. Adolphe. 1992. Toxic effects and detection of oxygen free radicals on cultured articular chondrocytes generated by menadione. Free Radic. Res. Commun. 17(4):279.

Murrell, T.G. 1991. Epidemiology and biochemical support for a theory on the cause and prevention of breast cancer. Med. Hypotheses. 36(4):389.

Nakayama, T., Y. Noda, Y. Goto, and T. Mori. 1994. Effects of visible light and other environmental factors on the production of oxygen radicals by hamster embryos. Theriogenology 41:499.

Natsuyama, S., Y. Noda, K. Narimoto, and T.Mori. 1993a. Role of protein supplements in the culture of mouse embryos. Theriogenology. 40(1):149.

Natsuyama, S., Y. Noda, M. Yamashita, Y. Nagahama, and T. Mori. 1993b. Superoxide dismutase and thioredoxin restore defective p34cdc2 kinase activation in mouse two-cell block. Biochim. Biophy. Acta. 1176(1-2):90.

Noda, Y., H. Matsumoto, Y. Umaoka, K. Tatsumi, J. Kishi, and T. Mori. 1991. Involvement of superoxide radicals in the mouse two-cell block. Mol. Biol. Reprod. 28(4)356.

Nonogaki T., Y. Noda, K. Narimoto, Y. Umaoka, and T. Mori. 1991. Protection from oxidative stress by thioredoxin and superoxide dismutase of mouse embryos fertilized in vitro. Hum. Reprod. 6(9):1305.

Nonogaki, T., Y. Noda, K.Narimoto, Y.Umaoka, and T. Mori. 1992. Effects of superoxide dismutase on mouse in vitro fertilization and embryo culture system. J. Assist. Reprod. Genet. 9(3):274.

Pacifici, R.E., and K.J. Davies. 1991. Protein, lipid and DNA repair Systems in oxidative stress: The free-radical theory of aging revisited. Gerontology 37(1-3):166.

Paller, M.S., and H.S. Jacob. 1994. Cytochrome P-450 mediates tissue-damaging hydroxly radical formation during reoxygenation of the kidney. Proc. Nat. Acad. Sci. USA. 91(15):7002.

Parrish, J.J., J.L. Susko-Parrish, M.L. Leibfried-Rutledge, E.S. Critser, W.H. Eyestone, and N.L. First. 1986. Bovine in vitro fertilization with frozen-thawed semen. Theriogenology 25(4):591.

Parshad, R., K.K. Sanford, G.M. Jones, F.M. Price, and W.G. Taylor. 1977. Oxygen and light effects on chromosomal aberrations in mouse cells in vitro. Exp. Cell. Res. 104:199.

Reiter, R.J. 1993. Interactions of pineal hormone melatonin with oxygen-centered free radicals: A brief review. Braz. J. Med. Biol. Res. 26(11):1141.

Rosenkrans, C.F., Jr., and N.L. First. 1991. Culture of bovine zygotes to the blastocyst stage: Effects of amino acids and vitamins. Theriogenology 35:266.

Slater, T.F. 1972. Free Radical Mechanisms in Tissue Injury. Pion Limited, London, England.

Sugiyama, M., X. Lin, and M. Costa. 1991. Protective effect of vitamin E against chromosomal aberrations and mutation induced by sodium chromate in chinese hamster V79 cells. Mut. Res. 260:19.

Umaoka, Y., Y. Noda, K. Narimoto, and T. Mori. 1992. Effects of oxygen toxicity on early development of mouse embryos. Mol. Reprod. Dev. 31(1):28.

Vincent, F., M. Corral, N. Defer, and M. Adolphe. 1991. Effects of oxygen free radicals on articular chondrocytes in culture: c-myc and c-Ha-ras messenger RNAs and proliferation kinetics. Exp. Cell. Res. 192(2):333.

Warren, J.S., K.J. Johnson, and P.A. Ward. 1987. Oxygen radicals in cell injury and cell death. Pathol. Immunopathol. Res. 6:301.

Wright, R.J. Jr., and K.R. Bondioli. 1981. Aspects of in vitro fertilization and embryo culture in domestic animals. J. Anim. Sci. 53:702.

Yuan, H., and Z. Zhang. 1992. Some factors affecting sister-chromatid differentiation (SCD) and sister-chromatid exchange (SCE) in hordeum vulgare. Mut. Res. 272(2):125.

Zieger, M.A., D.J. Glofcheski, J.R. Lepock, and J. Kruuv. 1991. Factors influencing survival of mammalian cells exposed to hyperthermia: Effects of hepes, free radicals, and H2O2 under light and dark conditions. Cryobiology 28(1):8.

Zimmerman, W.F., and S. Keys. 1991. Effects of the antioxidants dithiothreitol and vitamin E on phospholipid metabolism in isolated rod outer segments. Exp. Eye Res. 52(5):607.