OXIDATIVE STRESS MECHANISMS WITHIN THE DEVELOPING PORCINE OOCYTE AND THE EFFECTS OF ANTIOXIDANT SUPPLEMENTATION

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ABSTRACT

Oxidative stress contributes to inadequate *in vitro* maturation of porcine oocytes which leads to a failure of successful fertilization and embryo development. Therefore, the overall objective of this research was to characterize the mechanisms of oxidative stress in maturing oocytes and determine how oocytes alleviate oxidative stress with the assistance of supplemental antioxidants. A preliminary study was conducted to evaluate the effects of glutathione (GSH), N-acetyl-cysteine (NAC), and N-acetyl-cysteine-amide (NACA) supplemented to the maturation medium on intracellular GSH concentrations, nuclear maturation, fertilization success and embryo development. Antioxidants GSH, NAC and NACA (1.0 mM) were supplemented to the media during oocyte maturation. Intracellular GSH concentrations were recorded at 48 h of maturation and nuclear maturation and fertilization were analyzed 12 h after IVF. Embryo development was analyzed at 48 h and 144 h after IVF or intracytoplasmic sperm injection (ICSI). Supplementation of antioxidants had no effect on intracellular levels of GSH, nuclear maturation or fertilization traits. Blastocyst formation for NAC (35.0 ± 7.4%) and NACA (40.0 ± 7.4%) supplementation were higher ($P < 0.05$) than the control (20.0 ± 7.4%) and GSH supplemented (20 ± 7.4%) oocytes. The same pattern was seen for ICSI-derived embryos: blastocyst formation for NAC (22.0 ± 5.9%) and NACA (25.0 ± 4.6%) supplementation were higher ($P < 0.05$) than the un-supplemented (10.0 ± 6.0%) oocytes. There were no differences between NAC and NACA supplementation and there were no differences between the cleavage rates for any of the treatment groups. These results indicate that supplementing 1.0 mM of NAC or NACA to the oocyte maturation medium and the ICSI medium increased the percentage of viable embryos reaching the blastocyst stage of development, and could warrant further investigation. The next study was conducted to evaluate the effects of different concentrations of NAC supplemented to the

maturation medium on embryo development. Comparisons of significant concentrations of NAC and NACA on embryo development were evaluated for nuclear maturation, fertilization success and embryo development. Concentrations of NAC (0, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0 mM) were supplemented to maturing oocytes and embryo development was analyzed at 48 h and 144 h post-fertilization. There were no differences between cleavage rates for any of the treatment groups. Blastocyst formation for 1.5 mM NAC (56.5 ± 9.2%) was significantly higher ($P < 0.05$) than all other supplementations. There were no differences in nuclear maturation or fertilization when comparing 1.5 mM NAC and 1.5 mM NACA supplementation to the maturation media. There was no difference between cleavage rates of 1.5 NAC and 1.5 mM NACA supplementation to the maturation media. Blastocyst formation for 1.5 mM NAC (44.4 ± 4.7%) and 1.5 mM NACA (46.2 ± 3.4%) supplementation were significantly higher ($P < 0.05$) than the control (32.1 ± 6.2%) oocytes. These results indicate that supplementing 1.5 mM of NAC or NACA to the oocyte maturation medium increased the percentage of viable embryos reaching the blastocyst stage of development and could be used during the oxidative stress experiments. In the final study, the mechanisms of oxidative stress in maturing oocytes were studied in addition to evaluating the effects of antioxidant supplementation to the media. This study focused on superoxide dismutase (SOD), GSH peroxidase, catalase and intracellular GSH concentrations with respect to DNA fragmentation evaluated using the single cell Comet assay. Results indicate that when SOD was inhibited, the GSH peroxide levels and length of DNA migration significantly increased ($P < 0.05$). Catalase levels significantly decreased ($P < 0.05$) and intracellular GSH remained unchanged. When GSH peroxidase was inhibited, the SOD levels and catalase levels significantly decreased ($P < 0.05$) but the intracellular GSH and DNA migration length significantly increased ($P < 0.05$). The supplementation of 1.5 mM NAC and 1.5 mM NACA had multiple effects on the enzyme levels. Specifically, supplementation of 1.5 mM NAC or 1.5 mM NACA significantly decreased ($P < 0.05$) the length of DNA migration when other enzymes were inhibited compared to no antioxidant supplementation. These results indicate that antioxidant supplementation may alleviate the free radicals associated with oxidative stress in the maturing porcine oocyte. In conclusion, supplementing the antioxidants NAC or NACA to the oocyte

maturation media does not have negative effects on IVF or embryo culture. Supplementation of NACA increases the number of oocytes reaching the blastocyst stage of development. Glutathione, SOD, catalase, and GSH peroxidase are all required to be functional during oocyte development to alleviate oxidative stress on the oocyte. Antioxidants enhance the enzyme activity during oocyte maturation and may even contribute to protecting the oocyte when enzyme activity is impaired.

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REVIEW OF LITERATURE

In vitro production of pigs

Research within the agricultural, biotechnological and medical sectors has seen an increased interest in molecular technologies and cell biology, coinciding with society's keen interest in stem cell research. The pig has become a leading model for researchers due to its physiological similarities to humans [1]. Pigs are a suitable model for organ transplantation, transgenic production and various areas of reproductive research. Additionally, they were the first livestock species for which genome sequencing began and they were the fourth mammal (human, mouse, rat) to be gene sequenced [2]. In order for the pig to remain a suitable model for research purposes, large quantities of viable embryos need to be produced in an efficient, cost effective manner. Currently, the *in vitro* system is becoming more defined; however, the progression from oocyte maturation to developing embryo is not efficient. Research is aimed at increasing the efficiency of this system through elucidating the reproductive and cellular mechanisms associated with the gametes and general reproductive processes. The *in vitro* system can be broken down into three broad steps: oocyte maturation, fertilization, and embryo culture.

In vitro oocyte maturation is a process in which oocytes are aspirated from ovarian follicles and matured in a chemically defined medium until they are ready for fertilization. During maturation the oocyte undergoes nuclear maturation and cytoplasmic maturation, both of which will be discussed in greater detail. The fertilization step includes preparing oocytes and spermatozoa for fertilization as well as the actual process of fertilization. This step appears to have the most noticeable inadequacies, and therefore draws the attention of a majority of the research. Embryo culture refers to the culturing of the zygote after fertilization, to the endpoint set forth by the researcher. This endpoint ranges from the 2-cell stage for microinjection purposes, 4-cell stage for embryo transfer studies, or blastocyst for stem cell and embryonic development research.

Defining the actual beginning and end points of each step is an arduous task because *in vivo* the reproductive processes occur along a continuum; meaning, the inadequacies of the system at a particular point are likely a result of a different problem prior to the observation. For example, if there is a problem with embryos progressing from the 4-cell to the 8-cell stage of development, the problem may not be at that step, but perhaps something that happened or failed to happen during fertilization or during oocyte maturation [3]. With this in mind the aim of this literature review is to present the research pertaining to *in vitro* maturation and briefly discuss fertilization and embryo development.

The mechanisms of oocyte maturation are highly complex and are usually broken down into those of nuclear maturation and cytoplasmic maturation. Nuclear maturation consists of that which occurs within the nucleus of an oocyte, whereas cytoplasmic maturation encompasses the events that occur outside of the nucleus. It is important to note that both of these processes begin during oogenesis and are greatly influenced by the oocyte-follicle interaction during development. When studying *in vitro* oocyte maturation, it is crucial to consider at what stage the oocytes were removed from the follicles and the potential effects of that act. To progress in a logical manner, nuclear maturation will be discussed, followed by cytoplasmic maturation.

The primordial germ cells in females will differentiate prenatally into oogonia and undergo mitotic replication, becoming primary oocytes. These primary oocytes will become "arrested" at the dictyotene stage of meiotic prophase until ovulation. More notably, during this time of arrest, the follicle surrounding the oocyte will undergo drastic changes, creating a unique relationship with the oocyte which is essential for the life of the oocyte.

An oocyte is competent when it is able to resume meiosis [4]. *In vivo*, competency is obtained after the preovulatory surge of LH and the disruption of the gap junctions between the follicle and the oocyte. *In vitro,* the disruption of the gap junctions occurs

manually when the oocyte is aspirated from the follicle. This observation led to the hypothesis that an inhibitor was responsible for blocking the resumption of meiosis which was passed between the follicular cells and the oocyte [4]. This inhibitor is made up of at least a purine base and hypoxanthine; however, the exact structure and mechanisms of this oocyte maturation inhibitor (OMI) have yet to be determined [5]. The decrease in OMI is not solely responsible for the resumption of meiosis; the levels of cAMP in the oocyte are thought to be the dominating factor [6]. High levels of cAMP found in the oocyte, produced by the follicle, inhibit the resumption of meiosis. Once the levels of cAMP decrease, corresponding to the release of the oocyte from the follicle, meiosis resumes.

The removal of these inhibitors allows for the activation of cyclins, kinases, and phosphatases. Active transcription and translation is required in order for the chromatin to condense and the germinal vesicle to breakdown in pigs [7]. Cyclin B-p34cdc2, one of the most studied cyclins, is known as maturation promoting factor (MPF) and activates an enzymatic cascade resulting in the germinal vesicle breakdown, chromosome condensation and spindle formation. Low MPF concentrations are found in preantral oocytes, which do not typically resume nuclear maturation *in vitro* [8]. In the larger, preovulatory follicles the MPF activity is higher, indicating the oocyte is able to continue maturation [9]. Other cyclins involved in meiotic resumption are the mitogen-activated protein kinases (MAPK), which lead to the activation of proteins involved with cellular division [10].

The gonadotropin surge causes the dissociation of the follicular cells and the oocyte. This in turn causes a decrease in OMI and cAMP concentrations within the oocyte. Removal of the inhibitors causes the activation of multiple cyclins, MPF and MAPK which all activate proteins. These proteins are responsible for the germinal vesicle breakdown, chromosome condensation, and cell division/polar body extrusion. At the end of nuclear maturation, the oocyte is arrested in metaphase II of meiosis until fertilization occurs (Figure 1-1).

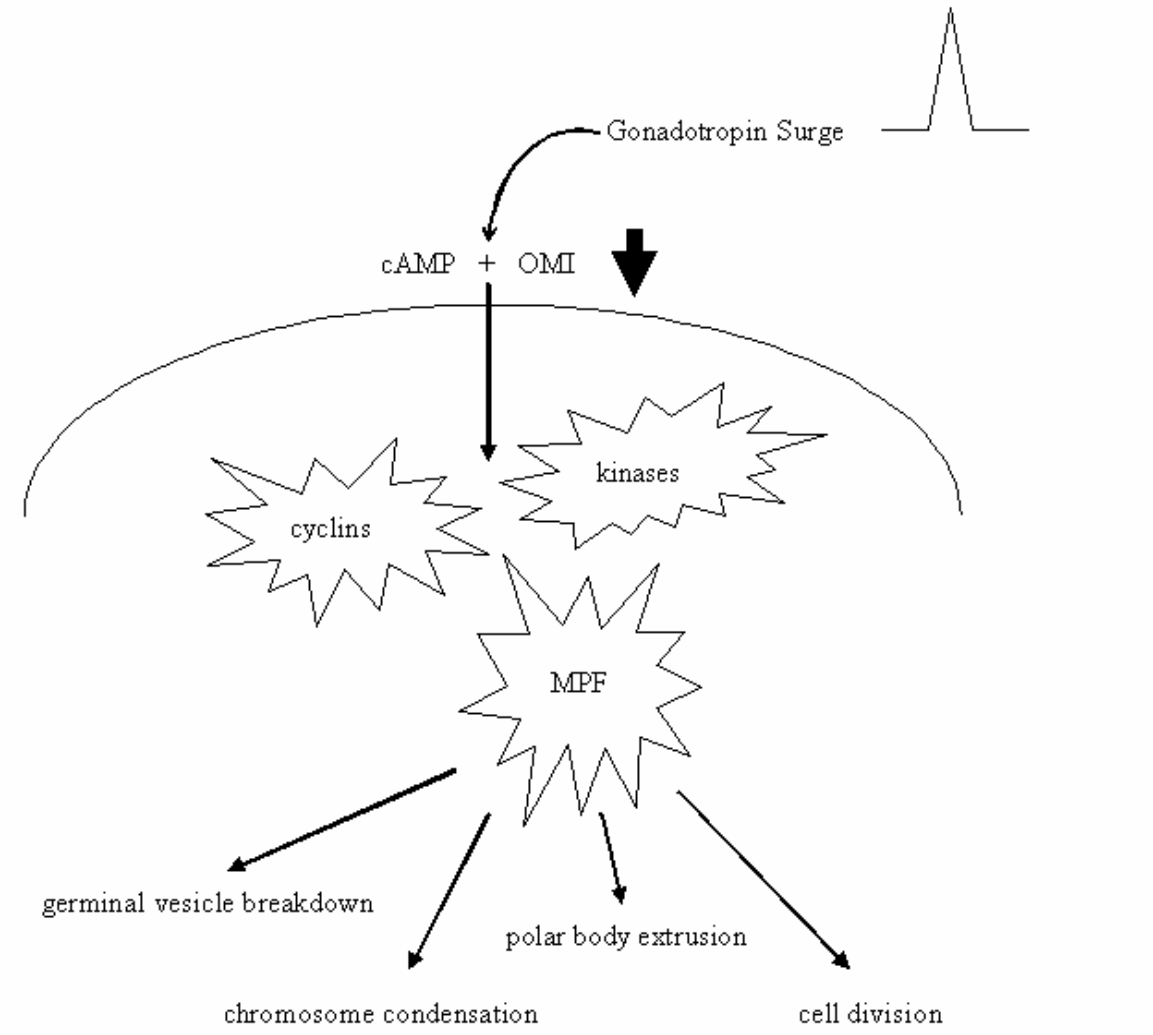

Figure 1-1. Molecular mechanisms involving nuclear maturation and resumption of meiosis

Early during follicle development, functional gap junctions form between the follicle and the oocyte. These complexes remain intact throughout oocyte maturation, until they become dissociated during the gonadotropin surge. The relationship between the

follicular cells and the oocyte is crucial for oocyte growth and metabolic maintenance. The ionic and electrotonic coupling between the two cell types along with the passage of nucleosides, amino acids and phospholipids is what allows cytoplasmic maturation to occur.

The span of time that the oocyte resides in the follicle can be divided into two phases. During the first phase the follicle and oocyte (30 μm) increase in size at approximately the same rate, whereas during the second phase the oocyte remains at its full size (120 μm) and the follicle continues to increase in size [7]. The rate of oocyte growth is proportional to the number of granulosa cells coupled to the oocyte [11]. This is understandable, as the more surface area in contact with the granulosa cells, the more substances can be transported into the oocyte for development.

Mitochondria are synthesized once the oocyte becomes competent, however the small degree of inner membrane folding, or cristae, indicates that the mitochondria have a low level of activity, or none at all [4]. Mitochondria function in producing the majority of the energy for the oocyte in the form of ATP. The energy requirements of the oocyte prior to activation are minimal, as indicated by the low degree of cristae folding. Once the oocyte is activated the mitochondria increase the degree of cristae folding, indicating a need for energy during fertilization.

The profile of RNA synthesis within the oocyte is high during initial oocyte growth; however, it declines as the oocyte reaches its final size. All of the RNA is native, and therefore not carried in from other sources [12]. Of the total mRNA synthesized, only about 15% is associated with ribosomes, whereas the rest is stored until future use. Evidence suggests that much of the RNA is used for the fabrication of the zona pellucida surrounding the oocyte and is supported by the observation that when fertilization occurs, the total amount of RNA in the oocyte has been significantly depleted [4].
Cortical granules are formed during cytoplasmic maturation from the Golgi apparatus. They originate in the cis-Golgi and eventually migrate from the trans-face to the cell membrane where they remain until oocyte activation. When the oocyte becomes fully

mature, the cortical granules migrate to directly beneath the plasma membrane in preparation for exocytosis during fertilization.

Signals and factors are required from the granulosa cells to complete cytoplasmic maturation and oocyte growth. The surrounding granulosa cells acquire these factors; however, the granulosa cells do not need to be in direct contact with the oocyte, as part of this occurs after ovulation [13]. This indicates that the signals from the granulosa cells are paracrine in action [14]. The exact signal(s) responsible for oocyte growth is unknown, but many promotion factors have been identified such as c-kit, c-kit ligand and stem cell factor [15].

Fertilization *in vitro* (IVF) and subsequent embryo culture should closely resemble the environment and timing that occurs *in vivo*. The *in vitro* conditions are inferior to those *in vivo* as evidenced by low rates of male pronuclear (MPN) formation and a high incidence of polyspermic penetration found during fertilization [16]. Polyspermic fertilized embryos have fewer inner cell mass numbers and abnormal cleavage patterns [17]. Research has shown that using oocytes surrounded by cumulus cells (compared to denuded oocytes) significantly increases the success of IVF [18]. The use of frozen-thawed boar spermatozoa is preferred for boar selection and repeatability purposes, however it has significantly reduced motility and viability compared to fresh semen [19]. The actual reason behind why the IVF system is inadequate is unknown. Studies suggest that it could be a result of inadequate cortical granule dispersion [20] or overall decrease in zona pellucida thickness and perivitelline space [21].

Swine embryo culture is able to produce 4-cell stage embryos capable of being implanted into recipients and carried to term [22] in addition to developing to the hatching blastocyst stage *in vitro* [23]. However, the developmental competence of *in vitro* derived offspring is significantly lower than natural offspring with respect to growth rate and weight [24]. Additionally, *in vitro* derived embryos have lower and delayed cleavage rates and asynchronous pronucleus development compared to *in vivo* derived embryos

[25] and the number of nuclei of *in vitro* derived blastocysts is significantly lower than *in vivo* blastocysts.

Antioxidants

Originally, an antioxidant was considered anything that prevented the consumption of oxygen. In the realm of science, an antioxidant is a molecule that retards the oxidation of another. Oxidation is half of any redox reaction, reduction being the other half. During oxidation, a molecule (known as the reductant) loses an electron to some product, which gains that electron. Concurrently, during reduction, a molecule (known as the oxidant) gains an electron from the reductant. Taken together, these reactions are known as a redox reaction (Figure 1-2).

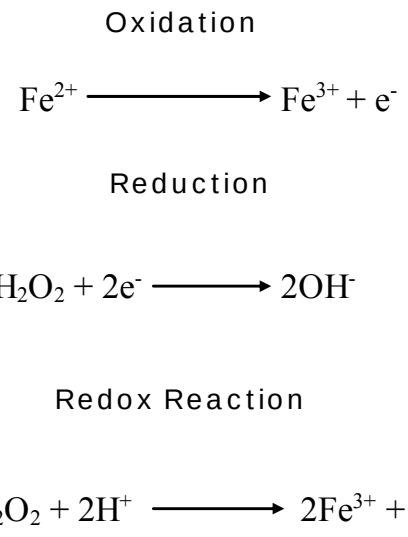

Figure 1-2. General equations comprising a redox reaction.

Thus, antioxidants prevent oxidation by gaining an electron and can be referred to as a reducing agent. Any electrons remaining as a result of a redox reaction are known as free radicals. If the level of free radicals in a cell becomes elevated beyond the ability of the

cell to adapt, the free radicals become detrimental to the cell. In biological systems, these free radicals are known as reactive oxygen species (ROS). Antioxidants are able to alleviate the buildup of ROS by removing the free radicals. Similar to most biochemical reactions, antioxidants are generally specific to an ROS; therefore, when considering any aerobic organism, there are numerous types and quantities of antioxidants. When the physiological level of antioxidants is too low or inhibited, oxidative stress occurs, which may damage and/or kill cells, organs, and individuals.

The importance and benefits of antioxidants have infiltrated into society and could be considered common knowledge. Through avenues such as advertisements and the popular press, antioxidants can be obtained over the counter as medicine, additives in food products, or naturally occurring, as is the case with most teas [26] and wines [27]. The scientific community, long aware of the benefits, is just starting to utilize antioxidants to their fullest potential [28]. Antioxidants are traditionally known to scavenge for free radicals and ROS in cells which reduces potential DNA and membrane damage, but a new role of antioxidants is beginning to emerge. Because antioxidants act as reducing agents, they in turn alter the intracellular redox potential, which changes the redox state of the cell [28]. This change in redox state regulates the activity of transcription factors [29], which regulate apoptosis and normal cell functions, thus giving antioxidants much more control over a cell than previously thought.

Research is showing that antioxidants have some role in protecting the body against almost every disease and physiological function. Antioxidants have been shown to prevent neurological diseases including Parkinson's, Alzheimer's, and amyotrophic lateral sclerosis [30] in addition to preventing heart problems [31]. Recently antioxidants have infiltrated into the reproductive sector and have been implicated in improving embryo implantation [32]. Most of the responses and treatments associated with inflammation use some type of antioxidants as well [33]. Although most antioxidants have a broad spectrum of applications, this review will focus towards those associated with reproduction: glutathione (GSH) and N-acetyl-cysteine (NAC). An NAC derivative, N-acetyl-cysteine-amide (NACA) will also be discussed.

L-γ-glutamyl-L-cysteinyl-glycine, generally known as GSH has two structural characteristics, a sulfhydryl group and a γ-glutamyl linkage making it the most abundant intracellular thiol and γ-glutamyl compound (Figure 1-3) [34]. Glutathione is a tripeptide, non-protein thiol, and is found in all living cells in the form of a sulfhydryl group (GSH) or as a disulfide (GSSG). The GSH is the predominant form, accounting for 99.5% of the total GSH detected [35]. The conversion of GSSG to GSH is readily catalyzed by the enzyme glutathione reductase allowing GSH to act as a major intracellular reductant (Figure 1-4) [36, 37].

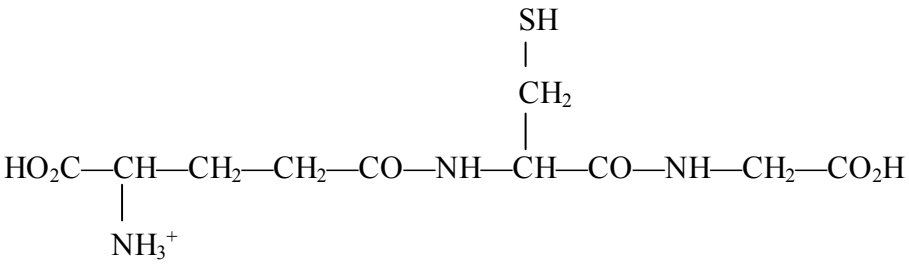

Figure 1-3. Structure of L-γ-glutamyl-L-cysteinyl-glycine, generally known as GSH.

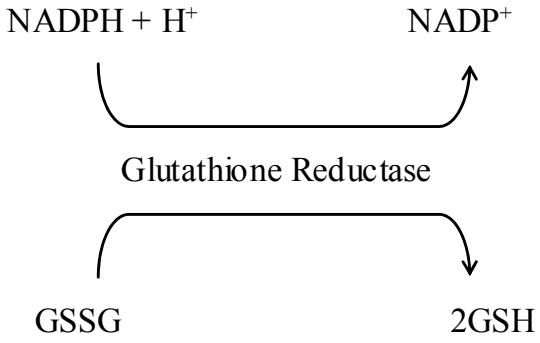

Figure 1-4. Conversion of GSSG to GSH.

The two primary roles of GSH are as an amino transporter [37] and as a cellular protectant against oxidative stress [38]. Amino acids (most commonly cysteine) are transported into the cell using the γ-glutamyl cycle, which is also responsible for GSH synthesis. The γ-glutamyl cycle is vital for cell survival, as γ-glutamyl transpeptidase is

responsible for transporting amino acids from outside the cell to the inside by attaching it to a γ-glutamyl amino acid carrier. Concurrently, GSH is being synthesized by GSH synthetase and replenished by the reduction of GSSG to GSH catalyzed by GSH reductase and NADPH or NADH as the hydrogen donor (Figure 1-5) [39].

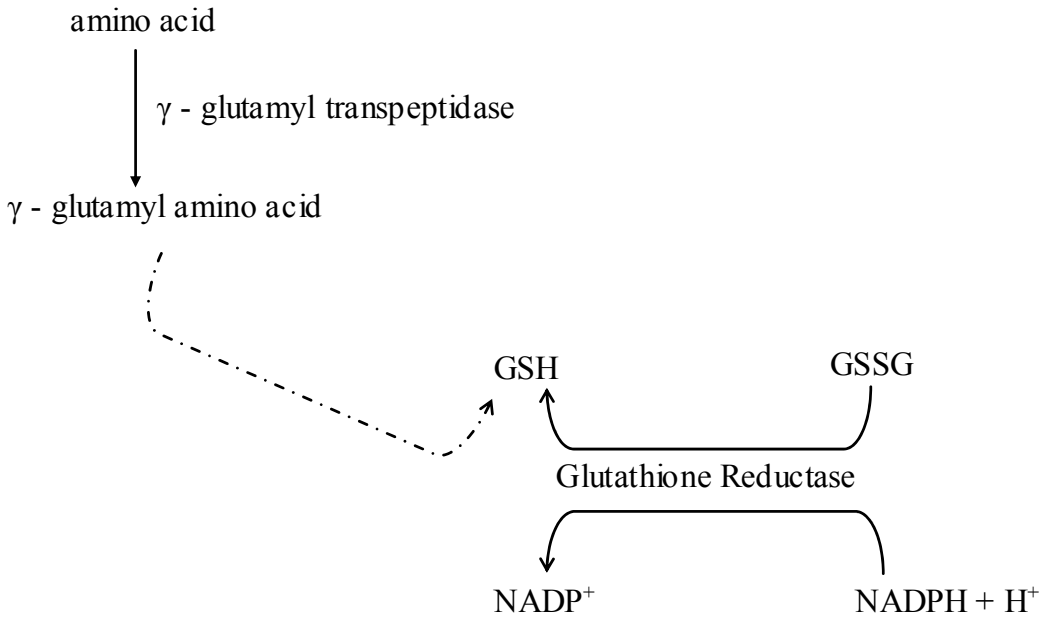

Figure 1-5. γ - glutamyl cycle and GSH synthesis.

Glutathione reacts with the oxidants and is readily oxidized to GSSG, whereas the oxidants are reduced to non-toxic metabolites. The GSSG is then reduced back to GSH to maintain equilibrium within the cell. Therefore, GSH provides a reducing

environment for biological systems and acts as a protectant against oxidative stress. This is apparent in most cell types [38], including neurons [40] and gametes [41].

Elevated levels of intracellular GSH increase the success of oocyte maturation, IVF and subsequent embryo development [42]. Research also suggests that elevated levels of GSH in cells may reduce the incidence of apoptosis and cell degeneration [42, 43]. During fertilization, GSH causes the cytoplasm to be in an oxidation-reduction state [44]. This environment promotes the decondensing sperm to develop a MPN [45] by breaking the disulfide bonds of protamine that hold the chromatin in a condensed conformation. Glutathione has been implicated in regulating DNA and protein synthesis and amino acid transport inside maturing oocytes [46]. Supplementation of GSH during oocyte maturation and IVF increases the success rate of fertilization and embryo development [47, 48]. Scientists are currently studying the role of GSH in preventing and alleviating oxidative stress during oocyte maturation [49].

N-acetyl-cysteine is a cysteine molecule with an acetyl group attached to its nitrogen atom. The thiol (sulfhydrl) group allows NAC to be classified as an antioxidant able to scavenge free radicals (Figure 1-6). Research has shown that NAC reduces cystine to cysteine, thus modulates GSH biosynthesis [50] and has the ability to supply its sulfhydryl group to enhance glutathione-S-transferase activity [51], which attaches glutathione to various compounds.

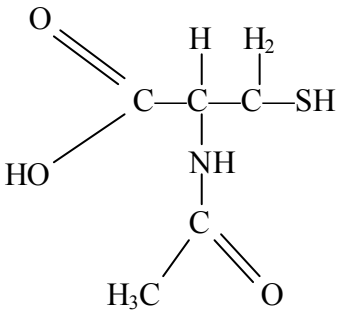

Figure 1-6. Chemical structure of N-acetyl-cysteine

The beneficial effects of NAC are widespread. High concentrations of NAC protect systemic hemodynamics, kidney function, and moderate anti-inflammatory mechanisms [52]. Current research suggests that NAC is able to repair damaged DNA and treat lymphomas by reducing reactive oxygen species [53]. Damaged liver and lung tissue were shown to repair themselves when supplemented with NAC by modulating gene expression [54].

N-acetyl-cysteine has been implicated to assist in reproductive processes as well, either in the genetics [55] or protection against oxidative stress [56]. Supplementation of NAC during the later stages of embryo development has shown to decrease the incidence of early fetal death [56]. Surprisingly, when NAC is supplemented during early embryo development it reduces the success of embryonic survival [57]. Research suggests that NAC has an effect on nuclear factor-κ B (NF-κB) activity [57, 49, 58] and that it is stage dependent. During the early stages of embryonic development NAC interferes with NF-κB activity whereas during the later stages of embryonic development NAC enhances NF-κB activity.

N-acetyl-cysteine-amide is a derivative of NAC with an amide group attached. The amide addition neutralizes the otherwise negatively charged carboxyl group allowing it to permeate most physiological membranes (Figure 1-7). Research shows that NACA is able to replenish intracellular GSH and defend cells against oxidation [59, 60]. The NACA acted on oxidized GSH (GSSG) and regenerated it into the reduced form (GSH). This would hypothetically be a viable treatment for oxidation-mediated diseases such as multiple sclerosis and encephalitis [61] due to its ability to cross physiological barriers, unlike its counterpart NAC.

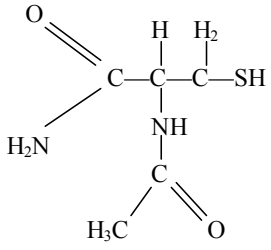

Figure 1-7. Chemical structure of N-acetyl-cysteine-amide

Oxidative Stress

In any living cell, there is an equilibrated system that keeps the generation of oxygen species in balance. When the system is compromised and the available antioxidants can not keep the oxygen concentration within appropriate levels, ROS are generated and accumulated. When an overabundance of ROS is produced and the cell cannot adapt, a phenomenon known as oxidative stress occurs. In the normal functioning cell, one of the roles of antioxidants is to scavenge the free radicals and eliminate or neutralize them, thus detoxifying the cell. The combined effect of the various antioxidants present in a cell and the multitude of enzymes and mechanistic pathways that exist to keep the cell in a favorable environment, provide a complex system to combat oxidative stress throughout the life cycle of a cell [28].

Prolonged oxidative stress is responsible for pathological ailments such as cancer, arthritis, and multiple neurological diseases [43]. Most of these types of diseases: Alzheimer's, Friedreich's ataxia, amylotrophic lateral sclerosis, Parkinson's, and Huntington's disease involve ROS and mitochondrial dysfunction, which can both be linked to GSH homeostasis imbalance [30]. Oxidative stress has broad effects on the reproductive system and has been implicated in impairing the functionality of ovarian steroidogenesis and ovulation [62], embryogenesis [63], and implantation and maintenance of pregnancy [33]. Perhaps more importantly and pertinent is that recently the effects of oxidative stress during oocyte maturation have been studied [49, 64].

During bovine oocyte maturation, the antioxidants and enzymes involved in scavenging ROS are located in the follicular cells and the oocyte. Removal of the follicular cells increases the level of oxidative stress up to a certain point in time during maturation [64]. Prior to 2001, it was unclear whether or not the mechanisms required to alleviate oxidative stress were even present inside the oocytes. Further research demonstrated that supplementing antioxidants to the different *in vitro* media was not adequate to alleviate oxidative stress [49]. The effects were substance specific whereby particular amino acids were more beneficial than generic antioxidants. There are undoubtedly still many

uncertainties with respect to how oxidative stress is handled during oocyte maturation and the mechanisms by which ROS are eliminated.

The general mechanisms that are believed to be conserved across cell types all begin with the generation of the ROS. The ROS are formed as a result of a stressor on the cell or as a byproduct of normal cell processes; this is often a direct oxidation of some oxygen species (Figure 1-8) [65]. If the ROS concentrations become elevated beyond the ability of the cell to neutralize or dispose of them, the ROS will cause oxidative damage through DNA breakdown, apoptosis, necrosis, and/or protein and lipid degradation (Figure 1-8) [66].

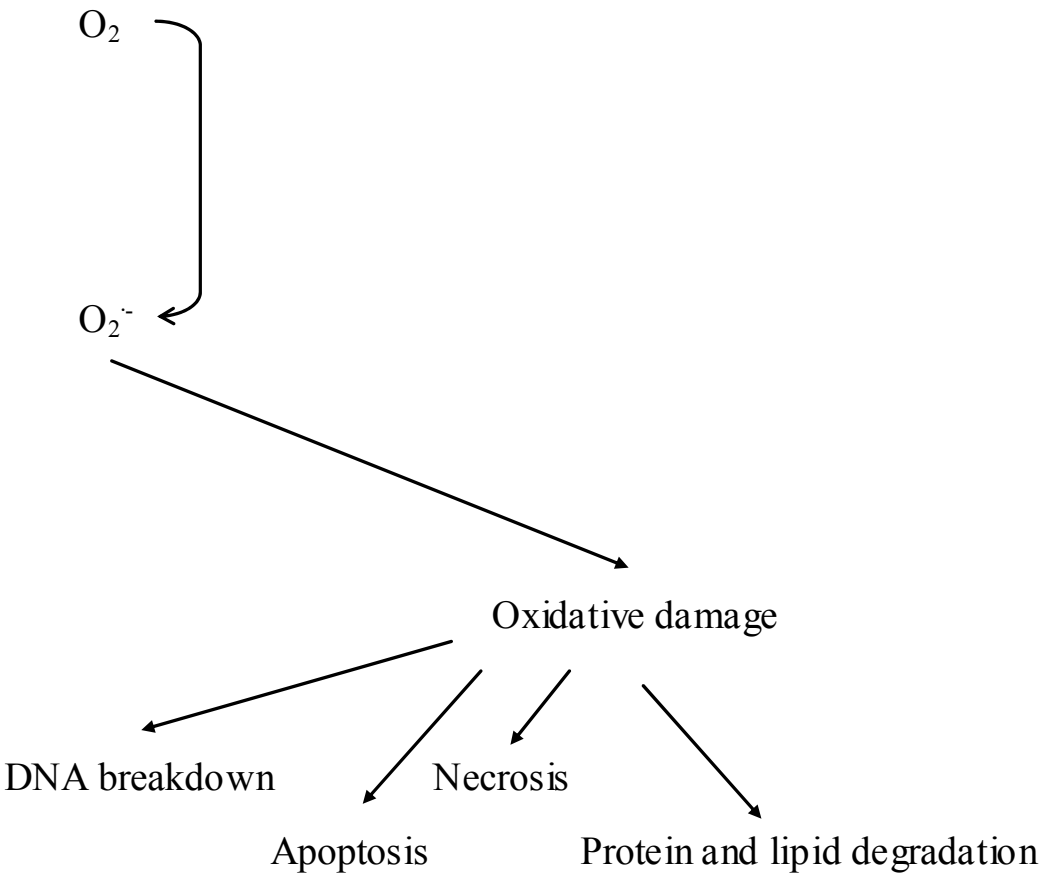

Figure 1-8. The cellular effects of oxidative damage.

Superoxide dismutase (SOD) catalyzes the dismutation of the ROS into hydrogen peroxide and molecular oxygen [67, 68]. In competent cells, there should be adequate levels of SOD to avoid the damaging effects of ROS. (Figure 1-9) [69].

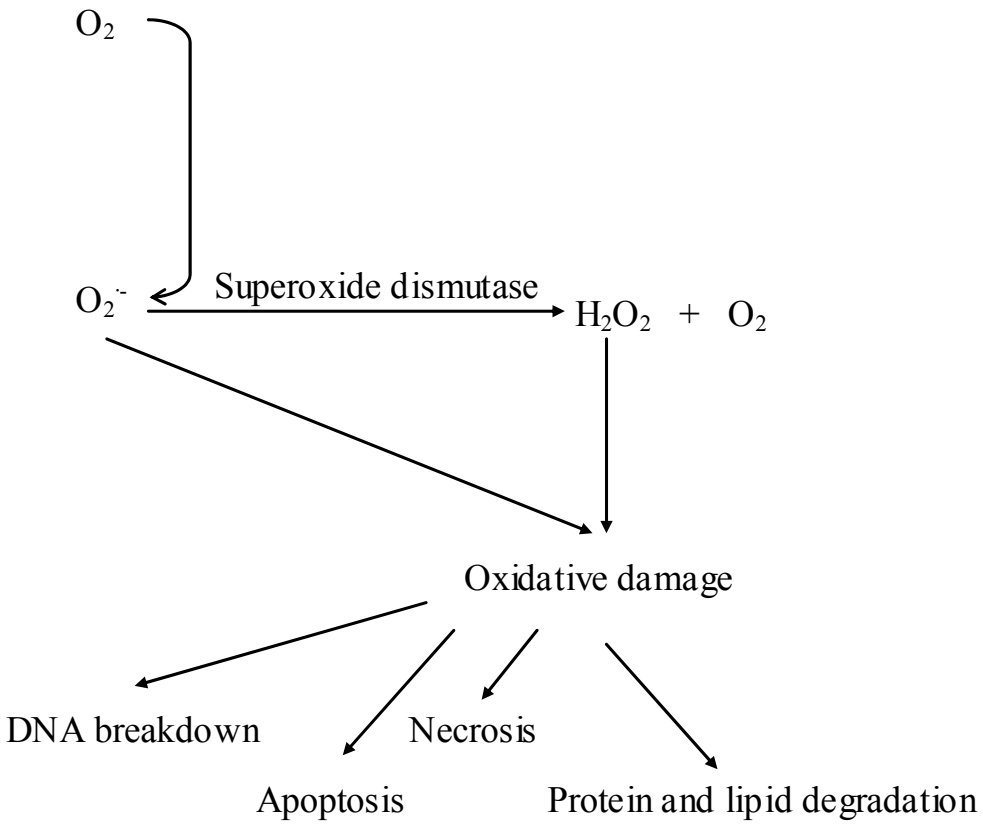

Figure 1-9. Mechanisms of SOD and hydrogen peroxide.

Although hydrogen peroxide is detrimental in most intracellular environments, it is far less damaging than free radical oxygen. The hydrogen peroxide can be decomposed into water and molecular oxygen by two different mechanistic pathways. One pathway utilizes catalase as the catalyst for the decomposition of hydrogen peroxide (Figure 1-10) [70]. The other, more complex pathway involves the GSH cycle.

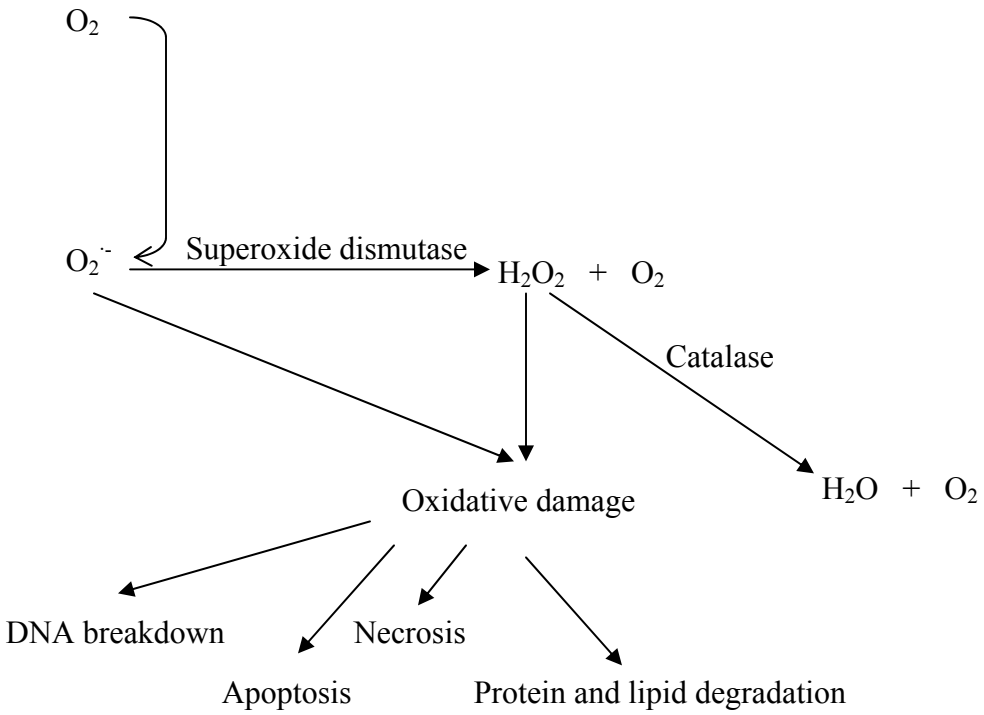

Figure 1-10. Mechanisms of catalase

Hydrogen peroxide is decomposed into water and molecular oxygen by GSH peroxidase which requires GSH as a co-factor. When GSH peroxidase oxidizes GSH to GSSG, hydrogen peroxide is broken down into water and molecular oxygen (Figure 1-11) [71]. Glutathione reductase is then responsible for reducing GSSG back to GSH, thus recycling GSH as the primary intracellular antioxidant [72].

Since GSH is a wide ranging and frequent used antioxidant, it has the potential to become the limiting factor in oxidative stress. If the intracellular concentration of GSH drops below the physiological level and stress occurs, the cell may not be able to compensate and, hence, deteriorate [43]. This is applicable to oocyte maturation in swine as well, as

shown through a series of published studies within the past ten years. The first study showed that surrounding follicular cells protect the developing oocytes against oxidative stress by increasing the intracellular concentrations of GSH in addition to reducing the incidence of DNA breakdown and apoptosis [73]. The subsequent study provided evidence that supplementing the maturation medium with antioxidants provides a more optimal environment by reducing the amount of ROS and perhaps compensating for inadequate maturation environment or diminished follicular cell function [74]. The final study elucidated the specific effects of the follicular fluid on maturing oocytes. The SOD levels in the fluid are responsible for reducing DNA damage and increasing cytoplasmic maturation competence [75].

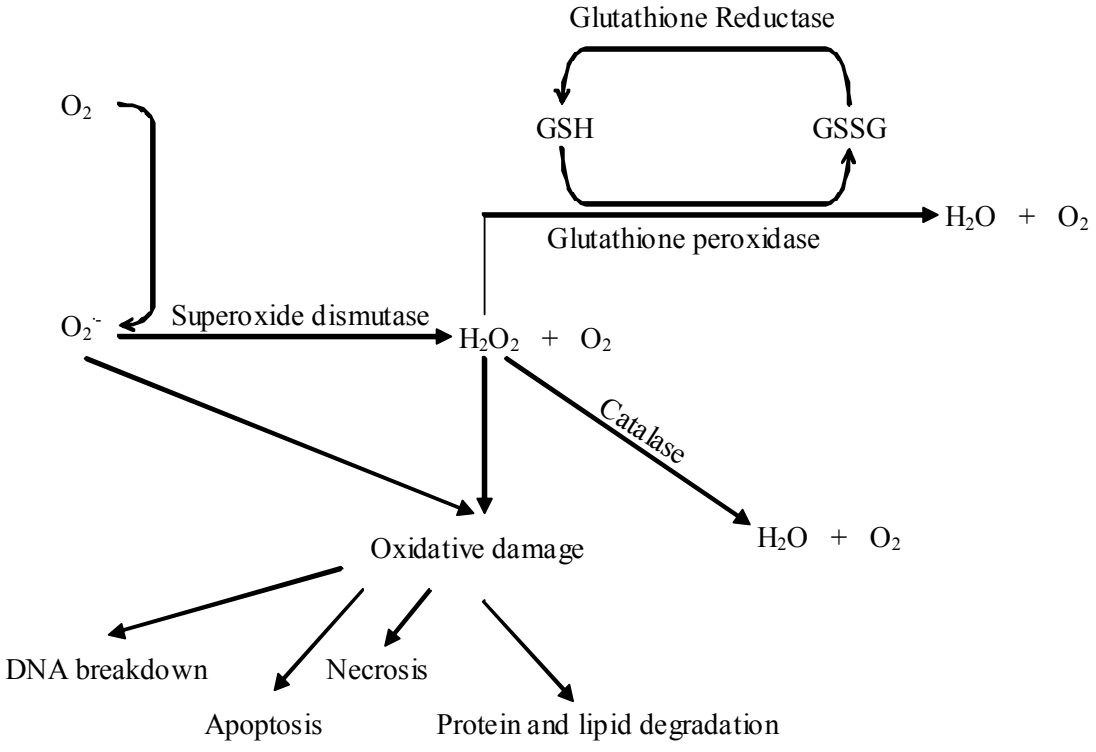

Figure 1-11. Role of GSH during oxidative stress.

It is often overlooked that oxidative stress is not absolute, just like any other biological process. A cell is a constantly adapting system and for the most part can adjust to survive in stressful situations and environments. Oxidative stress is only noticeable in extreme instances when the imminent fate is cell death or apoptosis.

Research Objectives

An increase in viable oocytes at the end of maturation equates to potentially more embryos being produced, it was therefore deemed acceptable to, 1) perform preliminary studies to determine the viability of using antioxidants during maturation in addition to various fertilization methods (Chapter 2), and 2) determine the concentration of antioxidant supplementation to oocytes to elicit optimal *in vitro* fertilization results and viable embryos (Chapter 3).

In considering the problems addressed during the review of literature, the objectives of the research were to then, 1) characterize the mechanisms of oxidative stress in maturating oocytes (Chapter 4), and 2) determine how oocytes alleviate oxidative stress (Chapter 4). With the ever increasing public interest in antioxidants, additional research was conducted to determine the effects of antioxidant supplementation to maturing oocytes (Chapter 4).

Literature Cited

1. Abeydeera LR. In vitro production of embryos in swine. Theriogenology 2002; 57:256-273.
2. Karsenty E, Barillot E, Tosser-Klopp G, Lahbib-Mansais Y, Milan D, Hatey F, Cirera S, Sawera M, Jørgensen CB, Chowdhary B, Fredholm M, Wimmers K, Ponsuksili S, Davoli R, Fontanesi L, Braglia S, Zambonelli P, Bigi D, Neuenschwander S, Gellin J. The GENETPIG database: a tool for comparative mapping in pig (*Sus scrofa)*. Nucleic Acids Res 2003; 31:138-141.
3. Abeydeera LR, Wang WH, Cantley TC, Rieke A, Day BN. Coculture with follicular shell pieces can enhance the developmental competence of pig oocytes after in vitro fertilization: relevance to intracellular glutathione. Biol Reprod 1998; 58:213-218.
4. Szöllösi D. Oocyte maturation. In: Thibault C, Levasseur MC, Hunler RHF (eds), Reproduction in Mammals and Man. Paris: Ellipses; 1993: 307-325.
5. Eppig JJ, Downs SM. The role of purines in the maintenance of meiotic arrest in mammalian oocytes. Prog Clin Biol Res 1988; 267:103-113.
6. Schultz R. Molecular aspects of oocyte growth and maturation. In: Rossant J, Pedersen RA (eds), Experimental Approaches to Mammalian Embryonic Development. Cambridge: University Press; 1987: 195-237.
7. Hunter MG. Oocyte maturation and ovum quality in pigs. Rev Reprod 2000; 5:122-130.
8. Christmann L, Jung T, Moor RM. MPF components and meiotic competence in growing pig oocytes. Mol Reprod Dev 1994; 38:85-90.
9. Hirao Y, Tsuji Y, Miyano T, Okano A, Miyake M, Kato S, Moor RM. Association between p34cdc2 levels and meiotic arrest in pig oocytes during early growth. Zygote 1995; 3:325-332.
10. Wehrend A, Meinecke B. The meiotic cell cycle in oocytes of domestic animals. Reprod Domest Anim 1998; 33:289-297.

11. Herlands RL, Schultz RM. Regulation of mouse oocyte growth: probable nutritional role for intercellular communication between follicle cells and oocytes in oocyte growth. J Exp Zool 1984; 229:317-325.
12. Bachvarova R, Paynton BV. Gene expression during growth and meiotic maturation of mouse oocyte. Prog Clin Biol Res 1989; 267:67-85.
13. Mattioli M, Galeati G, Bacci ML, Seren E. Follicular factors influence oocyte fertilizability by modulating the intercellular cooperation between cumulus cells and oocyte. Gamete Research 1988; 21:223-232.
14. Cecconi S, Rossi G, De Felici M, Colonna R. Mammalian oocyte growth in vitro is stimulated by soluble factor(s) produced by preantral granulosa cells and by Sertoli cells. Mol Reprod Dev 1996; 44:540-546.
15. Tanikawa M, Harada T, Mitsunari M, Onohara Y, Iwabe T, Terakawa N. Expression of c-kit messenger ribonucleaic acid in human oocyte and presence of soluble c-kit in folliciluar fluid. J Clin Endocrinol Metab 1998; 83:1239-1242.
16. Hunter MG. Oocyte maturation and ovum quality in pigs. Rev Reprod 2000; 5:122-130.
17. Han YM, Wang WH, Abeydeera LR, Petersen AL, Kim JH, Murphy C, Day BN, Prather RS. Pronuclear location before the first cell division determines ploidy of polyspermic pig embryos. Biol Reprod 1999; 61:1340-1346.
18. Coy P, Martinez E, Ruiz S, Vazquez JM, Roca J, Gadea J. Environment and medium volume influence in vitro fertilisation of pig oocytes. Zygote 1993; 1:209-213.
19. Wang WH, Niwa K, Okuda K. In-vitro penetration of pig oocytes matured in culture by frozen-thawed ejaculated spermatozoa. J Reprod. Fertil 1991; 93:491-496.
20. Cran DG, Cheng WTK. The cortical reaction in pig oocytes during in vivo and in vitro fertilization. Gamete Research 1986; 13:241-251.
21. Wang WH, Abeydeera LR, Prather RS, Day DN. Morphologic comparison of ovulated and in vitro-matured porcine oocytes, with particular reference to polyspermy after in vitro fertilization. Mol Reprod Dev 1998; 49:308-316.

22. Mattioli M, Bacci ML, Galeati G, Seren E. Developmental competence of pig oocytes matured and fertilized in vitro. Theriogenology 1989; 31:1201-1207.
23. Yoshida M, Ishizaki Y, Kawagishi H, Bamba K, Kojima Y. Effects of pig follicular fluid on maturation of pig oocytes in vtiro and on their subsequent fertilizing and developmental capacity in vitro. J Reprod. Fertil 1992; 95:481-488.
24. Kikuchi K, Kashiwazaki N, Noguchi J, Shimada A, Takahashi R, Hirabayashi M, Shino M, Ueda M, Kaneko H. Developmental competence, after transfer to recipients, of porcine oocytes matured, fertilized, and cultured in vitro. Biol Reprod 1999; 60:336-340.
25. Laurincik J, Rath D, Niemann H. Differences in pronucleus formation and first cleavage following in vitro fertilization between pig oocytes matured in vivo and in vitro. J Reprod Fertil 1994; 102:277-284.
26. Siddiqui IA, Afaq F, Adhami VM, Ahmad N, Mukhtar H. Antioxidants of the beverage tea in promotion of human health. Antioxid Redox Signal 2004; 6:571-582.
27. Bertelli AA. Wine, research and cardiovascular disease: Instructions for use. Atherosclerosis published 24 May 2007; PMID: 17531243.
28. Brash DE, Havre PA. New careers for antioxidants. Proc Natl Acad Sci USA 2001; 99:13969-13971.
29. Sun Y, Oberley LW. Redox regulation of transcriptional activators. Free Radical Biol Med 1996; 21:335-348.
30. Schulz JB, Lindenau J, Seyfried J, Dichgans J. Glutathione, oxidative stress and neurodegneration. Er J Biochem 2000; 267:4904-4911.
31. Dhalla NS, Saini HK, Tappia PS, Sethi R, Mengi SA, Gupta SK. Potential role and mechanisms of subcellular remodeling in cardiac dysfunction due to ischemic heart disease. J Cardiovasc Med 2007; 8:238-50.
32. Ornoy A. Embryonic oxidative stress as a mechanism of teratogenesis with special emphasis on diabetic embryopathy. Reprod Toxicol 2007; 24:31-41.
33. Myatt L, Cui X. Oxidative stress in the placenta. Histochem Cell Biol 2004; 122:369-382.

34. Meister A, Tate SS. Glutathione and related γ-glutamyl compounds: biosynthesis and utilization. Annu Rev Biochem 1976; 45:559-604.
35. Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 1985; 113:548-555.
36. Jocelyn PC. Glutathione metabolism in animals. Biochem Soc Symp 1959; 17:43-65.
37. Meister A. Amino acids and glutathione. Proc Biochemical Symp 1982; 5-27.
38. Kosower NS, Kosower EM. Protection of membranes by glutathione. In: Flohe L, Benohr C, Sies H (eds), Glutathione. New York: Academic Press, Inc.; 1973: 216-226.
39. Beutler E. Glutathione reductase. In: Flohe L, Benohr C, Sies H (eds), Glutathione. New York: Academic Press, Inc.; 1973: 109-113.
40. Muller WE, Laplanche JL, Ushijima H, Schroder HC. Novel Approaches in diagnosis and therapy of Creutzfeldt-Jakob disease. Mech Ageing Dev 2000; 116:193-218.
41. Yoshida M, Ishigaki K, Nagai T, Chikyu M, Pursel VG. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol Reprod 1993; 49:89-94.
42. Whitaker BD, Knight JW. Exogenous γ-glutamyl cycle compounds supplemented to in vitro maturation medium influence in vitro fertilization, culture, and viability of porcine oocytes and embryos. Theriogenology 2004, 62:311-322.
43. Hayes JD, McLellan LI. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radical Research 1999; 31:273-300.
44. Nagai T. The improvement of in vitro maturation systems for bovine and porcine oocytes. Theriogenology 2001; 55:1291-1301.
45. Sawai K, Funahashi H, Niwa K. Stage-specific requirement of cysteine during in vitro maturation of porcine oocytes for glutathione synthesis associated with male pronuclear formation. Biol Reprod 1997; 57:1-6.

46. Yamauchi N, Nagai T. Male pronuclear formation in denuded porcine oocytes after in vitro maturation in the presence of cysteamine. Biol Reprod 1999; 61:828-833.
47. Urdaneta A, Jimenez AR, Paramio MT, Izquierdo D. Cysteamine, glutathione and ionomycin treatments improve in vitro fertilization of prepubertal goat oocytes. Zygote 2004; 12:277-284.
48. Funahashi H, Sano T. Select antioxidants improve the function of extended boar semen stored at 10 °C. Theriogenology 2005; 63:1605-1616.
49. Ali AA, Bilodeau JF, Sirard MA. Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. Theriogenology 2003; 59:939-949.
50. Issels RD, Nagele A, Eckert KG, Wilmanns W. Promotion of cystine uptake and its utilization for glutathione biosynthesis induced by cysteamine and N-acetylcysteine. Biochem Pharmacol 1988; 37:881-888.
51. Nakata K, Kawase M, Ogino S, Kinoshita C, Murata H, Sakaue T, Ogata K, Ohmori S. Effects of age on levels of cysteine, glutathione and related enzyme activities in livers of mice and rats and an attempt to replenish hepatic glutathione levels of mouse with cysteine derivatives. Mech Ageing Dev 1996; 90:195-207.
52. Schaller G, Pleiner J, Mittermayer F, Posch M, Kapiotis S, Wolzt M. Effects of N-acetylcysteine against systemic and renal hemodynamic effects of endotoxin in healthy humans. Crit Care Med 2007; 35:1869-1875.
53. Reimann M, Loddenkemper C, Rudolph C, Schildhauer I, Teichmann B, Stein H, Schlegelberger B, Dorken B, Schmitt CA. The Myc-evoked DNA damage response accounts for treatment resistance in primary lymphomas in vivo. Blood published 11 June 2007; PMID: 17562874.
54. Jin X, Wang L, Wu HS, Zhang L, Wang CY, Tian Y, Zhang JH. N-acetylcysteine inhibits activation of toll-like receptor 2 and 4 gene expression in the liver and lung after partial hepatic ischemia-reperfusion injury in mice. Hepatobiliary Pancreat Dis Int 2007; 6:284-289.

55. Pfeffer U, Ferrari N, Dell'Eva R, Indraccolo S, Morini M, Noonan DM, Albini A. Molecular mechanisms of action of angiopreventive anti-oxidants on endothelial cells: microarray gene expression analyses. Mutat Res 2005; 591:198-211.
56. Xu DX, Chen YH, Wang H, Zhao L, Wang JP, Wei W. Effect of N-acetylcysteine on lipopolysaccharide-induced intra-uterine fetal death and intra-uterine growth retardation in mice. Toxicol Sci 2005; 88:525–533.
57. Nishikimi A, Mukai J, Yamada M. Nuclear translocation of nuclear factor kappa B in early 1-cell mouse embryos. Biol Reprod 1999; 60:1536-1541.
58. Shao DZ, Lee JJ, Huang WT, Liao JF, Lin MT. Inhibition of nuclear factor-kappa B prevents staphylococcal enterotoxin A-induced fever. Mol Cell Biochem 2004; 262:177-185.
59. Grinberg L , Fibach E, Amer J, Atlas D. N-acetylcysteine amide, a novel cell-permeating thiol, restores cellular glutathione and protects human red blood cells from oxidative stress. Free Radic Biol Med 2005; 38:136–145.
60. Wu W, Goldstein G, Adams C, Matthews RH, Ercal N. Separation and quantification of N-acetyl-L-cysteine and N-acetyl-cysteine-amide by HPLC with fluorescence detection. Biomed Chromatogr 2006; 20:415-422.
61. Offen D, Gilgun-Sherki Y, Barhum Y, Benhar M, Grinberg L, Reich R, Melamed E, Atlas D. A low molecular weight copper chelator crosses the blood–brain barrier and attenuates experimental autoimmune encephalomyelitis. J Neurochem 2004; 89:1241–1251.
62. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reproductive Biology and Endocrinology 2005; 3:28-47.
63. Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE, Thornton J, Agarwal A. Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil Steril 2004; 82:593-600.
64. Cetica PD, Pintos LN, Dalvit GC, Beconi MT. Antioxidant enzyme activity and oxidative stress in bovine oocyte in vitro maturation. IUBMB Life 2001; 51:57-64.
65. Nicholls DG, Budd SL. Mitochondria and neuronal survival. Physiol Rev 2000; 80:315-360.

66. Fiers W, Beyaert R, Declercq W, Vandenabeele P. More than one way to die: apoptosis, necrosis and reactive oxygen damage. Oncogene 1999; 18:7719-7730.
67. McCord JM, Fridovich I. Superoxide dismutase: an enzymic function for erythrocuprein (hemocuprein). J Biol Chem 1969; 244:6049-6055.
68. Cavanagh EM, Inserra F, Ferder L, Romano L, Ercole L, Fraga CG. Superoxide dismutase and glutathione peroxidase activities are increased by enalapril and captopril in mouse liver. FEBS Letters 1995; 361:22-24.
69. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972; 247:3170-3175.
70. Aebi, H. Catalase in vitro. Methods Enzymol 1984; 105:121-126.
71. Günzler WA, Kremers H, Flohé L. An improved coupled test procedure for glutathione peroxidase (EC 1-11-1-9-) in blood. Z Klin Chem Klin Biochem 1974; 12:444-448.
72. Meister A. Amino acids and glutathione. Proceedings of the Biochemical Symposium 1982; 5-27.
73. Tatemoto H, Sakurai N, Muto N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during in vitro maturation: role of cumulus cells. Biol Reprod 2000; 63:805–810.
74. Tatemoto H, Ootaki K, Shigeta K, Muto N. Enhancement of developmental competence after in vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-O-glucoside during in vitro maturation. Biol Reprod 2001; 65:1800-1806.
75. Tatemoto H, Muto, N, Sunagawa I, Shinjo A, Nakada T. Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid. Biol Reprod 2004; 71:1150-1157.

CHAPTER TWO

PRELIMINARY STUDIES

ABSTRACT

This study was conducted to evaluate the effects of glutathione (GSH), N-acetyl-cysteine (NAC), and N-acetyl-cysteine-amide (NACA) supplemented to the maturation medium on intracellular GSH concentrations, nuclear maturation, fertilization success and embryo development. Antioxidants GSH, NAC and NACA (1.0 mM) were supplemented to the media during oocyte maturation. Intracellular GSH concentrations were recorded at 48 h of maturation and nuclear maturation and fertilization were analyzed 12 h after IVF. Embryo development was analyzed at 48 h and 144 h after IVF or intracytoplasmic sperm injection (ICSI). Supplementation of antioxidants had no effect on intracellular levels of GSH, nuclear maturation or fertilization traits. Blastocyst formation for NAC (35.0 ± 7.4%) and NACA (40.0 ± 7.4%) supplementation were higher ($P < 0.05$) than the control (20.0 ± 7.4%) and GSH (20.0 ± 7.4%) supplemented oocytes. The same pattern was seen for ICSI-derived embryos: blastocyst formation for NAC (22.0 ± 5.9%) and NACA (25.0 ± 4.6%) supplementation were higher ($P < 0.05$) than the un-supplemented (10.0 ± 6.0%) oocytes. There were no differences between NAC and NACA supplementation and there were no differences between the cleavage rates for any of the treatment groups. These results indicate that supplementing 1.0 mM of NAC or NACA to the oocyte maturation medium and the ICSI medium increased the percentage of viable embryos reaching the blastocyst stage of development.

INTRODUCTION

The ever-increasing importance of antioxidants is apparent throughout the scientific community and society. Antioxidants have roles in a wide variety of areas including skin repair [1], diabetes [2], and heart function [3]. Antioxidant supplementation has

beneficial effects on basic reproductive processes [4], oocyte maturation [5], and embryo development [6].

Antioxidants such as glutathione (GSH) are potent reducing agents for sustaining favorable *in vitro* environments during oocyte and embryo development. A large facet of pig *in vitro* research is to find media supplementations that increase intracellular GSH during oocyte maturation. An increase in intracellular GSH improves IVF and embryo development [7, 8]. Glutathione is synthesized by the γ-glutamyl cycle, therefore supplementing the γ-glutamyl cycle compound L-α-aminobutyrate and reducing agent β-mercaptoethanol into the *in vitro* maturation medium, increases the intracellular GSH concentrations, decreases the occurrence of polyspermic penetration and increases embryo development traits during *in vitro* culture [9].

The techniques of intracytoplasmic sperm injection (ICSI), pronuclear microinjection and genetic diagnosis of embryos result in a considerable loss of embryos compared to non-manipulated embryos, perhaps due to an enhanced level of oxidative stress [10]. High success rates *in vitro* could lead to *in vivo* applications to reduce infertility and increase embryo viability. Increasing the efficacy of *in vitro* embryo production has applications to a number of procedures including production of transgenic animals, as well as serving as a model for examining the potential effects of oxidative stress on infertility in humans.

Recently, Novia Pharmaceuticals Ltd. (Haifa Bay, Israel) produced a low molecular weight copper chelator, N-acetyl-cysteine-amide (NACA) that is able to cross the Blood-Brain-Barrier and target neural tissue/cells [11]. This compound is GSH-like in activity and derived from N-acetyl-cysteine (NAC). If NACA is shown to have no adverse side effects, it could be used to treat a multitude of diseases. If NACA is more potent than NAC, GSH and other γ-glutamyl cycle compounds, and can penetrate into the developing embryo, perhaps NACA will improve *in vitro* reproductive techniques including oocyte maturation, IVF and embryo development. Therefore, the objective of these preliminary studies was to determine the effects of GSH, NAC and NACA supplementation to the media during porcine oocyte maturation.

MATERIALS AND METHODS

Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO). The oocyte maturation medium was tissue culture medium 199 (TCM199) with Earle's salts (Invitrogen, Carlsbad, CA) supplemented with 5 μg/mL FSH, 1 μg/mL insulin, 50 ng/mL Gentamicin sulfate, 10 ng/mL Epidermal Growth Factor (EGF), and 10% fetal calf serum. The medium used for IVF was a modified Tris-buffered medium (mTBM) formulated by Abeydeera and Day [12]. The medium used to perform ICSI was a HEPES-buffered Tyrode medium (HbT) [13] containing 10% polyvinylpyrrolidone (PVP). The embryo culture medium was North Carolina State University (NCSU) 23 medium [14] containing 4% BSA (A 8022). The NACA was supplied by Novia Pharmaceuticals Ltd. (Haifa Bay, Israel).

Maturation of Oocytes

Oocytes aspirated from mature follicles (3-6 mm diameter) were obtained commercially (BoMed, Madison, WI) and shipped overnight in TCM199. Only oocytes observed with uniform ooplasm and compact cumulus cells 22 h after initial placement in media were selected. The selected oocytes were washed three times and randomly placed (45-50 oocytes/well) into 500 μL of previously equilibrated TCM199 under mineral oil without FSH and fetal calf serum supplementation. Oocytes were incubated at 39 °C in an atmosphere of 5% CO_2 for an additional 18 to 26 h. After incubation, cumulus cells were removed from the oocytes by repeat pipetting in TCM199 containing 0.1% hyaluronidase. Oocytes observed with uniform ooplasm were then washed and stored (30-50 oocytes/well) in mTBM under mineral oil until IVF.

Glutathione Assay

The concentration of intracellular GSH was determined using the 5,5'-dithio-bis(2-nitrobenzoic acid) – GSH disulfide (DTNB-GSSG) reductase recycling assay as described by Anderson [15]. Matured denuded oocytes were washed in 0.2 M sodium phosphate buffer containing 10 mM EDTA (pH 7.2). Approximately 20 oocytes were transferred to a micro-centrifuge tube and stored at –80 °C. Oocytes were thawed and 5 µL of 1.25 M H_3PO_4 was added to each tube and the oocytes were ruptured using a blunt glass rod. The oocytes were then frozen and stored again at –80 °C until the day of the assay. For each sample, 0.3 mM NADPH and 6.0 mM DTNB were incubated at 30 °C for 15 min. The sample was added to the cuvette followed by 250 U/mL of GSH reductase to initiate the reaction. The change in absorbance was determined using a Genesis 20 spectrophotometer (Thermo Spectronic, Rochester, NY) at 412 nm for 3 min. The concentration of intracellular GSH was determined from a standard curve where the concentration of GSH was plotted against the rate of change in absorbance at 412 nm.

Spermatozoa Preparation and IVF

Approximately 3 h before the completion of maturation, a frozen semen pellet (Swine Genetics International Ltd., Cambridge, IA) was thawed in Dulbecco phosphate buffered saline containing 0.1% BSA, 75 µg/mL potassium penicillin and 50 µg/mL streptomycin sulfate at 39 ºC and centrifuged at 36.3 x *g* for 5 min. The semen was then washed twice at 553 x *g* for 5 min. After washing, the spermatozoa pellet was re-suspended with IVF medium to a concentration of 4 x 10^5 spermatozoa/mL and incubated for 2.5 h at 39 ºC in 5% CO_2 before 50 µL was added to each group of oocytes. The final concentration was approximately 2,000 spermatozoa/oocyte.

Examination of Fertilization

About 12 h after IVF, oocytes were mounted and fixed with 25% (v:v) acetic acid in ethanol at room temperature. After 48 h of fixation, oocytes were stained with 1% orcein

in 45% (v:v) acetic acid and examined using a phase-contrast microscope at 400X magnification. Oocytes were characterized by visualization of polyspermic penetration and male pronuclear (MPN) formation.

Intracytoplasmic Sperm Injection

Groups of 10 oocytes were centrifuged at 15,000 x *g* in HbT and placed in a microdrop on an inverted dish. Frozen-thawed sperm were then placed in an adjacent microdrop. Manipulation was carried out in 10 µL droplets of HbT under parafin oil using Narishige manipulators and a Nikon inverted microscope equipped with Hoffman modulator optics. The oocytes were stabilized with a holding pipette with an outer diameter of about 200 µm and an inner diameter of about 50 µm. The sperm were injected using a PiezoDrill micropipette (Prime Tech, Tsukuba, Japan) with an outer diameter of 8 to 9 µm and an inner diameter of 6 µm (Humagen, Charlottesville VA). The polar body of the oocyte was placed at 6 or 12 o'clock and the point of injection was at 3 o'clock. Individual oocytes were penetrated by the injecting micropipette and a small amount of cytoplasm was drawn into the micropipette to ensure penetration of the oocyte. Then the cytoplasm together with one spermatozoon and a small amount of medium was injected into the oocyte. Immediately following ooplasmic injection, the injection pipette was withdrawn quickly and the oocyte released from the holding pipette to reduce the intracytoplasmic pressure.

Embryo Culture

After ICSI or 4 to 6 h of IVF, the zygotes were washed three times in embryo culture medium, placed (20 zygotes/well) into 500 µL of previously equilibrated culture medium under mineral oil, and incubated at 39 °C in an atmosphere of 5% CO_2. Embryos were evaluated for cleavage and blastocyst formation under a stereomicroscope at 48 and 144 h of culture respectively.

Experiment I: Comparison between GSH, NAC and NACA supplementation on intracellular GSH concentrations, IVF and embryo culture

The effect of supplementing GSH, NAC and NACA to the maturation media and the subsequent effects during IVF and on embryo development were studied. The intracellular concentration of GSH was determined after 48 h oocyte maturation. The concentration used for all supplements was 1.0 mM based on recommendations from Novia Pharmaceuticals Ltd. The endpoints measured were the number of oocytes at the metaphase II stage of nuclear maturation, number of oocytes penetrated, number of oocytes penetrated with a MPN, number of polyspermic oocytes, and the number of embryos undergoing cleavage by 48 h post-IVF and those at the blastocyst stage by 144 h post-IVF.

Experiment II: Comparison between NAC and NACA supplementation on ICSI

This experiment studied the differences between supplementing 1.0 mM NAC or NACA to the maturation medium and the ICSI medium. The endpoints measured were the number of embryos undergoing cleavage by 48 h and those at the blastocyst stage by 144 h after ICSI.

Statistical Analysis

Data were analyzed by one-way ANOVA using the PROC ANOVA procedures of SAS (SAS Institute, Cary, NC) because the data were balanced in all experiments. When there was a significant effect of treatment, significant differences were determined using LSMEANS statement and Tukey adjustment for multiple comparisons. The effects included in the model for intracellular GSH concentrations in Experiment I were treatment, sample and replications. The sample was defined as the contents of the assay tube and a replicate was the number of tubes analyzed. Replicate effects were not significant ($P > 0.05$) and deleted from the final model. The effects included in the model for IVF and embryo development in Experiment I were treatment, well and replication.

The well was defined as all the contents within one well of a plate and replication was the total number of wells analyzed. Replicate and well effects were not significant ($P > 0.05$) and deleted from the final model. In Experiment II, the model included the main effects of treatment, well and replication. Replicate and well effects were not significant ($P > 0.05$) and deleted from the final model. In all analyses, a probability of less than 0.05 ($P < 0.05$) was considered significant.

RESULTS

Experiment I: Comparison between GSH, NAC and NACA supplementation on intracellular GSH concentrations, IVF and embryo culture

Intracellular concentrations of GSH are shown in Table 2-1. Overall, 1.0 mM supplementation of any antioxidant did not increase the intracellular GSH compared to the control (3.20 ± 5.03 pmol GSH/oocyte). Additionally, there were no differences between the supplementations ($P > 0.10$).

The kinetics and fertilization traits of oocytes 12 h after fertilization are shown in Table 2-2. The percent of oocytes reaching the metaphase II stage of nuclear maturation was not different between the treatment groups. Supplementation of antioxidants did not change the percentage of oocytes penetrated, MPN formation, or incidence of polyspermic penetration ($P > 0.05$).

There were no differences between the treatment groups when observing cleavage rates at 48 h post-IVF (Figure 2-1). However, by 144 h post-IVF the percentages of embryos reaching the blastocyst stage in the 1.0 mM NACA supplemented group (40.0 ± 7.4%) and 1.0 mM NAC supplemented group (35.0 ± 7.4%) were significantly higher ($P < 0.05$) than the control (20.0 ± 7.4%) and 1.0 mM GSH supplemented groups (20.0 ± 7.4%) (Figure 2-1). There were no differences between the 1.0 mM NACA and 1.0 mM NAC supplemented groups or between the control and 1.0 mM GSH treatment groups.

Experiment II: Comparison between NAC and NACA supplementation on ICSI

There were no differences in cleavage rates at 48 h after ICSI between the treatment groups (Figure 2-2). There was no difference between the 1.0 mM NAC (22.0 ± 5.9%) and 1.0 mM NACA (25.0 ± 4.6%) treatment groups by 144 h after ICSI. However, there was a difference ($P < 0.05$) between the antioxidant supplemented groups (1.0 mM NAC and 1.0 mM NACA) and the control (10.0 ± 6.0%) when observing blastocyst development (Figure 2-2).

Table 2-1. Intracellular concentrations of GSH after oocyte maturation in Experiment I. Treatment is the antioxidant supplemented to the maturation media or control. Data are expressed as mean ± SE. The sample size was 60 oocytes per treatment group.

Treatment	**Intracellular Glutathione / oocyte (pmol) ± SE**
Control	3.20 ± 5.03
NACA	4.18± 5.03
NAC	5.99 ± 5.03
GSH	7.03 ± 5.03

Table 2-2. Effects of antioxidants during oocyte maturation on nuclear maturation and fertilization in Experiment I. Treatment is the antioxidant supplemented to the maturation media or control. Data are expressed as mean ± SE.

	Treatment		
	Control	**NAC**	**NACA**
Number oocytes examined	20	20	20
% oocytes at metaphase II stage	85.0 ± 6.2	80.0 ± 6.2	90.0 ± 6.2
% oocytes penetrated	80.0 ± 3.4	85.0 ± 3.4	85.0 ± 3.4
% oocytes with male pronucleus[a]	87.5 ± 5.9	88.2 ± 5.9	82.4 ± 5.9
% polyspermic oocytes[a]	31.3 ± 7.9	29.4 ± 7.9	23.5 ± 7.9

[a] Percentage of the number of oocytes penetrated.

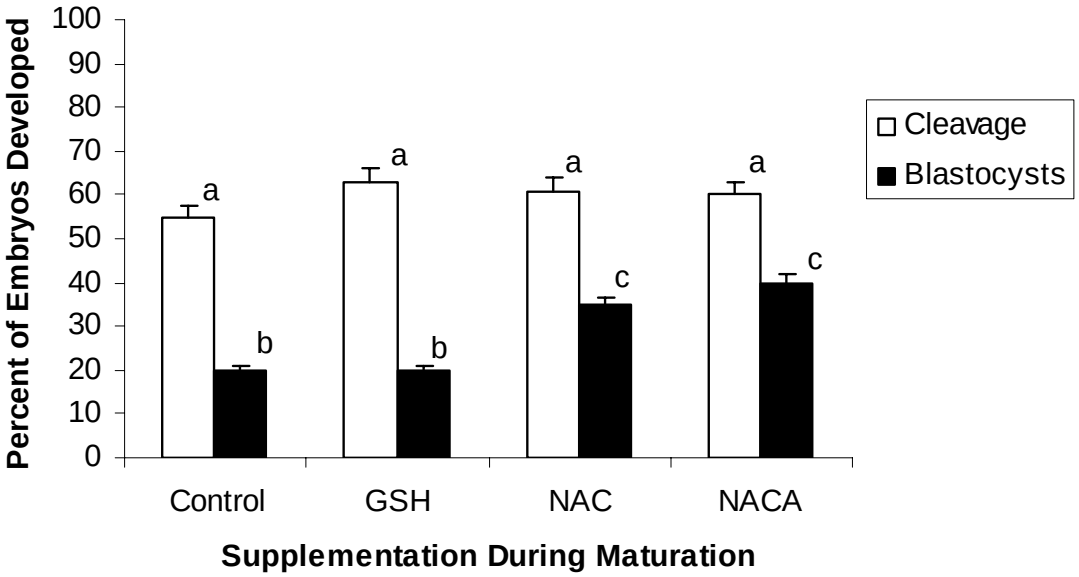

Figure 2-1. Effect of antioxidant supplementation during oocyte maturation on development of embryos in Experiment I. Data are expressed as mean ± SE. The sample size was 50 oocytes per treatment. [a,b,c] Means with different superscripts differ at least $P < 0.05$. Differences between cleavage and blastocyst percentages are not comparable.

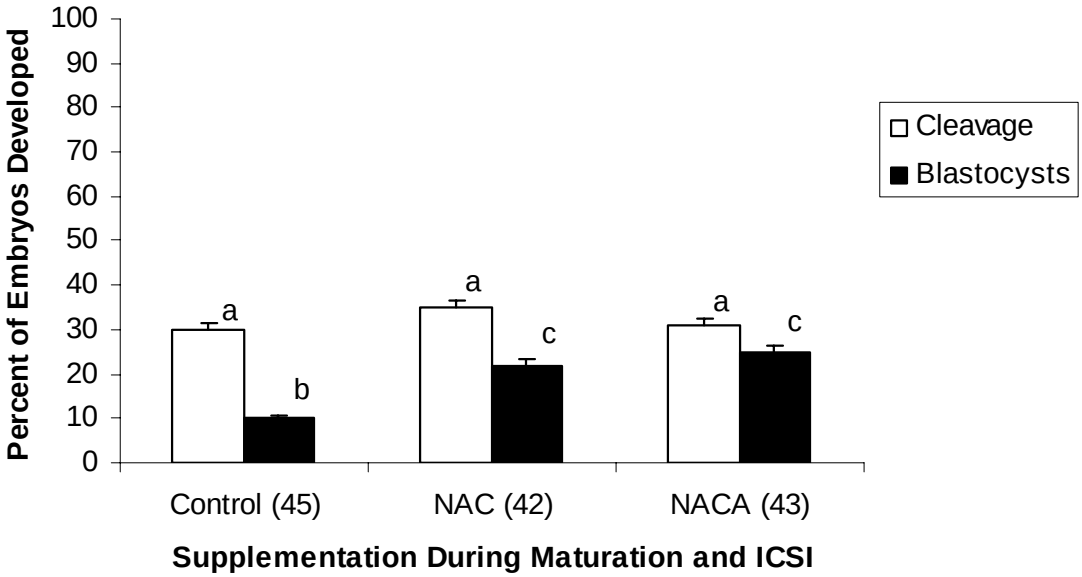

Figure 2-2. Effect of antioxidant supplementation during oocyte maturation and ICSI on development of embryos in Experiment II. Data are expressed as mean ± SE. The sample size is in parenthesis next to each treatment. [a,b,c] Means with different superscripts differ at least $P < 0.05$. Differences between cleavage and blastocyst percentages are not comparable.

DISCUSSION

Our results show that supplementing either 1.0 mM NAC or NACA to the maturation medium increased the percentage of blastocysts formed by 144 h ($P < 0.05$) of embryo culture compared to the control and 1.0 mM GSH supplemented groups (Experiment I). There was no difference between 1.0 mM NAC and 1.0 mM NACA supplementation, probably due to the structural similarities between the two antioxidants [11]. However, there was no difference between the treatment groups during early embryo development at the 2-cell stage ($P > 0.10$). There was no difference in intracellular levels of GSH after oocyte maturation or nuclear maturation and fertilization parameters ($P > 0.05$). Previous studies have shown that antioxidants in general have elicited increased levels of GSH by the end of oocyte maturation [16, 17]. One explanation for the discrepancy between our results and other reports could be the concentration of the antioxidants we used (1.0 mM) was based on manufacturer recommendations and could be too low. It is important to be able to supplement the maturation media so that an increase in intracellular GSH is observed. Intracellular GSH is beneficial during fertilization because it aids in MPN formation and DNA decondensation [18]. Supplementation or creation of a cysteine-rich environment for the oocytes has been shown to increase fertilization success [19, 20]. Since we found no difference during IVF it is evident that supplementation with 1.0 mM of GSH, NAC, or NACA does not create a cysteine-rich environment. However, supplementation of 1.0 mM NAC or 1.0 mM NACA does increase embryo development to the blastocyst stage and demonstrates one of the beneficial effects of antioxidants.

Although IVF is the preferred method of fertilization in pigs because of time, labor and cost, ICSI is becoming a more popular option to provide embryos [21]. The ability to obtain large quantities of viable embryos utilizing ICSI has increased the marketability of pigs as a research model and has increased interest of the scientific community [22]. The success rate of obtaining viable embryos using ICSI is low because it involves the physical puncturing of the zona pellucida. Supplementation to the ICSI medium with antioxidants to alleviate the stress associated with breaking the zona pellucida has shown to be beneficial [23]. Our results (Experiment II) indicate that supplementation of 1.0

mM NAC or 1.0 mM NACA to the oocyte maturation and ICSI media may alleviate the stress associated with ICSI because a higher percentage of embryos reached the blastocyst stage compared to the control group. There was no difference between any of the treatment groups when observing rates of cleavage and no difference between the 1.0 mM NAC and 1.0 mM NACA treatment groups during the duration of the experiment

As a preliminary study, our results indicate that supplementing 1.0 mM NAC or 1.0 mM NACA to the maturation medium will increase the percentage of IVF- and ICSI-derived embryos reaching the blastocyst stage ($P < 0.05$) of development. Supplementation of 1.0 mM NAC or 1.0 mM NACA did not decrease the success of fertilization or intracellular levels of GSH. Further studies need to be conducted to determine the optimal dose of the antioxidants and their specific mechanism of action during oocyte maturation.

LITERATURE CITED

1. Wondrak GT. Let the sun shine in: mechanisms and potential for therapeutics in skin photodamage. Curr Opin Investig Drugs 2007; 8:390-400.
2. McCune LM, Johns T. Antioxidant activity relates to plant part, life form and growing condition in some diabetes remedies. J Ethnopharmacol 2007; 112:461-469.
3. Bertelli AA. Wine, research and cardiovascular disease: Instructions for use. Atherosclerosis published 24 May 2007; PMID: 17531243.
4. Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE, Thornton J, Agarwai A. Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil Steril 2004; 82:593-600.
5. Tao Y, Zhou B, Xia G, Wang F, Wu Z, Fu M. Exposure to L-ascorbic acid or alpha-tocopherol facilitates the development of porcine denuded oocytes from metaphase I to metaphase II and prevents cumulus cells from fragmentation. Reprod Dom Anim 2004; 39:52–57.

6. Kitagawa Y, Suzuki K, Yoneda A, Watanabe T. Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos. Theriogenology 2004; 62:1186–1197.
7. Sawai K, Funahashi H, Niwa K. Stage-specific requirement of cysteine during in vitro maturation of porcine oocytes for glutathione synthesis associated with male pronuclear formation. Biol. Reprod 1997; 57:1-6.
8. Yamauchi N, Nagai T. Male pronuclear formation in denuded porcine oocytes after in vitro maturation in the presence of cysteamine. Biol Reprod 1999; 61:828-833.
9. Whitaker BD, Knight JW. Exogenous γ-glutamyl cycle compounds supplemented to in vitro maturation medium influence in vitro fertilization, culture, and viability of porcine oocytes and embryos. Theriogenology 2004, 62: 311-322.
10. Greco E, Romano S, Iacobelli M, Ferrero S, Baroni E, Minasi MG, Ubaldi F, Rienzi L, Tesarik J. ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment. Hum Reprod 2005; 20:2590-2594.
11. Offen D, Gilgun-Sherki Y, Barhum Y, Benhar M, Grinberg L, Reich R, Melamed E, Atlas D. A low molecular weight copper chelator crosses the blood-brain barrier and attenuates experimental autoimmune encephalomyelitis. J Neurochemistry 2004; 89:1241-1251.
12. Abeydeera LR, Day BN. In vitro penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. Theriogenology 1997; 48:537-544.
13. Prather, RS, Boice ML, Gibson J, Hoffman KE, Parry TW. In vitro development of embryos from Sinclair miniature pigs: a preliminary report. Theriogenology 1995; 43:1001-1007.
14. Petters RM, Wells KD. Culture of pig embryos. J Reprod Fertil Suppl 1993; 48:61-73.
15. Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 1985; 113:548-555.

16. Nagai T. In vitro maturation and fertilization of pig oocytes. Anim Reprod Sci 1996; 42:153-163.
17. Yoshida M. Role of glutathione in the maturation and fertilization of pig oocytes in vitro. Mol Reprod Dev 1993; 35:76-81.
18. de Matos DG, Furnus CC. The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development: effect of β-mercaptoethanol, cysteine and cysteine. Theriogenology 2000; 53:761-771.
19. Meister A, Tate SS. Glutathione and the related γ-glutamyl compounds: biosynthesis and utilization. Ann Rev Biochem 1976; 45:559-604.
20. Nagai T. The improvement of in vitro maturation systems for bovine and porcine oocytes. Theriogenology 2001; 55:1291-1301.
21. Martin MJ. Development of in vivo-matured porcine oocytes following intracytoplasmic sperm injection. Biol Reprod 2000; 63:109-112.
22. Kim NH, Shin JS, Kim C, Jun SH, Lee HT, Chung KS. Fertilization and in vitro development of porcine oocytes following intracytoplasmic injection of round spermatid or round spermatid nuclei. Theriogenology 1999; 51:1141–1149.
23. Kishida R, Lee ES, Fukui Y. In vitro maturation of porcine oocytes using a defined medium and developmental capacity after intracytoplasmic sperm injection. Theriogenology 2004; 62:1663–1676.

CHAPTER THREE

EFFECTS OF DOSE AND ANTIOXIDANT SUPPLEMENTATION TO *IN VITRO* MATURATED OOCYTES

ABSTRACT

This study was conducted to evaluate the effects of different concentrations of N-acetyl-cysteine (NAC) supplemented to the maturation medium on embryo development. Comparisons of significant concentrations of NAC and N-acetyl-cysteine-amide (NACA) NACA on embryo development were evaluated for nuclear maturation, fertilization success and embryo development. Concentrations of NAC (0, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0 mM) were supplemented to maturing oocytes and embryo development was analyzed at 48 h and 144 h post-fertilization. There were no differences between cleavage rates for any of the treatment groups. Blastocyst formation for 1.5 mM NAC (56.5 ± 9.2%) was significantly higher ($P < 0.05$) than all other supplementations. There were no differences in nuclear maturation or fertilization when comparing 1.5 mM NAC and 1.5 mM NACA supplementation to the maturation media. There was no difference between cleavage rates of 1.5 NAC and 1.5 mM NACA supplementation to the maturation media. Blastocyst formation for 1.5 mM NAC (44.4 ± 4.7%) and 1.5 mM NACA (46.2 ± 3.4%) supplementation were significantly higher ($P < 0.05$) than the control (32.1 ± 6.2%) oocytes. These results indicate that supplementing 1.5 mM of NAC or NACA to the oocyte maturation medium increased the percentage of viable embryos reaching the blastocyst stage of development.

INTRODUCTION

The scientific and medical communities are aware of the multiple roles that antioxidants have in protecting the body against a wide spectrum of diseases. Antioxidants are nontoxic compounds that scavenge free radicals, most notably reactive oxygen species.

Antioxidants reduced the risk of various types of cancer [1] as well as Creutzfeldt-Jakob disease [2] and may play a role in the treatment and prevention of Parkinson's disease, multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, and malaria [3]. Currently, the role of antioxidants in alleviating oxidative stress during reproductive processes is being evaluated [4] and these compounds may be involved in protecting against defective embryo development. Reactive oxygen species may originate from embryo metabolism and/or the surrounding environment [5] and induce early embryonic developmental block and retardation [6].

Research has shown that the primary antioxidant in oocytes and embryos is glutathione (GSH), which provides a reducing environment for oocytes and embryos to protect against oxidative damage. Glutathione is a water-soluble tripeptide (L-γ-glutamyl-L-cysteinyl-glycine), considered the most abundant intracellular small thiol molecule. The thiol sulfhydryl group is a potent reducing agent, making it an ideal wide-spectrum antioxidant. Low levels of GSH often lead to decreased numbers of competent oocytes during fertilization. However, low percentages of competent oocytes are also observed in some cases when the average levels of GSH are present [7]. This provides evidence that GSH is not the only compound responsible for completing cytoplasmic maturation in pigs. Oocyte maturation is often broken down into two steps: nuclear and cytoplasmic maturation. Nuclear maturation is often adequate *in vitro*; however, cytoplasmic maturation is usually incomplete, as characterized by low levels of GSH and the inability of the oocyte to complete successful fertilization [8]. These observations suggest that other antioxidants warrant further investigation.

Since cysteine is one of the amino acid building blocks of GSH, it is the common media supplementation in order to increase the intracellular concentrations of GSH [9]. There are two transport systems that cysteine can utilize to enter the oocyte. Cysteine is easily oxidized into cystine which is transported into the oocyte via the cysteine-glutatmate (Xc^-) system [10]. The Xc^- system is a sodium independent anionic amino acid antiporter system. When there is an overabundance of cystine, the Xc^- system becomes overloaded and GSH production is not efficient. The other transport system is where cysteine is

transported directly into the oocyte using the alanine-serine-cysteine (ASC) system, a sodium dependent neutral amino acid transport system [10]. Although the ASC system is the more direct route of entry, its activity is substantially less than that of the Xc^- system. Using a derivative of cysteine, such as N-acetyl-cysteine (NAC) would hypothetically have the same effect as cysteine, but be able to enter the oocyte at a more efficient rate and thus increase the intracellular concentrations of GSH.

N-acetyl-cysteine facilitated intracellular GSH biosynthesis by reducing extracellular cystine to cysteine [11], or by supplying sulfhydryl groups that can stimulate GSH synthesis and enhance glutathione-S-transferase activity [12]. N-acetyl-cysteine is a potent free radical scavenger as a result of its nucleophilic actions on reactive oxygen species and can be considered as a supplement to alleviate GSH depletion and free radical formations during oxidative stress [13].

Surprisingly, current research in the bovine indicates that NAC supplementation has either no effect or a detrimental effect on oocyte development [14, 15]. To our knowledge, the effects of NAC supplementation on pig oocyte maturation have not been studied. In consideration of previous results in the bovine, a suitable derivative of NAC will be considered. N-acetyl-cysteine-amide (NACA) is a NAC derivative developed by Novia Pharmaceuticals Ltd. (Haifa Bay, Israel) that is able to cross the Blood-Brain-Barrier and target neural tissue/cells [3]. This compound is glutathione-like in activity and composition and, if shown to have no adverse side effects, could be used to treat a multitude of diseases.

The present study was conducted to investigate the effects of various concentrations of NAC supplemented to the maturation medium on embryo development, followed by comparing the effects of NAC and NACA on fertilization and embryo development.

MATERIALS AND METHODS

Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO). The oocyte maturation medium was tissue culture medium 199 (TCM199) with Earle's salts (Invitrogen, Carlsbad, CA) supplemented with 5 μg/mL FSH, 1 μg/mL insulin, 50 ng/mL Gentamicin sulfate, 10 ng/mL Epidermal Growth Factor (EGF), and 10% fetal calf serum. The medium used for IVF was a modified Tris-buffered medium (mTBM) formulated by Abeydeera and Day [12]. The embryo culture medium was North Carolina State University (NCSU) 23 medium [14] containing 4% BSA (A 8022). The NACA was supplied by Novia Pharmaceuticals Ltd. (Haifa Bay, Israel).

Maturation of Oocytes

Oocytes aspirated from mature follicles (3-6 mm diameter) were obtained commercially (BoMed, Madison, WI) and shipped overnight in TCM199. Only oocytes observed with uniform ooplasm and compact cumulus cells 22 h after initial placement in media were selected. The selected oocytes were washed three times and randomly placed (45-50 oocytes/well) into 500 μL of previously equilibrated TCM199 under mineral oil without FSH and fetal calf serum supplementation. Oocytes were incubated at 39 °C in an atmosphere of 5% CO_2 for an additional 18 to 26 h. After incubation, cumulus cells were removed from the oocytes by repeat pipetting in TCM199 containing 0.1% hyaluronidase. Oocytes observed with uniform ooplasm were then washed and stored (30-50 oocytes/well) in mTBM under mineral oil until IVF.

Spermatozoa Preparation and IVF

Approximately 3 h before the completion of maturation, a frozen semen pellet (Swine Genetics International Ltd., Cambridge, IA) was thawed in Dulbecco phosphate buffered saline (DPBS) containing 0.1% BSA, 75 μg/mL potassium penicillin and 50 μg/mL

streptomycin sulfate at 39 ºC and centrifuged at 36.3 x *g* for 5 min. The semen was then washed twice at 553 x *g* for 5 min. After washing, the spermatozoa pellet was re-suspended with IVF medium to a concentration of 4 x 10^5 spermatozoa/mL and incubated for 2.5 h at 39 ºC in 5% CO_2 before 50 µL was added to each group of oocytes. The final concentration was approximately 2,000 spermatozoa/oocyte.

Examination of Fertilization

About 12 h after IVF, oocytes were mounted and fixed with 25% (v:v) acetic acid in ethanol at room temperature. After 48 h of fixation, oocytes were stained with 1% orcein in 45% (v:v) acetic acid and examined using a phase-contrast microscope at 400X magnification. Oocytes were characterized by visualization of polyspermic penetration and male pronuclear (MPN) formation.

Embryo Culture

After 4 to 6 h of IVF, the zygotes were washed three times in embryo culture medium, placed (20 zygotes/well) into 500 µL of previously equilibrated culture medium under mineral oil, and incubated at 39 °C in an atmosphere of 5% CO_2. Embryos were evaluated for cleavage and blastocyst formation under a stereomicroscope at 48 and 144 h of culture, respectively.

Experiment 1: Comparison of different NAC concentrations on embryo development

The effect of supplementing different concentrations of NAC to the maturation media on embryo development was studied. The NAC concentrations used were 0 (control), 0.5, 1.0, 1.5, 2.0, 2.5, and 5.0 mM. The endpoints measured were the number of zygotes undergoing cleavage by 48 h post-IVF and those at the blastocyst stage by 144 h post-IVF.

Experiment II: Comparison between NAC and NACA supplementation on IVF and embryo development.

The effect of supplementing NAC and NACA to the maturation media and the subsequent effects during IVF and on embryo development were studied. The concentration used for both supplements was 1.5 mM based on results from Experiment I. The endpoints measured were the number of oocytes at the metaphase II stage of nuclear maturation, number of oocytes penetrated, number of oocytes penetrated with a MPN, number of polyspermic oocytes, and the number of embryos undergoing cleavage by 48 h post-IVF and those at the blastocyst stage by 144 h post-IVF.

Statistical Analysis

In general, data were analyzed by one-way ANOVA using the PROC GLM procedures of SAS (SAS Institute, Cary, NC) because the data were unbalanced. However, the IVF traits used balanced data and PROC ANOVA procedures were used instead. When there was a significant effect of treatment, significant differences were determined using LSMEANS statement and Tukey adjustment for multiple comparisons. The effects included in the model for embryo development in Experiment I were treatment, well and replication. The well was defined as all the contents within one well of a plate and replication was the total number of wells analyzed. Replicate and well effects were not significant ($P > 0.05$) and deleted from the final model. The effects included in the model for IVF and embryo development in Experiment II were treatment, well and replication. Replicate and well effects were not significant ($P > 0.05$) and deleted from the final model. In all analyses, a probability of less than 0.05 ($P < 0.05$) was considered significant.

RESULTS

Experiment I: Comparison of different NAC concentrations on embryo development

Embryo development data for all concentrations of NAC are shown in Figure 3-1. The 2.5 and 5.0 mM NACA supplemented group had significantly lower cleavage rates at 48 h post-IVF (Figure 3-1) compared to the other treatments. By 144 h post-IVF the 1.5 mM NACA supplemented group had a significantly higher ($P < 0.05$) percentage of embryos reaching the blastocyst stage (56.5 ± 9.2%) than all other treatment groups (Figure 3-1). There were no differences in the percentage of embryos reaching blastocyst stage between the control (30.8 ± 7.1%), 0.5 mM NAC (22.9 ± 3.6%), 1.0 mM NAC (34.0 ± 8.1%), 2.0 mM NAC (19.8 ± 11.4%), 2.5 mM NAC (16.4 ± 12.1%), and 5.0 mM NAC (20.0 ± 13.1%) (Figure 3-1).

Experiment II: Comparison between NAC and NACA supplementation on IVF and embryo development.

Based on the results from Experiment I, 1.5 mM NAC supplementation elicited the only significant increase ($P < 0.05$) in blastocyst formation compared to the other doses and therefore was the dose used in Experiment II. Experiment II compared 1.5 mM NAC and 1.5 mM NACA supplementation to the maturation medium on IVF and embryo development. The kinetics and fertilization traits of oocytes 12 h after fertilization are shown in Table 3-1. The percent of oocytes reaching the metaphase II stage of nuclear maturation was not different between the treatment groups. Supplementation of antioxidants did not change the percentage of oocytes penetrated, MPN formation, or incidence of polyspermic penetration (Table 3-1).

There was no difference between the treatment groups when observing cleavage rates at 48 h post-IVF (Figure 3-2). However, by 144 h post-IVF the percentages of embryos reaching the blastocyst stage in the antioxidant supplemented groups were significantly higher ($P < 0.05$) compared to the control group (32.1 ± 6.2%) (Figure 3-2). There was

no difference between the number of embryos reaching the blastocyst stage from the 1.5 mM NAC treatment group (44.4 ± 4.7%) compared to the number of embryos reaching the blastocyst stage from the 1.5 mM NACA treatment group (46.2 ± 3.4%) (Figure 3-2).

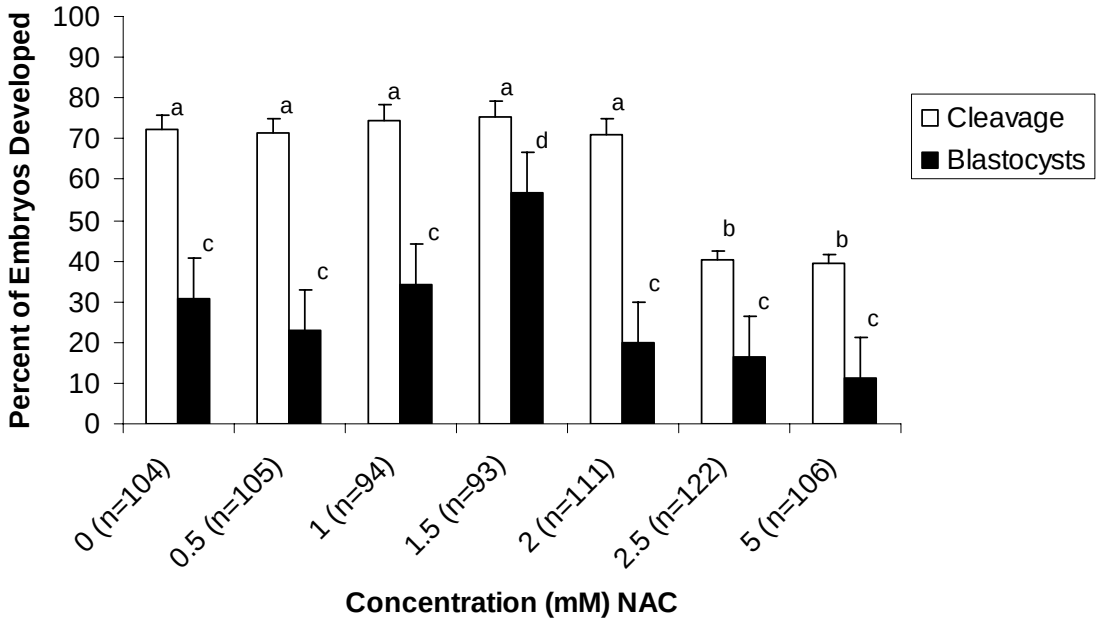

Figure 3-1. Effect of different NAC concentrations supplemented during oocyte maturation on embryo development in Experiment I. Data are expressed as mean ± SE. The sample size is in parenthesis next to each treatment. [a,b,c,d] Means with different superscripts differ at least $P < 0.05$. Differences between cleavage and blastocyst percentages are not comparable.

Table 3-1. Effects of antioxidants during oocyte maturation on nuclear maturation and fertilization in Experiment II. The treatment is the antioxidant supplemented (1.5 mM) and the control treatment is no supplementation. Data are expressed as mean ± SE.

Treatment	Control	NAC	NACA
Number oocytes examined	25	25	25
% oocytes at metaphase II stage	80.0 ± 4.1	84.0 ± 4.1	88.0 ± 4.1
% oocytes penetrated	92.0 ± 2.3	96.0 ± 2.3	92.0 ± 2.3
% oocytes with male pronucleus[a]	82.6 ± 5.1	83.3 ± 5.1	87.0 ± 5.1
% polyspermic oocytes[a]	21.7 ± 4.6	20.8 ± 4.6	26.1 ± 4.6

[a] Percentage of the number of oocytes penetrated.

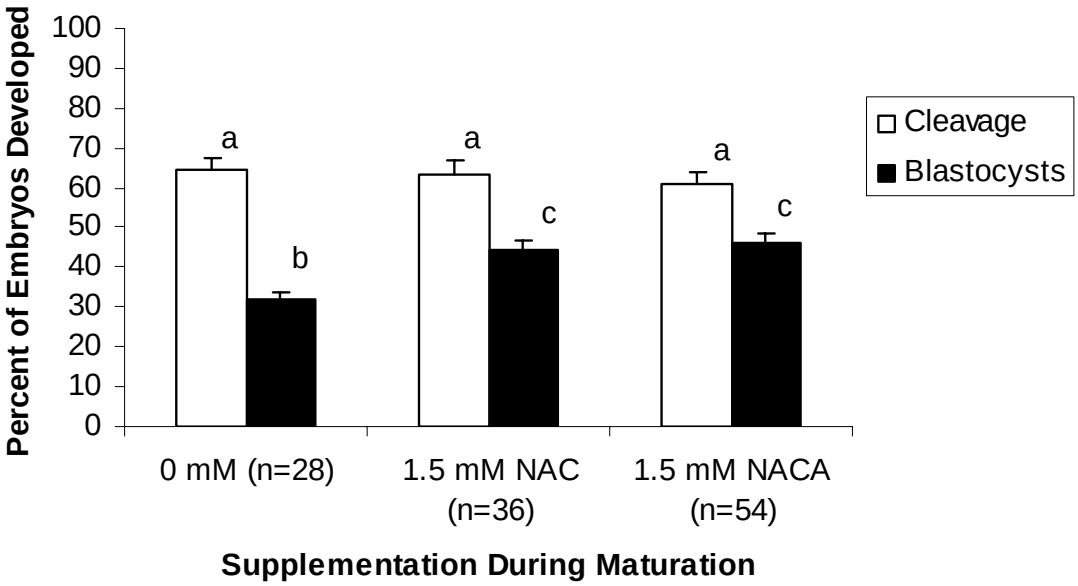

Figure 3-2. Effect of antioxidant supplementation during oocyte maturation on embryo development in Experiment II. Data are expressed as mean ± SE. The sample size is in parenthesis next to each treatment. [a,b,c] Means with different superscripts differ at least $P < 0.05$. Differences between cleavage and blastocyst percentages are not comparable.

DISCUSSION

Our results show that supplementing 1.5 mM NAC to the oocyte maturation media significantly increased ($P < 0.05$) the percent of embryos reaching the blastocyst stage of development by 144 h post-IVF compared to the other doses of NAC (0, 0.5, 1.0, 2.0, 2.5, 5.0 mM) (Experiment I).

Although antioxidants are known to alleviate oxidative stress in reproductive cases in a clinical setting [18], very little research has been done with antioxidant supplementation to pig oocytes [19]. The antioxidant NAC has been studied in the bovine [14, 15], but to the best of our knowledge this is the first study looking at NAC supplementation to the media in pigs. Therefore, we first supplemented increasing concentrations of NAC to the maturation medium to observe the effects on embryo development (Experiment I). The only significant difference observed in percent of embryos cleaving by 48 h post-IVF was the 2.5 and 5.0 mM supplemented groups (Figure 3-1). In general, our results are in agreement with previous research [14, 15] that demonstrated that NAC has no effect on embryo development. Our data during the blastocyst stage showed otherwise: 1.5 mM NAC supplementation had 56.5 ± 9.2% of embryos reaching the blastocyst stage which was significantly higher ($P < 0.05$) than those observed in the control (30.8 ± 7.1%), 0.5 mM NAC (22.9 ± 3.6%), 1.0 mM NAC (34.0 ± 8.1%), 2.0 mM NAC (19.8 ± 11.4%), 2.5 mM NAC (16.4 ± 12.1%), and 5.0 mM NAC (20.0 ± 13.1%) treatments (Figure 3-1). One explanation for the differences between our results and those reported previously is that both previous research groups used 0.6 mM of NAC and our best response was observed at over twice that, 1.5 mM NAC (Figure 3-1). It is important to note that any concentrations higher than 1.5 mM resulted in similar results found in concentrations less than 1.5 mM. This could indicate that the amount of NAC or antioxidant supplemented needs to be a very precise addition or excessive amounts could be toxic to the cells.

Using 1.5 mM as the supplementation concentration, we compared NAC to a derivative, NACA, evaluating nuclear maturation, fertilization and embryo development (Experiment II). There were no significant differences between treatment groups when

evaluating nuclear maturation (Table 3-1). Supplementation of 1.5 mM NAC or 1.5 NACA did not reduce the penetration rate or MPN during fertilization compared to the control group (Table 3-1). Previous research has shown that most antioxidants are detrimental to the fertilization process when administered during IVF [20]. We supplemented the antioxidants during maturation, which has shown to increase the cytoplasmic maturation process in developing oocytes, and leading to higher success rates of IVF and embryo culture [21]. The supplementation of 1.5 mM NAC and 1.5 mM NACA did not lower the success of IVF compared to the control group (Table 3-1).

Similar to the results obtained in Experiment I, at 48 h post-IVF there was no difference in the percent of embryos cleaved between the treatment groups (Experiment II, Figure 3-2). At 144 h post-IVF there was no difference between the 1.5 mM NAC (44.4 ± 4.7%) and 1.5 mM NACA (46.2 ± 3.4%) supplementation when evaluating blastocyst formation. Supplementation of 1.5 mM NAC and NACA had significantly higher ($P < 0.05$) percentages of embryos reaching the blastocyst stage compared to the control group (32.1 ± 6.2%) (Figure 3-2). Our observations that show supplementing antioxidants increase embryo development, are in agreement with previous studies [22, 23].

Adequate levels of GSH are required in matured oocytes to be able to form a MPN during fertilization [24]. In favorable conditions, GSH is transported from the surrounding cumulus cells through the gap junctions into the oocyte to support MPN formation [25]. However, *in vitro* conditions are usually sub-optimal and alternative mechanisms to increase intracellular GSH are required. N-acetyl-cysteine supplementation has shown to increase cystine uptake in ovarian cells [26] as well as endothelial cells [27, 28] and has the ability to stimulate GSH synthesis [29]. The NAC derivative, NACA is thought to cross the cell membrane more easily due to its high lipophilicity [13]. N-acetyl-cysteine-amide has been shown to synthesize GSH inside red blood cells [30]. These findings suggest that supplementing oocytes during maturation with either NAC or NACA will increase the intracellular levels of GSH and thus improve IVF and embryo culture. Our results provide evidence that 1.5 mM NAC or NACA supplementation does not reduce

fertilization success (Table 3-1) and increase the percentage of embryos reaching the blastocyst stage (Figures 3-1 and 3-2).

In conclusion, this study shows that supplementation of 1.5 mM NAC or NACA during oocyte maturation does not decrease IVF success and increases ($P < 0.05$) the percent of embryos reaching the blastocyst stage of development compared to oocytes not matured with antioxidants.

LITERATURE CITED

1. Brash DE, Havre PA. New careers for antioxidants. Proc Natl Acad Sci USA 2002; 99:13969-13971.
2. Muller WE, Laplanche JL, Ushijima H, Schroder HC. Novel approaches in diagnosis and therapy of Creutzfeldt-Jakob disease. Mech Ageing Dev 2000; 116:193-218.
3. Offen D, Gilgun-Sherki Y, Barhum Y, Benhar M, Grinberg L, Reich R, Melamed E, Atlas D. A low molecular weight copper chelator crosses the blood-brain barrier and attenuates experimental autoimmune encephalomyelitis. J Neurochem 2004; 10.1111:1-11.
4. Bedaiwy MA, Falcone T, Mohamed MS, Aleem AA, Sharma RK, Worley SE, Thornton J, Agarwai A. Differential growth of human embryos in vitro: role of reactive oxygen species. Fertil Steril 2004; 82:593-600.
5. Goto Y, Noda Y, Mori T, Nakano M. Increased generation of reactive oxygen species in embryos cultured in vitro. Free Radic Biol Med 1993; 15:69–75.
6. Guerin P, El Mouatassim S, Menezo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update 2001; 7:175–189.
7. Yoshida M, Ishigaki K, Pursel VG. Effect of maturation media on male pronucleus formation in pig oocytes matured in vitro. Mol Reprod Dev 1992; 31:68-71.

8. Abeydeera LR. In vitro production of embryos in swine. Theriogenology 2002; 57:256-273.
9. Abeydeera LR, Wang WH, Cantley TC, Rieke A, Day BN. Coculture with follicular shell pieces can enhance the developmental competence of pig oocytes after in vitro fertilization: relecance to intracellular glutathione. Biol Reprod 1998; 58:213-218.
10. Toshida M, Takahasi K. Changes in glutathione and transport activity of cysteine and cysteine during maturation, fertilization and development in pig ova. In: Lauria A, Gandolfi F, Enne G, Gianaroli L (eds), Gametes: Development and Function. Serono: Serono Symposia; 1998: 621.
11. Issels RD, Nagele A, Eckert KG, Wilmanns W. Promotion of cystine uptake and its utilization for glutathione biosynthesis induced by cysteamine and N-acetylcysteine. Biochem Pharmacol 1988; 37:881–888.
12. Nakata K, Kawase M, Ogino S, Kinoshita C, Murata H, Sakave T, Ogata K, Ohmori S. Effects of age on levels of cysteine, glutathione and related enzyme activities in livers of mice and rats and an attempt to replenish hepatic glutathione levels of mouse with cysteine derivatives. Mech Ageing Dev 1996; 90:195–207.
13. Wu W, Goldstein G, Adams C, Matthews RH, Ercal N. Separation and quantification of N-acetyl-L-cysteine and N-acetyl-cysteine-amide by HPLC with fluorescence detection. Biomedical Chromatogr 2006; 20:415–422.
14. Ali AA, Bilodeau JF, Sirard MA. Antioxidant requirements for bovinbe oocytes varies during in vitro maturation, fertilization and development. Theriogenology 2003; 59:939-949.
15. de Matos DG, Furnus CC, Moses DF. Glutathione synthesis during in vitro maturation of bovine oocytes: role of cumulus cells. Biol Reprod 1997; 57:1420-1425.
16. Abeydeera LR, Day BN. In vitro penetration of pig oocytes in a modified Tris-buffered medium: effect of BSA, caffeine and calcium. Theriogenology 1997; 48:537-544.
17. Petters RM, Wells KD. Culture of pig embryos. J Reprod Fertil Suppl 1993; 48:61-73.

18. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol and Endocrin 2005; 3:28-48.
19. Niemann H, Rath D. Progress in reproductive biotechnology in swine. Theriogenology 2001; 56:1291-1304.
20. Yoshida M. Role of glutathione in the maturation and fertilization of pig oocytes in vitro. Mol Reprod Dev 1993; 35:76-81.
21. Abeydeera LR, Wang WH, Cantley TC, Prather RS, Day BN. Presence of β-mercaptoethanol can increase the glutathione content of pig oocytes matured in vitro and the rate of blastocyst development after in vitro fertilization. Theriogenology 1998; 50:747–756.
22. Tatemoto H, Muto N, Sunagawa I, Shinjo A, Nakada T. Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid. Biol Reprod 2004; 71:1150-1157.
23. Tao Y, Zhou B, Xia G, Wang F, Wu Z, Fu M. Exposure to L-ascorbic acid or a-tocopherol facilitates the development of porcine denuded oocytes from metaphase I to metaphase II and prevents cumulus cells from fragmentation. Reprod Dom Anim 2004; 39:52–57.
24. Yoshida M, Ishigaki K, Nagai T, Chikyu M, Pursel VG. Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of oocytes to form male pronucleus. Biol Reprod 1993; 49:89-94.
25. Mori T, Amano T, Shimizu H. Roles of gap junctional communication of cumulus cells in cytoplasmic maturation of porcine oocytes cultured in vitro. Biol Reprod 2000; 62:913-919.
26. Meier T, Issels RD. Promotion of cyst(e)ine uptake. Methods Enzymol 1995; 252:103-112.
27. Sen U, Moshal KS, Singh M, Tyagi N, Tyagi SC. Homocysteine-induced biochemical stress predisposes to cytoskeletal remodeling in stretched endothelial cells. Mol Cell Biochem 2007; 302:133-143.
28. Cotgreave I, Moldeus P, Schuppe I. The metabolism of N-acetylcysteine by human endothelial cells. Biochem Pharmacol 1991; 42:13-16.

29. Yim CY, Hibbs JB Jr, McGregor JR, Galinsky RE, Samlowski WE. Use of N-acetyl cysteine to increase intracellular glutathione during the induction of antitumor responses by IL-2. J Immunol 1994; 152:5796-5805.
30. Grinberg L, Fibach E, Amer J, Atlas D. N-acetylcysteine amide, a novel cell-permeating thiol, restores cellular glutathione and protects human red blood cells from oxidative stress. Free Radic Biol Med 2005; 38:136–145.

CHAPTER FOUR

MECHANISMS OF OXIDATIVE STRESS IN OOCYTES AND THE ROLE OF ANTIOXIDANTS

ABSTRACT

The mechanisms of oxidative stress in maturing oocytes were studied in addition to evaluating the effects of antioxidant supplementation to the media. This study focused on superoxide dismutase (SOD), glutathione (GSH) peroxidase, catalase and intracellular GSH concentrations with respect to DNA fragmentation evaluated using the single cell Comet assay. Results indicate that when SOD was inhibited, the GSH peroxide levels and length of DNA migration significantly increased ($P < 0.05$). Catalase levels significantly decreased ($P < 0.05$) and intracellular GSH remained unchanged. When GSH peroxidase was inhibited, the SOD levels and catalase levels significantly decreased ($P < 0.05$) but the intracellular GSH and DNA migration length significantly increased ($P < 0.05$). The supplementation of 1.5 mM N-acetyl-cysteine (NAC) and 1.5 mM NAC-amide (NACA) had multiple effects on the enzyme levels. Specifically, supplementation of 1.5 mM NAC or 1.5 mM NACA significantly decreased ($P < 0.05$) the length of DNA migration when other enzymes were inhibited compared to no antioxidant supplementation. These results indicate that antioxidant supplementation may alleviate the free radicals associated with oxidative stress in the maturing porcine oocyte.

INTRODUCTION

The *in vitro* production of pig embryos is not efficient and has many shortcomings. Research has shown that the main problems associated with the inefficient production of embryos are high rates of polyspermic penetration during IVF and a low rate of male pronuclear (MPN) formation [1]. It is thought that these problems arise from poor cytoplasmic maturation during oocyte maturation [2].

There have been many advances that target the defined conditions of the maturation system to assist in cytoplasmic maturation and produce more viable embryos. The supplementation of growth factors like epidermal growth factor (EGF) increases MPN formation during IVF [3]. Other supplements shown to have similar effects as well as improve embryo development include, but are not limited to: co-culture with follicular shell pieces [4], gonadotropins [5], IGF-I [6], and glucocorticoids [7]. Most research is directed at increasing the concentration of intracellular glutathione (GSH) by the end of oocyte maturation [8]. High levels of GSH are thought to enhance MPN formation during IVF [9] by breaking the disulfide bond of protamine in the sperm head [10]. Surprisingly, with such a large emphasis placed on increasing the intracellular GSH concentrations, the ability to form a MPN and decrease polyspermic penetration has not changed proportionately to the effort. Perhaps emphasis should be placed more on the physical conditions and the environment and their effect on the oocyte rather than direct manipulation.

Glutathione is a thiol tri-peptide that provides cells with a reductive environment and protects against oxidative damage [11]. Oxidative stress on oocytes hinders nuclear and cytoplasmic maturation [12] and may even cause cell death [13]. The formation of reactive oxygen species (ROS) as a result of oxidative stress disrupts proper cell function [14] and research is starting to focus on reducing oxidative stress and ROS within the oocyte in the bovine [12]. The biochemical mechanisms and oxidative stress pathways have not been extensively studied in the porcine system. Additionally, although researchers routinely supplement media with various compounds including antioxidants [15], no research has been done to see how they affect the oxidative stress pathways within the maturing oocyte.

Therefore, the objectives of this research were to 1) determine the mechanisms of oxidative stress in maturating oocytes, 2) determine how the oocytes alleviate oxidative stress, and 3) determine how antioxidants affect the oxidative stress mechanisms.

MATERIALS AND METHODS

Media

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO). The oocyte maturation medium was tissue culture medium 199 (TCM199) with Earle's salts (Invitrogen, Carlsbad, CA) supplemented with 5 μg/mL FSH, 1 μg/mL insulin, 50 ng/mL Gentamicin sulfate, 10 ng/mL EGF, and 10% fetal calf serum. The NACA was supplied by Novia Pharmaceuticals Ltd. (Haifa Bay, Israel).

Maturation of Oocytes

Oocytes aspirated from mature follicles (3-6 mm diameter) were obtained commercially (BoMed, Madison, WI) and shipped overnight in TCM199. Only oocytes observed with uniform ooplasm and compact cumulus cells 22 h after initial placement in media were selected. The selected oocytes were washed three times and randomly placed (45-50 oocytes/well) into 500 μL of previously equilibrated TCM199 under mineral oil without FSH and fetal calf serum supplementation. Oocytes were incubated at 39 °C in an atmosphere of 5% CO_2 for an additional 18 to 26 h. After incubation, cumulus cells were removed from the oocytes by repeat pipetting in TCM199 containing 0.1% hyaluronidase. Oocytes observed with uniform ooplasm were then washed and stored.

Glutathione Assay

The concentration of intracellular GSH was determined using the 5,5'-dithio-bis(2-nitrobenzoic acid) – GSH disulfide (DTNB-GSSG) reductase recycling assay as described by Anderson [16]. Matured denuded oocytes were washed in 0.2 M sodium phosphate buffer containing 10 mM EDTA (pH 7.2). Approximately 20 oocytes were transferred to a micro-centrifuge tube and stored at –80 °C. Oocytes were thawed and 5 μL of 1.25 M H_3PO_4 was added to each tube and the oocytes were ruptured using a blunt glass rod. The oocytes were then frozen and stored again at –80 °C until the day of the

assay. For each sample, 0.3 mM NADPH and 6.0 mM DTNB were incubated at 30 °C for 15 min. The sample was added to the cuvette followed by 250 U/mL of GSH reductase to initiate the reaction. The change in absorbance was determined using a Genesis 20 spectrophotometer (Thermo Spectronic, Rochester, NY) at 412 nm for 3 min. The concentration of intracellular GSH was determined from a standard curve where the concentration of GSH was plotted against the rate of change in absorbance at 412 nm.

Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was determined by following the reduction rate of cytochrome c utilizing the xanthine-xanthine oxidase system as described by Flohé and Ötting [17]. Mature denuded oocytes were prepared by freeze–thawing twice in distilled water, and then centrifuged at 4°C and 10,000 x *g* for 20 min. The supernatant was used to determine SOD activity by adding a 50 mM phosphate buffer (7.8 pH, .1 mM EDTA) containing 5 μM xanthine in 0.001 N sodium hydroxide and 2 μM cytochrome c. The reaction was initiated by the addition of 0.2 U/mL xanthine oxidase in 0.1 mM EDTA and the change in absorbance was determined using a Genesis 20 spectrophotometer (Thermo Spectronic, Rochester, NY) at 550 nm for 10 min. One unit was defined as the enzyme amount that inhibits the rate of cytochrome c reduction by 50%.

Catalase Activity

Catalase activity was determined by following the rate of hydrogen peroxide decomposition as described by Aebi [18]. Enzymatic extracts of denuded oocytes were obtained by extraction with 1% Triton X-100 in saline solution for 15 h at 4 °C and centrifuged at 4 °C and 10,000 x *g* for 20 min. The supernatant was used to determine catalase activity by adding to 30 mM hydrogen peroxide in 50 mM phosphate buffer (pH 7.0). The change in absorbance was determined using a DU-6 UV-Visible spectrophotometer (Beckman, Irvine, CA) at 240 nm for 3 min. One unit was defined as the reaction velocity constant of the first order (k) in s^{-1}.

Glutathione Peroxidase Activity

Glutathione peroxidase activity was determined by following the rate of NADPH oxidation as originally described by Gunzler et al. [19]. Matured denuded oocytes were prepared by freeze–thawing twice in distilled water, and centrifuged at 4 °C and 10,000 x *g* for 20 min. The supernatant was used to determine GSH peroxidase activity by measuring the oxidation rate of 0.15 nM NADPH in a reaction mixture containing 1 mM azide, 0.5 mM tert-butyl hydroperoxide, 50 mM phosphate buffer (pH 7.0) at 30 °C in the presence of 1 mM reduced GSH and 0.25 U/mL GSH reductase. The change in absorbance was determined using a Genesis 20 spectrophotometer (Thermo Spectronic, Rochester, NY) at 340 nm for 3 min. One unit was defined as 1 μmol of GSH oxidized/min = 1 μmol NADPH oxidized/min.

Oxidative Damage

Oocyte DNA fragmentation was analyzed using the single cell gel electrophoresis or "Comet" assay [20]. Oocytes were mixed with 2% low-melting agarose at 40 °C on a glass slide and covered. The slides were kept at 4 °C for 10 min to allow solidification of the agarose. The coverslip was removed and the slide was immersed in a lysing solution (1% N-lauroyl-sarcosine, 2.5 M NaCl, 10 mM EDTA, 10 mM Tris, pH 10, and 1% Triton X-100) for 1 h to lyse the cells and permit DNA unfolding. The slides were placed on a horizontal gel electrophoresis unit and equilibrated for 20 min in Tris-Borate-EDTA (TBE) electrophoresis buffer. Electrophoresis was conducted for 20 min at 50 V and then the slides were stained with 10 μg/mL 4, 6-diamidino-2-phenylindole (DAPI) for 10 min, washed with distilled water, and covered with a cover-slip. The cover-slips were sealed with clear nail polish and examined through a fluorescent microscope using a fluoroisothiocyanate filter set (FITC); wavelength of excitation, 470 ± 20 nm {470/40 nm}; wavelength of emission, 515 nm {long pass}.

Preliminary Study: Validation of inhibitors on oocyte viability

Prior to the study it was necessary to ensure that the inhibitors were specific and targeted only their respective enzymes and did not kill the oocytes. Iodoacetic acid (IA) is known to inactivate GSH peroxidase activity by alkylation of the selenium moiety at the active site [21]. Addition of diethyldithiocarbamate (DDC) inhibits both copper and zinc SOD and is widely used to study the role of ROS in many cells [22]. Therefore, we supplemented maturing oocytes with 50 mM DDC or 2 mM IA and observed development at the blastocyst stage after IVF.

Experiment 1: Mechanisms of oxidative stress and oocyte adaptability

The mechanisms of oxidative stress during oocyte maturation and how the oocyte responds were studied. The concentrations of GSH, SOD, GSH peroxidase and catalase were determined in addition to DNA fragmentation length in oocytes at the end of maturation. Oocyte responses were determined by supplementing with 50 mM DDC and with 2 mM IA.

Experiment 2: Effects of antioxidants on oxidative stress and oocyte adaptability

This experiment replicated the first experiment except that the antioxidants NAC or NACA (1.5 mM each) were supplemented to the maturation medium to determine the effects of antioxidant supplementation on oxidative stress mechanisms in oocytes

Statistical Analysis

Data in all experiments were analyzed by one-way ANOVA using the PROC ANOVA procedures of SAS (SAS Institute, Cary, NC) because the data were balanced in all cases. When there was a significant effect of treatment, significant differences were determined using LSMEANS statement and Tukey adjustment for multiple comparisons. The effects included in the model for the preliminary study of inhibitor validation were treatment and

well. Well effects were not significant ($P > 0.05$) and deleted from the final model. The effects included in the model for all assays in Experiment I and II were treatment, sample, and replication. The sample was defined as the contents of the assay tube and a replicate was the number of tubes analyzed. Replicate and sample effects were not significant ($P > 0.05$) and deleted from the final model. In all analyses, a probability of less than 0.05 ($P < 0.05$) was considered significant.

RESULTS

Preliminary Study: Validation of inhibitors on oocyte viability

The results from inhibitor supplementation to the oocyte maturation media are shown in Figure 4-1. The supplementation of 50 mM DDC to the maturation media significantly decreased ($P < 0.05$) the percent of embryos reaching the blastocyst stage by 144 h post-IVF (22 ± 8.1%) compared to the control (40 ± 8.1%). Similarly, the supplementation of 2 mM IA to the maturation media significantly decreased ($P < 0.05$) the percent of embryos reaching the blastocyst stage by 144 h post-IVF (15 ± 8.1%) compared to the control. There was no significant difference between the 50 mM DDC and 2 mM IA treatment groups ($P > 0.50$).

Experiment 1: Mechanisms of oxidative stress and oocyte adaptability

The levels of intracellular GSH concentrations are shown in Table 4-1. There were no differences in intracellular GSH concentrations between the control (3.58 ± 0.42 pmol GSH/oocyte) and 50 mM DDC supplementation (4.07 ± 0.42 pmol GSH/oocyte). The intracellular GSH concentrations from 2 mM IA supplementation (5.77 ± 0.42 pmol GSH/oocyte) were significantly higher ($P < 0.05$) than the control and 50 mM DDC treatments.

The average SOD units per oocyte at 48 h of oocyte maturation are shown in Table 4-1. There was no observable level of SOD in the 50 mM DDC treatment group (0 ± 0.05

Units/oocyte). The 2 mM IA treatment group had significantly higher ($P < 0.05$) SOD levels ($4.12 \times 10^{-2} \pm 0.05$ Units/oocyte) compared to the 50 mM DDC treatment group. The control had significantly higher ($P < 0.05$) SOD levels ($5.01 \times 10^{-2} \pm 0.05$ Units/oocyte) compared to the 2 mM IA treatment, and thus the 50 mM DDC treatment group.

The average GSH peroxidase units/oocyte at 48 h of oocyte maturation are shown in Table 4-1. There was no observable level of GSH peroxidase in the 2 mM IA treatment group (0 ± 0.0056 Units/oocyte). The control had significantly higher ($P < 0.05$) GSH peroxidase levels ($0.25 \times 10^{-4} \pm 0.0056$ Units/oocyte) compared to the 2 mM IA treatment group. The 50 mM DDC supplemented group had significantly higher ($P < 0.05$) GSH peroxidase levels ($1.28 \times 10^{-4} \pm 0.0056$ Units/oocyte) compared to the control, and thus the 2 mM IA treatment group.

The average catalase units at 48 h of oocyte maturation are shown in Table 4-1. There was no observable level of catalase in the 50 mM DDC treatment group (0 ± 0.002 Units/oocyte). The 2 mM IA treatment group was not significantly different ($P < 0.05$) compared to the 50 mM DDC treatment group. The control had significantly higher ($P < 0.05$) catalase levels ($0.41 \times 10^{-1} \pm 0.002$ Units/oocyte) compared to the 2 mM IA treatment group and the 50 mM DDC treatment group.

The average lengths of DNA migration in oocytes after 48 h of maturation, as determined by the Comet assay, are shown in Table 4-1. The Comet assay is based on the principle that the broken strands of DNA will migrate away from fixed oocyte and the measurement is taken from the edge of the migrated DNA to the center of the oocyte. The average length of DNA migration in the 2 mM IA treatment group (61.8 ± 4.2 μm) was significantly longer ($P < 0.05$) than the control (42.3 ± 4.2 μm). The average length of DNA migration in the 50 mM DDC treatment group (98.1 ± 4.2 μm) was significantly longer ($P < 0.05$) than both the control and 2 mM IA treatment group.

Experiment 2: Effects of antioxidants on oxidative stress and oocyte adaptability

The levels of intracellular GSH concentrations with 1.5 mM NAC and 1.5 mM NACA supplementation to the maturation media are shown in Table 4-1. When 1.5 mM NAC was supplemented, there were no differences in intracellular GSH concentrations between the control (4.53 ± 0.42 pmol GSH/oocyte), 50 mM DDC (5.2 ± 0.42 pmol GSH/oocyte), and 2 mM IA treatments (4.72 ± 0.42 pmol GSH/oocyte). When 1.5 mM NACA was supplemented, the control (4.28 ± 0.42 pmol GSH/oocyte) and 50 mM DDC treatment group (5.72 ± 0.42 pmol GSH/oocyte) had a significantly higher ($P < 0.05$) intracellular GSH concentration compared to the 2 mM IA treatment group (0.43 ± 0.42 pmol GSH/oocyte). There was no difference between the control and 50 mM DDC treatment group. Comparing between antioxidant supplementations, there were no significant differences except for the control and 1.5 mM NAC with 2 mM IA treatment were significantly ($P < 0.05$) higher than the 1.5 mM NACA with 2mM IA treatment.

The average SOD units at 48 h of oocyte maturation with 1.5 mM NAC and 1.5 mM NACA supplementation to the media are shown in Table 4-1. When 1.5 mM NAC was supplemented, there were no observable SOD levels in the 50 mM DDC treatment group (0 ± 0.05 Units/oocyte). The 2 mM IA treatment group had a significantly higher level ($P < 0.05$) of SOD (4.3×10^{-2} ± 0.05 Units/oocyte) compared to the control (3.83×10^{-2} ± 0.05 Units/oocyte). The control and 2 mM IA treatment group were both significantly higher ($P < 0.05$) than the 50 mM DDC treatment group. When 1.5 mM NACA was supplemented, there were no observable SOD levels in the 50 mM DDC treatment group (0 ± 0.05 Units/oocyte). The control had a significantly higher level ($P < 0.05$) of SOD (5.48×10^{-2} ± 0.05 Units/oocyte) compared to the 2 mM IA treatment group (5.1×10^{-2} ± 0.05 Units/oocyte). The control and 2 mM IA treatment groups were both significantly higher ($P < 0.05$) than the 50 mM DDC treatment group. Comparing between antioxidant supplementations, the average SOD levels were significantly higher ($P < 0.05$) in the 1.5 mM NACA supplemented group compared to the 1.5 mM NAC and control. The control was significantly higher ($P < 0.05$) than the 1.5 mM NAC supplemented group. The 1.5 mM NACA supplementation with 2 mM IA treatment was

significantly higher ($P < 0.05$) than the control and 1.5 mM NAC supplementation with 2 mM IA treatment when comparing average SOD levels.

The average GSH peroxidase units at 48 h of oocyte maturation with 1.5 mM NAC and 1.5 mM NACA supplementation to the media are shown in Table 4-1. When 1.5 mM NAC was supplemented, there were no observable GSH peroxidase levels in the 2 mM IA treatment group (0 ± 0.0056 Units/oocyte). The 50 mM DDC treatment group had a significantly higher level ($P < 0.05$) of GSH peroxidase ($2.23 \times 10^{-4} \pm 0.0056$ Units/oocyte) compared to the control ($0.46 \times 10^{-4} \pm 0.0056$ Units/oocyte). The control and 50 mM DDC treatment group were both significantly higher ($P < 0.05$) than the 2 mM IA treatment group. When 1.5 mM NACA was supplemented, there were no observable GSH peroxidase levels in the 2 mM IA treatment group (0 ± 0.0056 Units/oocyte). The control had a significantly higher level ($P < 0.05$) of GSH peroxidase ($7.79 \times 10^{-4} \pm 0.0056$ Units/oocyte) compared to the 50 mM DDC treatment group ($3.35 \times 10^{-4} \pm 0.0056$ Units/oocyte). The control and 50 mM DDC treatment group were both significantly higher ($P < 0.05$) than the 2 mM IA treatment group. Comparing between antioxidant supplementations, the average GSH peroxidase levels were significantly higher ($P < 0.05$) in the 1.5 mM NACA supplemented group compared to the 1.5 mM NAC and control. The 1.5 mM NAC supplementation with 50 mM DDC treatment was significantly higher ($P < 0.05$) than the control with 50 mM DDC treatment. The 1.5 mM NACA supplementation with 50 mM DDC treatment was significantly higher ($P < 0.05$) than the control and 1.5 mM NAC supplementation with 50 mM DDC treatment when comparing average GSH peroxidase levels.

The average catalase units at 48 h of oocyte maturation with 1.5 mM NAC and 1.5 mM NACA supplementation to the media are shown in Table 4-1. When 1.5 mM NAC was supplemented, the average catalase levels were significantly higher ($P < 0.05$) in the control ($2.04 \times 10^{-1} \pm 0.002$ Units/oocyte) compared to the 2 mM treatment group ($0.16 \times 10^{-1} \pm 0.002$ Units/oocyte). The control and 2 mM IA treatment group were both significantly higher ($P < 0.05$) than the 50 mM DDC treatment group ($0.01 \times 10^{-1} \pm 0.002$ Units/oocyte). When 1.5 mM NACA was supplemented, there were no observable

catalase levels in the control (0 ± 0.002 Units/oocyte). The 2 mM IA treatment group had a significantly higher level ($P < 0.05$) of catalase ($0.77 \times 10^{-1} \pm 0.002$ Units/oocyte) compared to the 50 mM DDC treatment group ($0.51 \times 10^{-1} \pm 0.002$ Units/oocyte). The 2 mM IA and 50 mM DDC treatment groups were both significantly higher ($P < 0.05$) than the control. Comparing between antioxidant supplementations, the average catalase levels were significantly higher ($P < 0.05$) in the control compared to the 1.5 mM NACA supplemented group. The average catalase units in the 1.5 mM NAC supplemented group were significantly higher ($P < 0.05$) than the control. The 1.5 mM NACA supplementation with 50 mM DDC treatment was significantly higher ($P < 0.05$) than the control and 1.5 mM NAC with 50 mM DDC treatment. The 1.5 mM NAC supplementation with 2 mM IA treatment was significantly higher ($P < 0.05$) than the control with 2 mM IA treatment. The 1.5 mM NACA supplementation with 2 mM IA treatment was significantly higher ($P < 0.05$) than the 1.5 mM NAC supplementation with 2 mM IA treatment group when comparing average catalase levels.

The average lengths of DNA migration in oocytes after 48 h of maturation with 1.5 mM NAC and 1.5 mM NACA supplementation to the media, as determined by the Comet assay, are shown in Table 4-1. When 1.5 mM NAC was supplemented, the average length of DNA migration was significantly longer ($P < 0.05$) in the 50 mM DDC treatment (86.4 ± 4.2 μm) compared to the 2 mM IA treatment group (52.3 ± 4.2 μm). The 50 mM DDC and 2 mM IA treatment groups were both significantly longer ($P < 0.05$) than the control (41.0 ± 4.2 μm). When 1.5 mM NACA was supplemented, the average length of DNA migration was not significantly longer between the 2 mM IA treatment (68.2 ± 4.2 μm) compared to the 50 mM DDC treatment group (64.3 ± 4.2 μm). The 50 mM DDC and 2 mM IA treatment groups were both significantly longer ($P < 0.05$) than the control (43.2 ± 4.2 μm). Comparing between antioxidant supplementations, the average length of DNA migration was significantly longer ($P < 0.05$) in the 1.5 mM NAC supplementation with 50 mM DDC treatment compared to the 1.5 mM NACA supplementation with 50 mM treatment. The control with 50 mM DDC treatment was significantly longer ($P < 0.05$) than the 1.5 mM NAC supplementation with 50 mM DDC treatment. The control and 1.5 mM NACA supplementation with 1

mM IA treatment were significantly longer ($P < 0.05$) than the 1.5 mM NAC supplementation with 2 mM treatment.

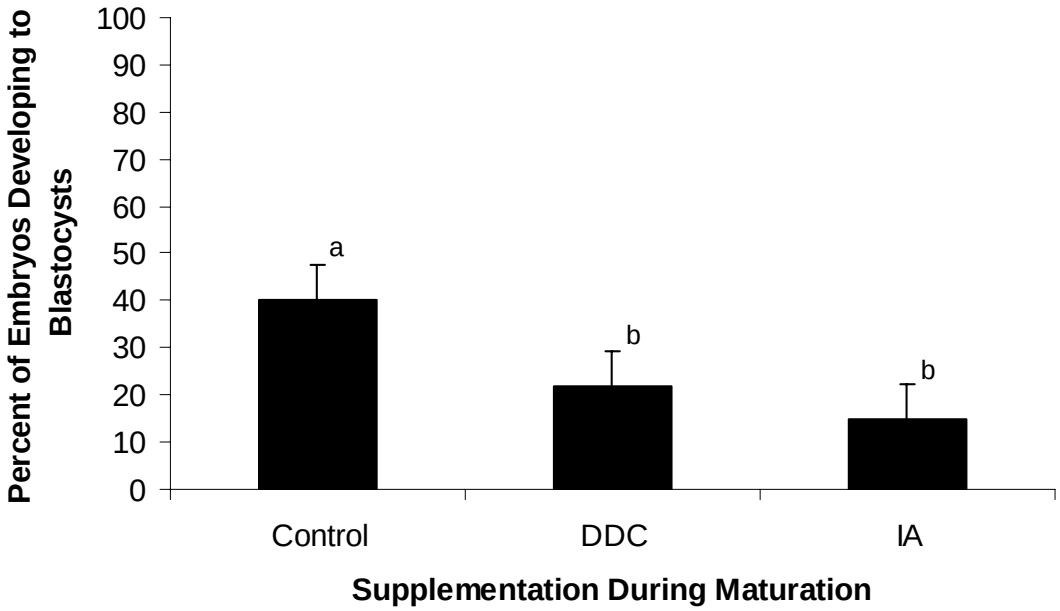

Figure 4-1. Effect of 50 mM diethyldithiocarbamate (DDC) and 2 mM iodoacetic acid (IA) supplementation during oocyte maturation on embryo blastocyst formation by 144 h post-IVF. Data are expressed as the mean ± SE. The sample size was 30 oocytes per treatment. [a,b] Means with different superscripts differ at least $P < 0.05$.

Table 4-1. Enzyme concentrations with specific supplements and inhibitors in 48 h matured oocytes.

Assay	Supplement[†]	Control	50 mM DDC	2 mM IA
Intracellular GSH concentrations (pmol GSH/oocyte oocyte ± SE)	None	3.58 ± 0.42^{Aa}	4.07 ± 0.42^{Aa}	5.77 ± 0.42^{Ab}
	1.5 mM NAC	4.53 ± 0.42^{Aa}	5.2 ± 0.42^{Aa}	4.72 ± 0.42^{Aa}
	1.5 mM NACA	4.28 ± 0.42^{Aa}	5.72 ± 0.42^{Aa}	0.43 ± 0.42^{Bb}
Average SOD levels (Units x 10^{-2}/oocyte ± SE)	None	5.01 ± 0.05^{Aa}	0.00 ± 0.05^{Ab}	4.12 ± 0.05^{Ac}
	1.5 mM NAC	3.83 ± 0.05^{Ba}	0.00 ± 0.05^{Ab}	4.30 ± 0.05^{Ac}
	1.5 mM NACA	5.48 ± 0.05^{Ca}	0.00 ± 0.05^{Ab}	5.10 ± 0.05^{Bc}
Average GSH peroxidase levels (Units x 10^{-4}/oocyte ± SE)	None	0.25 ± 0.0056^{Aa}	1.28 ± 0.0056^{Ab}	0.00 ± 0.0056^{Ac}
	1.5 mM NAC	0.46 ± 0.0056^{Aa}	2.23 ± 0.0056^{Bb}	0.00 ± 0.0056^{Ac}
	1.5 mM NACA	7.79 ± 0.0056^{Ba}	3.35 ± 0.0056^{Cb}	0.00 ± 0.0056^{Ac}
Average catalase levels (Units x 10^{-1}/oocyte ± SE)	None	0.41 ± 0.002^{Aa}	0.00 ± 0.002^{Ab}	0.02 ± 0.002^{Ab}
	1.5 mM NAC	2.04 ± 0.002^{Ba}	0.01 ± 0.002^{Ab}	0.16 ± 0.002^{Bc}
	1.5 mM NACA	0.00 ± 0.002^{Ca}	0.51 ± 0.002^{Bb}	0.77 ± 0.002^{Cc}
Average length of DNA migration (μm/oocyte ± SE)	None	42.3 ± 4.2^{Aa}	98.1 ± 4.2^{Ab}	61.8 ± 4.2^{Ac}
	1.5 mM NAC	41.0 ± 4.2^{Aa}	86.4 ± 4.2^{Bb}	52.3 ± 4.2^{Bc}
	1.5 mM NACA	43.2 ± 4.2^{Aa}	64.3 ± 4.2^{Cb}	68.2 ± 4.2^{Ab}

[†] Supplementations in rows are inhibitors and in columns are antioxidants. The sample size was 90 oocytes per treatment. Means with different superscripts differ at least $P < 0.05$ (upper case for columns, lower case for rows). Differences between assays are not comparable.

DISCUSSION

The profiles of enzymes associated with oxidative stress have been documented in the bovine oocytes [12, 23]. The action of SOD swine oocytes has been investigated [24]; however, how SOD interacts with the rest of the antioxidant system during oxidative stress remains unknown. To the best of our knowledge, this is the first comprehensive investigation directed at, 1) the mechanisms involved in the oocyte control of oxidative stress during maturation, and 2) how antioxidant supplementation affects these mechanisms and oocyte maturation.

Fully characterizing enzyme activity in maturing oocytes requires not only profiling enzyme levels during oocyte maturation, but inhibiting each enzyme and observing how the other enzymes and oocyte responds. Based on previous research, we limited the study by looking at the enzymes SOD [24], GSH peroxide [12], catalase [12], the intracellular antioxidant GSH [8], and DNA fragmentation using the "comet" assay [20]. Prior to the study, we validated the effects of the enzyme inhibitors on embryo development to ensure they did not cause the oocytes to die (Preliminary Study). Supplementation with 2 mM IA, a specific inhibitor of GSH peroxidase activity [21] and 50 mM DDC, a specific inhibitor of SOD [22] significantly reduced ($P < 0.05$) the percent of embryos reaching the blastocyst compared to the control (Figure 4-1). However, in all treatment groups there were observable blastocysts, indicating that the 2 inhibitors were not lethal to the oocytes and could be used for our study. Additionally, when 50 mM DDC was supplemented to the media, there were no observable levels of SOD (Table 4-1), illustrating the specificity of DDC as an SOD inhibitor. When 2 mM IA was supplemented to the media, there were no observable levels of GSH peroxidase (Table 4-1), illustrating the specificity of IA as a GSH peroxidase inhibitor.

When SOD was inhibited, the average levels of GSH peroxidase increased ($P < 0.05$) (Table 4-1). catalase decreased ($P < 0.05$) (Table 4-4), and DNA migration length increased ($P < 0.05$) (Table 4-1). This suggests that when SOD activity is impaired, the damage to the oocyte increases. The levels of intracellular GSH remained unchanged

(Table 4-1), perhaps indicating that GSH was not being synthesized in adequate amounts or able to respond to the increase in ROS formation due to oxidative stress. These observations give strong evidence that SOD acts directly on reducing free radical oxygen species into hydrogen peroxide and oxygen; which is in agreement with previous findings [25].

When GSH peroxidase was inhibited, the levels of SOD (Table 4-1) and catalase (Table 4-1) decreased ($P < 0.05$). Intracellular GSH levels (Table 4-1) and the length of DNA migration (Table 4-1) both increased ($P < 0.05$) when GSH peroxidase was inhibited. These observations suggest that SOD and catalase activities were decreased because the GSH levels were elevated. The GSH levels would be elevated because GSH peroxidase was not available to maintain the cellular balance of GSSG and GSH. However, as seen when SOD was inhibited, damage to the oocyte was apparent when considering the increased lengths of DNA fragments (Table 4-1). Compiling the observations from inhibiting SOD and GSH peroxidase suggests the enzymatic pathway in maturing porcine oocytes in Figure 5-2, in agreement with previous findings [25, 26]. de Cavanagh et al., [25] demonstrated that SOD and GSH peroxidase protect the cell against oxidative damage and inhibition of these specific enzymes without inhibiting catalase, damages the cell. Günzler et al., [26] also determined that targeting the inhibition of GSH peroxidase was specific and did not alter the activities of other cellular enzymes.

The second experiment investigated the oocyte response to 1.5 mM supplementation of NAC and NACA. Overall, the pattern observed previously (Experiment I) was not different than what was observed with either antioxidant supplementation. This indicates that supplementing the oocyte maturation medium with either 1.5 mM NAC or 1.5 mM NACA does not change what happened to the oocyte when SOD or GSH peroxidase is inhibited. The significant findings ($P < 0.05$) were found when making comparisons between NAC and NACA and the control. The profiles followed similar patterns, but the concentrations differed ($P < 0.05$), providing evidence that the antioxidants did influence oocyte maturation.

When supplementing the maturation media with 1.5 mM NAC, the average levels of SOD decreased ($P < 0.05$) compared to the control, but increased ($P < 0.05$) when GSH peroxidase was inhibited (Table 4-1). When 1.5 mM NACA was supplemented, the average SOD levels increased in all conditions ($P < 0.05$) (Table 4-1). This indicates that 1.5 mM NACA supplementation increases the average levels of SOD in maturing oocytes. This could be beneficial to the oocyte, because SOD reduces ROS [25].

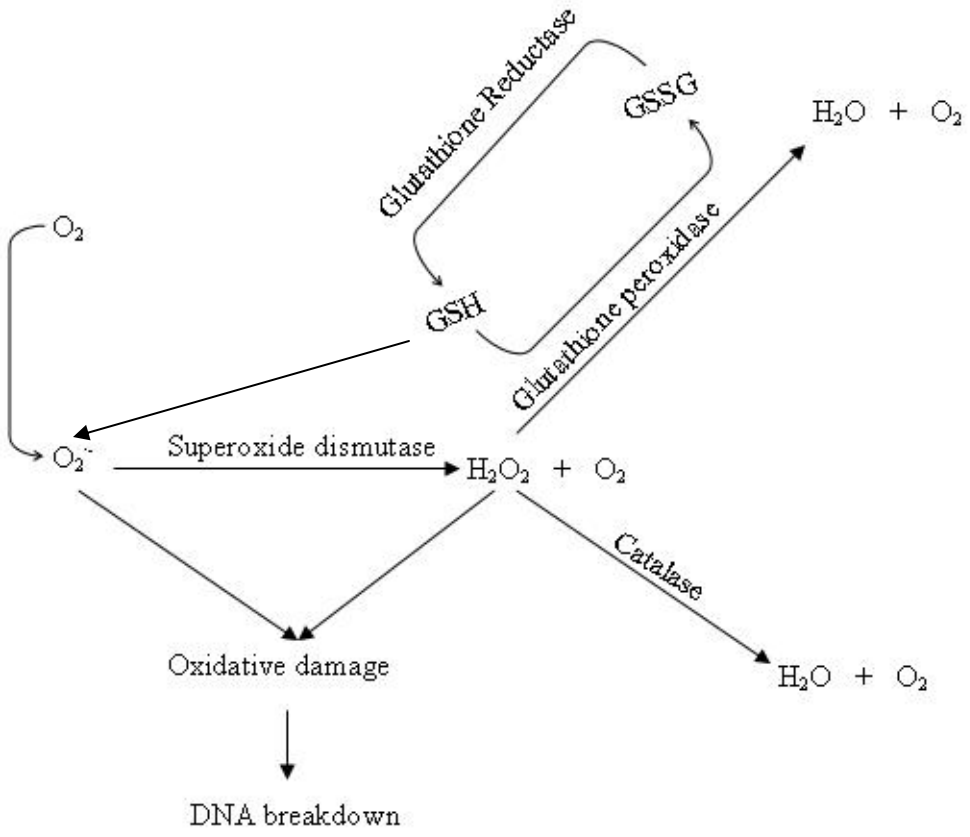

Figure 4-2. Proposed pathway of oxidative stress mechanisms in maturing porcine oocytes.

When supplementing the maturation media with 1.5 mM NAC or 1.5 mM NACA, the average levels of GSH peroxidase (Table 4-1) increased ($P < 0.05$) when SOD was inhibited. This would suggest that antioxidant supplementation may increase the intracellular levels of GSH and thus alleviate oxidative stress. However, we found the opposite: there was no significant increase in GSH when antioxidants were supplemented (Table 4-1). The only significant ($P < 0.05$) finding was the decrease in GSH with the 1.5 mM NACA supplementation and 2 mM IA treatment.

Supplementation of 1.5 mM NAC significantly increase ($P < 0.05$) the catalase levels, as did 1.5 mM NACA, with the exception of the uninhibited group (Table 4-1). As seen form our data and using our proposed pathway (Figure 4-1), catalase is one of two pathways to eliminate hydrogen peroxide, and also has a number of different uses in the cell [18]. Therefore, the measurements and assumptions that can be made about the role of catalase in maturing oocytes are limited.

Supplementing with either 1.5 mM NAC or NACA significantly reduced ($P < 0.05$) the length of DNA migration in the SOD and GSH peroxidase inhibited oocytes. This indicates that, 1) antioxidant supplementation may alleviate some of the DNA damage in oocytes caused by oxidative stress, and 2) antioxidant supplementation will not completely protect oocytes from unfavorable environments.

In conclusion, the mechanistic pathway that the oocyte utilizes to alleviate oxidative stress is very complex and includes many locations to target for regulation. Oxidative stress due to the accumulation of ROS is not an absolute phenomenon. There are a multitude of levels of oxidative stress so that the oocyte is able to adapt to the fluctuating environment. However, if the cell cannot adapt to high levels of oxidative stress, the ROS produced are detrimental to the cell [28]. The addition of either 1.5 mM NAC or NACA appears to alleviate some of the effects observed with oxidative stress. Both NAC and NACA have been shown to have antioxidant capabilities [29], however the differences between the two are varied, depending on environment of the oocyte. Therefore, it is difficult to determine if one antioxidant should be used over the other. It

is also apparent that no single antioxidant or substance for that matter is able to alleviate oxidative stress in maturing pig oocytes. This is an attribute to how complex the pathway for alleviating oxidative stress really is. Further research in elucidating the pathway and how to control it is needed to get a better understanding of oxidative stress in maturing oocytes.

LITERATURE CITED

1. Nagai, T. The improvement of in vitro maturation systems for bovine and porcine oocytes. Theriogenology 2001; 55:1291-1301.
2. Abeydeera, LR. In vitro production of embryos in swine. Theriogenology 2002 57:256-273.
3. Ding J, Foxcroft GR. Epidermal growth factor enhances oocyte maturation in pigs. Mol Reprod Dev 1994; 39:30-40.
4. Liu L, Dai Y, Moor RM. Role of secreted proteins and gonadotrophins in promoting full maturation of porcine oocytes in vitro. Mol Reprod Dev 1997; 47:191-199.
5. Illera MJ, Lorenzo PL, Illera JC, Petters RM. Development competence of immature pig oocytes under the influence of EGF, IGF-I, follicular fluid and gonadotropins during IVM-IVF processes. Int J Dev Biol 1998; 42:169-1172.
6. Xia P, Tekpetey FR, Armstrong DT. Effect of IGF-I on pig oocyte maturation, fertilization, and early embryonic development in vitro, and on granulosa and cumulus cell biosynthetic activity. Mol Reprod Dev 1994; 38:373-379.
7. Yang JG, Chen WY, Li PS. Effects of glucocorticoids on maturation of pig oocytes and their subsequent fertilizing capacity in vitro. Biol Reprod 1999; 60:929-936.
8. Whitaker BD, Knight JW. Exogenous γ-glutamyl cycle compounds supplemented to *in vitro* maturation medium influence *in vitro* fertilization, culture, and viability of porcine oocytes and embryos. Theriogenology 2004, 62:311-322.

9. Yoshida M, Ishigaki K, Nagai T, Chikyu M, Pursel VG. Glutathione concentration during maturation and after fertilization in pig oocytes relevance to the ability of oocytes to form male pronucleus. Biol Reprod 1993; 49:89-94.
10. Nagai T. In vitro maturation and fertilization of pig oocytes. Anim Reprod Sci 1996; 42:153-163.
11. Meister A, Tate SS. Glutathione and the related γ-glutamyl compounds: biosynthesis and utilization. Annu Rev Biochem 1976; 45:559-604.
12. Cetica PD, Pintos LN, Dalvit GC, Beconi MT. Antioxidant enzyme activity and oxidative stress in bovine oocyte in vitro maturation. IUBMB Life 2001; 51:57-64.
13. Del Corso A, Cappiello M, Mura U. Thiol dependant oxidation of enzymes: the last chance against oxidative stress. Int J Biochem 1994; 26:745-750.
14. Halliwell B, Gutteridge JM. Free radicals and antioxidant protection: mechanisms and significance in toxicology and disease. Human Toxicology 1988; 7:7-13.
15. Tatemoto H, Ootaki K, Shigeta K, Muto N. Enhancement of developmental competence after in vitro fertilization of porcine oocytes by treatment with ascorbic acid 2-*O*-α-glucoside during in vitro maturation. Biol Reprod 2001; 65:1800-1806.
16. Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 1985; 113:548-555.
17. Flohé L, Ötting F. Superoxide dismutase assays. Methods Enzymol 1984; 105:93-104.
18. Aebi, H. Catalase in vitro. Methods Enzymol 1984; 105:121-126.
19. Günzler WA, Kremers H, Flohé L. An improved coupled test procedure for glutathione peroxidase (EC 1-11-1-9-) in blood. Z Klin Chem Klin Biochem 1974; 12:444-448.
20. Tatemoto H, Sakurai N, Muto N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during in vitro maturation: role of cumulus cells. Biol Reprod 2000; 63:805-810.
21. Werz O, Szellas D, Henseler M, Steinhilber D. Nonredox 5-lipoxygenase

inhibitors require glutathione peroxidase for efficient inhibition of 5-lipoxygenase activity. Mol Pharmacol 1998; 54:445-451.

22. Dumay A, Rincheval V, Trotot P, Mignotte B, Vayssiere J. The superoxide dismutase inhibitor diethyldithiocarbamate has antagonistic effects on apoptosis by triggering both cytochrome *c* release and caspase inhibition. Free Radic Biol Med 2006; 40:1377-1390.
23. Ali AA, Bilodeau JF, Sirard MA. Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. Theriogenology 2003; 59:939-949.
24. Tatemoto H, Muto, N, Sunagawa I, Shinjo A, Nakada T. Protection of porcine oocytes against cell damage caused by oxidative stress during in vitro maturation: role of superoxide dismutase activity in porcine follicular fluid. Biol Reprod 2004; 71:1150-1157.
25. Cavanagh EMV, Inserra F, Ferder L, Romano L, Ercole L, Fraga CG. Superoxide dismutase and glutathione peroxidase activities are increased by enalapril and captopril in mouse liver. FEBS Letters 1995; 361:22-24.
26. Günzler WA, Kremers H, Flohé L. An improved coupled test procedure for glutathione peroxidase in blood. Z Klin Chem Klin Biochem 1974; 12:444-448.
27. Reimann M, Loddenkemper C, Rudolph C, Schildhauer I, Teichmann B, Stein H, Schlegelberger B, Dorken B, Schmitt CA. The Myc-evoked DNA damage response accounts for treatment resistance in primary lymphomas in vivo. Blood published 11 June 2007; PMID: 17562874.
28. Schulz JB, Lindenau J, Seyfried J, Dichgans J. Glutathione, oxidative stress and neurodegneration. Er J Biochem 2000; 267:4904-4911.
29. Grinberg L, Fibach E, Amer J, Atlas D. N-acetylcysteine amide, a novel cell-permeating thiol, restores cellular glutathione and protects human red blood cells from oxidative stress. Free Radic Biol Med 2005; 38:136–145.

CHAPTER SIX

SUMMARY

This dissertation consists of a series of studies designed to characterize the mechanisms of oxidative stress in maturing oocytes and determine how oocytes alleviate oxidative stress. The studies also addressed the effects of antioxidant supplementation on maturing oocytes. These results, which are based on the porcine model, can be applied to other species, including humans to explain the mechanisms and pathways inside maturing oocytes and add to the current research in medicine, transgenics, and reproductive biotechnology.

The first set of experiments (Chapter 2) determined that the two antioxidants, N-acetyl-cysteine (NAC) and N-acetyl-cysteine-amide (NACA) had no detrimental side effects on IVF, intracytoplasmic sperm injection or embryo development to the blastocyst stage. Intracellular glutathione (GSH) was not inhibited with the addition of either compound.

The next set of experiments (Chapter 3) determined the optimal dose of NAC supplementation to the maturing oocytes. The NAC was supplemented in 0.5 mM increments from 0 mM to 2.5 mM and 5.0 mM. Supplementation with 1.5 mM of NAC produced the highest percentage of blastocysts by 144 h embryo culture. The 1.5 mM NAC and 1.5 mM NACA supplementation were compared and there was no difference between the two during IVF or early embryonic development. By 144 h of embryo culture both supplementations had a higher percentage of embryos reaching the blastocyst stage compared to the control.

The final series of studies (Chapter 4) investigated the mechanistic pathway of the enzymes and compounds associated with oxidative stress in the pig oocyte. Free radicals accumulated from oxidative stress are converted to hydrogen peroxide by superoxide dismutase or water by GSH peroxidase. The GSH peroxidase is able to increase the pool

of intracellular GSH which reduces the amount of free radicals. Hydrogen peroxide is detrimental to the cell and is usually broken down by catalase into water and free oxygen.

The addition of 1.5 mM NAC or NACA to the maturation media reduced the action of the free radicals and reduced the activity of the enzymes. Antioxidants were able to alleviate oxidative stress without increasing DNA damage and enzyme activity, which is important for maintaining the homeostatic environment of the oocyte. The actions of NACA were confounding as they eliminated particular enzymatic activities. The lack of enzymatic activity would lead one to believe that the oocytes would not be viable; however, they survived like the control and in fact had higher percentages of embryos reaching the blastocyst stage.

Current research is aimed at increasing the efficiency of the *in vitro* system in swine through elucidating the reproductive and cellular mechanisms associated with the gametes and general reproductive processes. Additionally, the scientific community has long been aware of the benefits of antioxidants, however are just starting to utilize antioxidants to their fullest potential, especially in the reproductive field. The research and results in this dissertation contribute valuable information in being able to understand the mechanisms of oocyte maturation, how oocytes respond to oxidative stress, and the specific mechanisms of action of antioxidants.

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PUBLICATIONS

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ABSTRACTS

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