EXOGENOUS γ-GLUTAMYL CYCLE COMPOUND SUPPLEMENTATION TO *IN VITRO* MATURATION MEDIUM AND THE EFFECTS ON SUBSEQUENT *IN VITRO* FERTILIZATION AND CULTURE PARAMETERS OF PORCINE OOCYTES AND THEIR IMPACT ON EMBRYO VIABILITY

by

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ABSTRACT: High concentrations of intracellular glutathione enhance the *in vitro* production of porcine embryos. Six experiments were conducted to study the effects of varying concentrations of different supplements to the *in vitro* maturation (IVM) medium on *in vitro* fertilization (IVF) and *in vitro* culture (IVC), and evaluate subsequent embryo viability. In Exp. 1, 2, 3, and 4, porcine oocytes were matured in either 3.3 m*M* cysteine, 150 μ*M* cysteamine, 3.3 m*M* cysteine and 150 μ*M* cystemaine; 1.0 m*M* glycine, 2.5 m*M* glycine, 5.0 m*M* glycine; 1.0 m*M* L-glutamate, 2.5 m*M* L-glutamate, 5.0 m*M* L-glutamate; and 3.3 m*M* L-α-aminobutyrate, 25 μ*M* β-mercaptoethanol, 3.3 m*M* cysteine and 25 μ*M* β-mercaptoethanol, or 3.3 m*M* L-α-aminobutyrate and 25 μ*M* β-mercaptoethanol. After IVM (44 h), concentrations of intracellular glutathione (GSH) were determined using a colorimetric assay based on absorbency. The supplements that elicited significantly ($P < 0.05$) the greatest increase in GSH concentrations were 3.3 m*M* cysteine, 1.0 m*M* L-glutamate, 3.3 m*M* L-α-aminobutyrate, and 3.3 m*M* L-α-aminobutyrate with 25 μ*M* β-mercaptoethanol. In Exp. 5, oocytes matured with 3.3 m*M* L-α-aminobutyrate and 25 μ*M* β-mercaptoethanol had a significantly less ($P < 0.05$) occurrence of polyspermy and greater occurrence of MPN formation during IVF compared to the other treatment groups and a significantly greater percentage ($P < 0.05$) of embryos reaching the 2 cell developmental stage by 48 h post-IVF and blastocyst stage of development by 144 h post-IVF compared to the other treatment groups. In Exp. 6, treatment had no effect on the time of cell death. The times at which embryo mortality was significantly the greatest ($P < 0.05$) were located within the middle of IVC. The approximate time of the onset of cell death occurred between 24 to 42 h post-IVF with the greatest occurrence around 36 h. These results suggest that supplementing 3.3 m*M* L-α-aminobutyrate and 25 μ*M* β-mercaptoethanol into the IVM medium 1) increases intracellular GSH concentrations by the end of IVM, 2) decreases the occurrence of

polyspermy during IVF, 3) increases the MPN formation during IVF, and 3) increases embryo development parameters during IVC. Supplementation to the maturation media does not have an effect on cell death during embryo development. The onset of cell death appears to occur between 24 to 42 h post-IVF with the greatest occurrence around 36 h post-IVF. In order to increase the success of *in vitro* derived porcine embryos and offspring, the basic fundamentals of the system need to be fully understood.
Key Words: Porcine, Cell Death, Apoptosis, Glutathione, Oocyte, Embryo

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TABLE OF CONTENTS

CHAPTER I……………………………..……………..…………..…………..1
STATEMENT OF THE PROBLEM………………………....……………..1

CHAPTER II……………………………………………………..……………3
REVIEW OF THE LITERATURE……………………………...…….3
Introduction………………………………...………………...….3
In vitro maturation……………………………………....…………..4
In vitro fertilization…………………………......…………..…….9
In vitro culture………………………………..….................………11
Glutathione……………………………………..…......................…12
γ-Glutamyl cycle…………………………………...…………………14
Cell death………………………………….....……..………………17
Summary……………………………………..…......……………21

CHAPTER III…………………………………………………………………..23

EXOGENOUS γ-GLUTAMYL CYCLE COMPOUNDS INFLUENCE LEVELS OF INTRACELLULAR GLUATHIONE IN PORCINE OOCYTES……..………..23
Introduction………………………………………………………………....23
Materials and Methods……………………………………………..…………25
Experimental Results……………………..……………………….....………29
Discussion...33

CHAPTER IV…………………………………………………………..…….37

EXOGENOUS γ-GLUTAMYL CYCLE COMPOUNDS INFLUENCE *IN VITRO* FERTILIZATION AND CULTURE PARAMETERS IN PORCINE OOCYTES………………………………………………………………………37
Introduction……………………………………………………………………37
Materials and Methods………………..……………………………………….38
Experimental Results………………...............……………………………42
Discussion...48

CHAPTER V……………………………………..…………………………..50

EXOGENOUS L-α-AMINOBUTYRATE SUPPLEMENTED WITH β-MERCAPTOETHANOL INFLUENCE ON PORCINE EMBRYO VIABILITY…50
Introduction……………………………………………………………………50
Materials and Methods…………………………………………………………...51
Experimental Results………………………………………...............……56
Discussion...61

CHAPTER VI……………………………………………………………………..**63**
SUMMARY…..……………………………………………...………63
IMPLICATIONS……………………………………………………..……64

CHAPTER VII……………………………………………………………….…**65**
LITERATURE CITED……………………………………………......…65

APPENDIX A…………………………………………………………...............**77**
APPENDIX B...**80**
APPENDIX C...**82**
APPENDIX D...**84**
VITA…………………………..**85**

LIST OF FIGURES

FIGURE 1. Follicular and oocyte growth *in vivo*. The comparison of growth phase I and growth phase II between the follicle and the oocyte…………………………5
FIGURE 2. Meiotic resumption within the oocyte prior to fertilization. The LH surge from the pituitary causes the cumulus cells to synthesize hyaluronic acid (HA) and soluble oocyte factor (SOF). This breaks the gap junctions between the cumulus cells and the oocyte which eliminates oocyte maturation inhibitor (OMI) and reduces cyclic adenosine monophosphate (cAMP). Reduced levels of cAMP cause an increase in maturation promotion factor (MPF) concentrations which bind to histones and cause chromosomes to condense and break apart the nuclear membrane, resuming meiosis………...……..6
FIGURE 3. The biochemical structure of the tripeptide, non-protein thiol, glutathione (L-γ-glutamyl-L-cysteinyl-glycine). The two two structural characteristics are the sulfhydryl group and the γ-glutamyl linkage......…………………………………13
FIGURE 4. The γ-glutamyl cycle. Embedded in the membrane, γ-glutamyl transpeptidase transports an amino acid inside by attaching it to a glutathione (GSH) derived carrier, γ-glutamyl. The amino acid linked carrier is broken down by γ-glutamyl cyclotransferase and the amino acid is released into the cell. The carrier portion, 5-oxoproline, is converted to glutamic acid via 5-oxoprolinase, and is converted to GSH through a series of 3 ATP requiring enzymatic steps………...……16
FIGURE 5. Cell death by either apoptosis or necrosis. Necrotic cells lose membrane integrity and swell, and end with total cell lysis and organelle disintegration. Apoptotic cells experience membrane condensation and blebbing into small apoptotic bodies. Both pathways result in phagocytosis by macrophages………...18
FIGURE 6. Pathway of Apoptosis. When the death receptors are activated, inactive procaspases are cleaved to caspases. The Bcl-2 proteins of mitochondria act as ion channels, disrupting homeostasis, causing the release of cytochrome c. Cytochrome c activates Apaf-1 which converts procaspase-9 to caspase-9. Caspase-9 converts procaspase-3 to caspase-3, the main enzyme causes nuclear DNA fragmentation……………………..………………………………………20
FIGURE 7. Timeline of Events for Exp. 1, 2, 3, and 4. After being randomly assigned to different treatment groups, all oocytes were subjected to identical hormonal supplementation of 0.0005 units/mL FSH, 0.000625 units/mL LH, and 10 ng/mL epidermal growth factor and identical *in vitro* maturation (IVM) environments of 39°C in an atmosphere of 5% CO_2 at 0 h. Between 40 to 48 h post-hormone administration, oocytes were mixed with 0.1% hyaluronidase for 15 to 30 s to remove the cumulus cells and then washed in 0.2 *M* sodium phosphate buffer containing 10 m*M* EDTA (pH 7.2). The denuded oocytes were frozen in the 0.2 *M* sodium phosphate buffer containing 10 m*M* EDTA (pH 7.2) at -80° C until the day of the assay……………………………………..……………………......…28

FIGURE 8. Timeline of Events for Exp. 5. After being randomly assigned to different treatment groups, all oocytes were subjected to identical hormonal supplementation of 0.0005 units/mL FSH, 0.000625 units/mL LH, and 10 ng/mL epidermal growth factor and identical *in vitro* maturation (IVM) environments of 39°C in an atmosphere of 5% CO_2 at 0 h. Between 40 to 48 h post-hormone administration, oocytes were mixed with 0.1% hyaluronidase for 15 to 30 s to remove the cumulus cells and fertilized with 1 x 10^6 spermatozoa/mL in Tris-fertilization medium for 6 to 10 h. After *in vitro* fertilization (IVF), 5 to 15 ootids were stained with Hoechst 33342 and examined for spermatozoa penetration, polyspermy, and male pronuclear formation under a fluorescent microscope. The remaining zygotes were subject to identical *in vitro* culture (IVC) for 144 h post-IVF and examined for cleavage at 48 h and blastocyst formation at 144 h post-IVF..41
FIGURE 9. Percent of polyspermic oocytes after *in vitro* fertilization determined by Hoechst 33342 staining, comparing between treatment groups...........................44
FIGURE 10. Percent of oocytes with male pronucleus after *in vitro* fertilization determined by Hoechst 33342 staining, comparing between treatment groups....45
FIGURE 11. Percent of embryos reaching the 2-cell developmental stage (2CELL) 48 h post-*in vitro* fertilization (IVF) after *in vitro* maturation in different treatment groups and identical IVF...46
FIGURE 12. Percent of embryos reaching the blastocyst stage (BLAST) by 144 h post-*in vitro* fertilization (IVF) after *in vitro* maturation in different treatment groups and identical IVF...47
FIGURE 13. Timeline of Events for Exp. 6. After randomly being assigned to different treatment groups, all oocytes were subjected to identical hormonal supplementation of 0.0005 units/mL FSH, 0.000625 units/mL LH, and 10 ng/mL epidermal growth factor and identical *in vitro* maturation (IVM) environments of 39°C in an atmosphere of 5% CO_2 at 0 h. Between 40 to 48 h post-hormone administration, oocytes were mixed with 0.1% hyaluronidase for 15 to 30 s to remove the cumulus cells and fertilized with 1 x 10^6 spermatozoa/mL in Tris-fertilization medium for 6 to 10 h. After *in vitro* fertilization (IVF), embryos were subject to identical *in vitro* culture (IVC) for 144 h post-IVF and examined for cleavage at 48 h and blastocyst formation at 144 h post-IVF. Random samples of 3 to 6 embryos were taken every 6 h during IVC and examined for embryo viability using the fluorescent Annexin V-FITC assay.......……………………..…55
FIGURE 14. Embryo viability point system. Embryo viability was scored on a 5 point system determined from the observations of fluorescence from the assay: 0 = viable embryo/no embryonic cells fluorescing, 1 = initiation of embryo death/10% or less of embryonic cells fluorescing, 2 = up to 50% of the embryonic cells dead/up to 50% of embryonic cells fluorescing, 3 = more than half of the embryonic cells dead/50 to 80% of embryonic cells fluorescing, 4 = dead embryo/close to 100% of embryonic cells fluorescing...54
FIGURE 15. Viability score of porcine embryos every 6 h post-*in vitro* fertilization up to 60 h, determined by the Annexin V-FITC assay...58

FIGURE 16. Percent of embryos reaching the 2-cell developmental stage (2CELL) 48 h post-*in vitro* fertilization (IVF) after *in vitro* maturation in different treatment groups and identical IVF..........59
FIGURE 17. Percent of embryos reaching the blastocyst stage (BLAST) by 144 h post-*in vitro* fertilization (IVF) after *in vitro* maturation in different treatment groups and identical IVF..........60
FIGURE 18. Standard curve for intracellular glutathione (GSH) concentrations using the 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) – disulfide GSH (GSSG) reductase recycling assay..........83

LIST OF TABLES

TABLE 1. The overall analysis of variance for replication and treatment effects on pmol glutathione per oocyte per well....30
TABLE 2. Relative intracellular glutathione (GSH) concentrations expressed in pmol GSH per oocyte per well when compared to NCSU 23 maturation medium (CONTROL). Oocytes were matured for 40 to 48 h in CONTROL supplemented with 3.3 m*M* cysteine (CYS), 150 μ*M* cysteamine (CYSTE), or 3.3 m*M* CYS + 150 μ*M* CYSTE. The amount of GSH was then determined from the 5,5'-dithio-bis(2-nitrobenzoic acid) – GSH disulphide reductase recycling assay using a standard curve of concentration GSH versus rate of change in absorbency....30
TABLE 3. The overall analysis of variance for replication and treatment effects on pmol glutathione per oocyte per well....30
TABLE 4. Relative intracellular glutatione (GSH) concentrations expressed in pmol GSH per oocyte per well when compared to NCSU 23 maturation medium (CONTROL). Oocytes were matured for 40 to 48 h in CONTROL supplemented with 1.0 m*M* glycine (GLY), 2.5 m*M* GLY, or 5.0 m*M* GLY. The amount of GSH was then determined from the 5,5'-dithio-bis(2-nitrobenzoic acid) – GSH disulphide reductase recycling assay using a standard curve of concentration GSH versus rate of change in absorbency....31
TABLE 5. The overall analysis of variance for replication and treatment effects on pmol glutathione per oocyte per well....31
TABLE 6. Relative intracellular glutathione (GSH) concentrations expressed in pmol GSH per oocyte per well when compared to NCSU 23 maturation medium (CONTROL). Oocytes were matured for 40 to 48 h in CONTROL supplemented with 1.0 m*M* L-glutamate (GLU), 2.5 m*M* GLU, or 5.0 m*M* GLU. The amount of GSH was then determined from the 5,5'-dithio-bis(2-nitrobenzoic acid) – GSH disulphide reductase recycling assay using a standard curve of concentration GSH versus rate of change in absorbency....31
TABLE 7. The overall analysis of variance for replication and treatment effects on pmol glutathione per oocyte per well....32
TABLE 8 Relative intracellular glutathione (GSH) concentrations expressed in pmol GSH per oocyte per well when compared to NCSU 23 maturation medium (CONTROL). Oocytes were matured for 40 to 48 h in CONTROL supplemented with 3.3 m*M* cysteine (CYS), 3.3 m*M* L-α-aminobutyrate (aAB), 25 μ*M* β-mercaptoethanol (BME), 3.3 m*M* CYS and 25 μ*M* BME, or 3.3 m*M* aAB and 25 μ*M* BME. The amount of GSH was then determined from the 5,5'-dithio-bis(2-nitrobenzoic acid) – GSH disulphide reductase recycling assay using a standard curve of concentration GSH versus rate of change in absorbency....32
TABLE 9. The overall analysis of variance for replication and treatment effects on *in vitro* fertilization parameter spermatozoa penetration....43
TABLE 10. The overall analysis of variance for replication and treatment effects on *in vitro* fertilization parameter polyspermy....43
TABLE 11. The overall analysis of variance for replication and treatment effects on *in vitro* fertilization parameter male pronuclear formation....44

TABLE 12. The overall analysis of variance for replication and treatment effects on *in vitro* culture parameter 2-cell stage of development..45
TABLE 13. The overall analysis of variance for replication and treatment effects on *in vitro* culture parameter blastocyst stage of development.....................................46
TABLE 14. The overall analysis of variance for time and treatment effects on embryo viability score...57
TABLE 15. Least-square means (LSMEANS) ± standard error (SE) for overall viability score (0 = viable embryo, 1 = initiation of embryo death, 2 = up to 50% of the embryonic cells dead, 3 = more than half of the embryonic cells dead, 4 = dead embryo) at the different observational times post-*in vitro* fertilization................57
TABLE 16. Significant differences of embryo viability scores between observational times (h) post-*in vitro* fertilization...58
TABLE 17. The overall analysis of variance for replication and treatment effects on *in vitro* culture parameter 2-cell stage of development..59
TABLE 18. The overall analysis of variance for replication and treatment effects on *in vitro* culture parameter blastocyst stage of development......................................60

Abbreviations

The following abbreviations were used throughout this thesis:

ATP	adenosine triphosphate
BSA	bovine serum albumin
C	Celsius
d	day(s)
DNA	deoxyribonucleic acid
EDTA	ethylenediamine-tetraacetic acid
EGF	epidermal growth factor
Exp.	Experiment
FITC	fluorescein isothiocyanate
FSH	follicle-stimulating hormone
g	gram(s)
g	gravity
GH	growth hormone
GSH	glutathione
GSSG	glutathione disulfide
h	hour(s)
IVC	*in vitro* culture
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
L	liter
LH	luteinizing hormone
M	molar
min	minute(s)
mm	millimeter
m*M*	millimolar
mL	milliliter
mol	mole(s)
MPN	male pronucleus(ar)
NADH	reduced nicotinamide adenine dinucleotide
NCSU	North Carolina State University
nm	nanometer
pmol	picomole
s	second(s)
μg	microgram
μL	microliter
μm	micrometer
μ*M*	micromolar
yr	year(s)

CHAPTER I

STATEMENT OF THE PROBLEM

There have been many advances in the social, political, and scientific community in regard to biotechnology and transgenic research during the past decade. The porcine species is considered to be a suitable model for human research in several areas including fertilization, embryology, and xenotransplantation.

The *in vitro* system for producing viable porcine embryos has become increasingly defined during the last 15 yr. The *in vitro* system can be broken down into three broad steps: maturation, during which the oocyte is matured under the influence of hormones until it is capable of being fertilized; fertilization, when a spermatozoon penetrates the oocyte and the polar bodies are extruded; and culture, the progression from the one-cell zygote to the desired product, usually a hatching blastocyst.

However, three main problems still exist within the *in vitro* system: 1) a low rate of development and inferior quality of *in vitro* matured (IVM) – *in vitro* fertilized (IVF) oocytes-zygotes developing to the blastocyst stage; 2) a high rate of polyspermy during IVF; and 3) a low rate of male pronucleus formation (MPN) after fertilization. The success rate measured from the initial number of total oocytes to those reaching the blastocyst stage after fertilization is extremely low and therefore very inefficient.

The developmental competence of oocytes during IVM is far inferior to those undergoing maturation *in vivo.* The physiological appearance of *in vivo* oocytes is more uniform in regard to ooplasm distribution, zona pellucida integrity, and the surrounding cumulus cell compactness. It is thought that many of the necessary biochemical pathways to complete oocyte maturation prior to fertilization are incomplete or inadequate in the IVM oocytes. Post-fertilization, there is a high incidence of an enigmatic developmental block after the 2-cell embryo during *in vitro* culture (IVC) yet *in vivo*, it is rare for the 2-cell embryo not to cleave into the 4-cell embryo.

During IVF, the incidence of polyspermic penetration in oocytes is high. Having more than one spermatozoon fertilizing the oocyte causes none or more than one MPN to

form. Too many copies of nuclear material cause the zygote to die. Polyspermic penetration *in vivo* is a rare occurrence and very seldom documented.

After IVF of a monospermic oocyte, the head of the spermatozoon must decondense and begin to form the MPN. Male pronuclear formation is a critical step prior to the creation of the zygote. Inadequate IVM or IVF conditions may deteriorate the biochemical pathways of the spermatozoon or the egg and cause abnormal or nonexistent MPN, resulting in an unfertilized oocyte.

There have been many advances in the establishment of techniques and procedures using porcine oocytes for *in vitro* studies, however the *in vitro* protocol for producing viable porcine embryos is not fully defined. The entire *in vitro* system is complex in that whatever occurs or fails to occur in one step, most often will affect the outcome of the next step or subsequent steps. This causes the determination of the optimal environment for each step to be a very difficult and tedious process. A desired scenario would be to create a chemically defined medium for the *in vitro* laboratory setting that is easy to replicate, highly efficient, and repeatable. The quest to define this "ideal medium" is the basis of the extensive amount of media experimentation. Refinements of the *in vitro* system will enable researchers to increase the rate of success and obtain a higher percentage of oocytes ultimately reaching the blastocyst stage.

This project was designed to study the effects of varying concentrations of different supplements to the IVM media (Exp. 1, 2, 3, and 4), determine their impacts on IVF and IVC parameters (Exp. 5), and evaluate subsequent embryo viability during IVC with respect to time (Exp. 6).

CHAPTER II

REVIEW OF LITERATURE

Introduction

The production of transgenic livestock begins with the ability to efficiently produce large quantities of embryos at particular stages in development. Although the technology of manipulating the *in vitro* system has drastically improved during recent years, there are still many inadequacies. In particular, the porcine system is less technologically advanced than its bovine counterpart and therefore needs extensive research to further define the system.

The main focus of research has been trying to solve the problems of the low rate of development and inferior quality of IVM-IVF embryos developing to the blastocyst stage, the low rate of MPN formation, and the high rate of polyspermy during IVF (Abeydeera and Day, 1997; Dubuc and Sirard, 1995; Nagai, 2001).

An important event that must occur during porcine oocyte maturation is the adequate synthesis of glutathione (GSH) (Yoshida et al., 1993). Yoshida et al. (1993) demonstrated that high levels of GSH help promote the oocyte-sperm complex to develop a MPN. Yamauchi and Nagai (1999) further demonstrated that GSH functions in DNA and protein synthesis and amino acid transport inside the maturing oocytes. A lack of GSH in oocytes prior to fertilization results in the inability to form a MPN after fertilization. Glutathione promotes the breaking of the disulfide bond in protamine found in spermatozoa heads by making the oocyte cytoplasm into an oxidation-reduction state (Nagai, 2001). Adequate concentrations of intracellular GSH have beneficial effects on subsequent porcine embryo development (Abeydeera et al., 1998).

During development, seemingly random embryos undergo spontaneous cell death. Cell death can be classified as either necrotic or apoptotic. Necrosis results from exposure to extreme extrinsic factors where the cell loses its membrane integrity and disintegrates. Necrosis is also characterized by a loss of ion homeostasis and random DNA digestion. Apoptosis is known as “programmed cell death” and is a result of complex events mediated by multiple signal cascades. Apoptosis is characterized by

membrane blebbing into smaller bodies and non-random DNA fragmentation (Porter and Janicke, 1999). Currently it is thought that apoptosis is initiated to eliminate abnormal or defective embryos (Makarevich and Markkula, 2002).

Apoptosis and necrosis in embryos is also thought to be an indicator of poor maturation, fertilization and/or culture conditions and can be used as a screening mechanism for embryo quality (Soom et al., 2000; Byrne et al., 1999). Changes in the IVM media may cause the incidence of embryo death to diminish, causing an increase in the success rate of obtaining viable embryos.

***In vitro* maturation**

The IVM portion of the *in vitro* system is designed to closely resemble that which occurs *in vivo*. Currently, the conditions *in vitro* are inferior to those found *in vivo* as evidenced by low intracellular concentrations of GSH, inadequate oocyte maturation and the inability to assess/screen for superior oocytes (Nagai, 2001). Oocyte maturation correlates with follicular maturation and has been the topic of extensive research and observation.

Oocytes originate from the primordial germ cells found in the genital ridge lateral to the mesonephros between 22 to 24 d, but have been observed as early as 18 d in the porcine embryo (Black and Erickson, 1968). Germ cells undergo mitosis up through 50 d and then begin undergoing meiosis until approximately 35 d after parturition. The oocytes are arrested in the dictyate stage, a prolonged diplotene stage of the first meiotic division also known as the germinal vesicle stage. Primordial oocytes are surrounded by the primordial follicle, a single layer of squamous shaped granulosa cells. This describes the first phase of oocyte growth where growth of the oocyte and follicle are highly correlated. The second phase of growth is when the follicle continues to grow while the oocyte remains arrested until it reaches full adult size (115 μm) in the antral follicle (1.8 mm)(Figure 1).

During the second growth phase, oocytes quadruple in size and depend on somatic cell (granulosa) – oocyte interactions via gap junctions. The granulosa (cumulus) cells provide nucleotides, amino acids, phospholipids, substrates for energy

utilization, and maintain the ionic balance of the oocyte. Cumulus cells also protect oocytes against oxidative stress-induced apoptosis (Tatemoto et al., 2000).

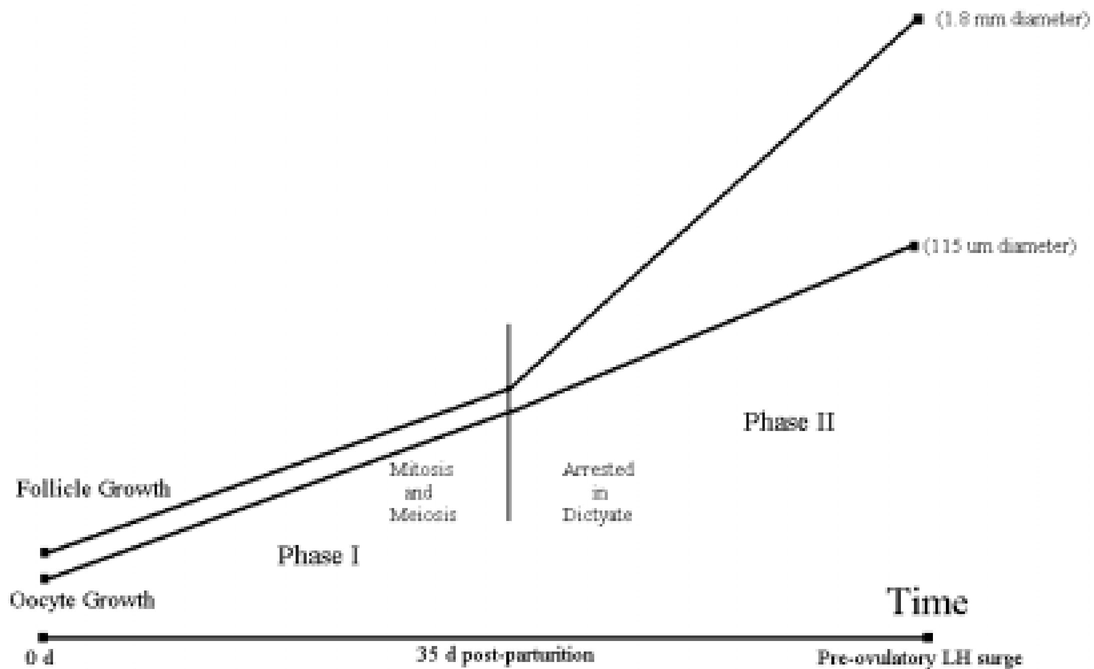


Figure 1. Follicular and oocyte growth *in vivo*. The comparison of growth phase I and growth phase II between the follicle and the oocyte.

The granulosa cells also act as paracrine centers for the oocytes and follicles by producing estrogen and other proliferative factors. Cumulus expansion is a result of synthesis and accumulation of the extracellular matrix component, hyaluronan (Kimura et al., 2002). The oocytes secrete cumulus-expansion-enabling factor that helps regulate cumulus cell expansion via FSH or EGF which is mediated by cyclic adenosine monophosphate (cAMP) (Singh et al., 1993; Coskun et al., 1995). Vaderhyden (1993) demonstrated by measuring cumulus cell size expansion under the influence of FSH alone and FSH with the oocyte, that cumulus-expansion-enabling factor is not required for cumulus expansion. As the follicle and oocyte mature, the number of binding sites for FSH and the binding capacity of FSH to the granulosa cells significantly increases until 6 d after culture (LaBarbera and Ryan, 1981). Additionally the oocyte secretes steroidogenic inhibitory factors that reduce the effects of gonadotropins on the oocyte.

This eliminates the possibility of premature resumption of meiosis and separation between the granulosa cells and the oocyte prior to the LH surge (Coskun et al., 1995).

In order for fertilization to occur, the oocyte must resume and complete meiosis. This is initially triggered by the LH surge from the anterior pituitary. The LH surge causes the cumulus cells to synthesize hyaluronic acid and soluble oocyte factor which breaks the gap junctions between the granulosa cells and the oocyte. This isolation eliminates the meiosis inhibitory substances such as oocyte maturation inhibitor and reduces the intracellular levels of cAMP, which causes cyclin to be produced. An increase in cyclin concentrations causes cyclin B-p34cdc2 or maturation promoting factor concentrations to increase, which bind to the histones of the oocyte and causes the chromosomes to condense and break apart the nuclear membrane, thus resuming meiosis (Hunter, 2000)(Figure 2).

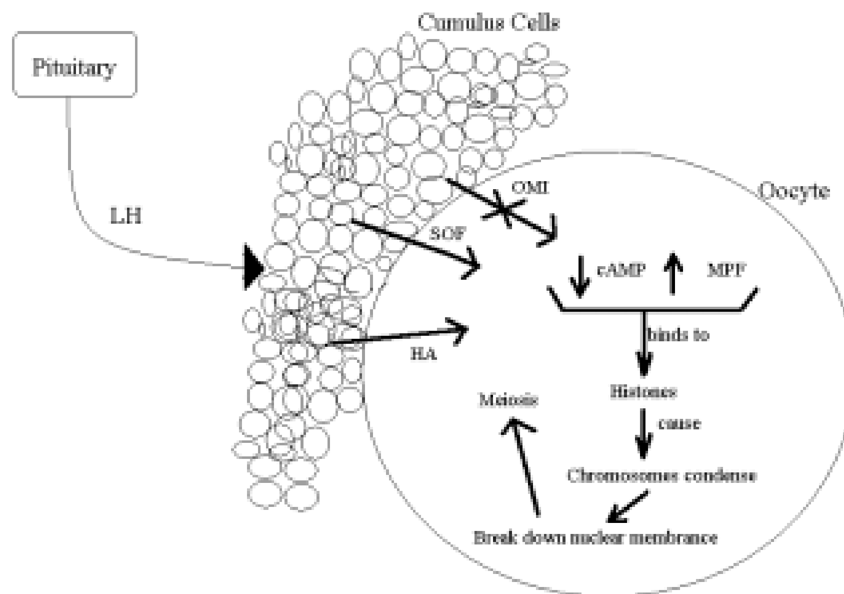


Figure 2. Meiotic resumption within the oocyte prior to fertilization. The LH surge from the pituitary causes the cumulus cells to synthesize hyaluronic acid (HA) and soluble oocyte factor (SOF). This breaks the gap junctions between the cumulus cells and the oocyte which eliminates oocyte maturation inhibitor (OMI) and reduces cyclic adenosine monophosphate (cAMP). Reduced levels of cAMP cause an increase in maturation promotion factor (MPF) concentrations which bind to histones and cause chromosomes to condense and break apart the nuclear membrane, resuming meiosis.

The IVM system is designed to increase the chance of oocyte maturation occurring without the presence of the follicle. The germinal vesicle breakdown and resumption of meiosis were first recognized *in vitro* by Edwards (1965), who observed that the maturation *in vitro* after the LH supplementation was similar to that *in vivo* and hypothesized that the observable blocks to meiosis and maturation were due to inadequacies of the IVM medium.

The developmental competence of oocytes depends upon the maturity of the follicle from which it was aspirated. As an antral follicle matures, it increases in volume. Motlik et al. (1984) observed that the ability of the porcine oocyte to resume meiosis (undergo germinal vesicle breakdown) is apparent in oocytes obtained from follicles greater than 0.8 mm in diameter. However, only oocytes obtained from follicles greater than 2.0 mm in diameter are able to extrude the first polar body after 48 h of IVM (Motlik et al., 1984; Perry et al., 1999). The nuclear transcriptional activity of oocytes obtained from follicles less than 2.0 mm is significantly decreased (Motlik et al., 1994). Cumulus cell expansion is significantly greater in cumulus-oocyte-complexes after the LH surge compared to before the LH surge (Torner et al., 1998).

These findings led researchers to supplement the maturation medium with follicular shell pieces/cells and/or fluid. Ding et al. (1992) found that oocytes co-cultured with follicular shells enhanced the MPN rate of formation during IVF. An increase in follicular size and diameter were significantly correlated to oocyte cytoplasmic maturation during IVM and the rate of MPN formation during IVF (Ding and Foxcroft, 1992; Ding and Foxcroft, 1994).

Supplementation of follicular cells in medium displayed a higher rate of fertilization and penetration of oocytes than oocytes incubated with only cumulus cells. Mattioli et al. (1988) observed that the follicular cell supplemented medium promoted MPN formation. They also examined whether the follicular shells acted on the oocytes directly or on the surrounding cumulus cells. Their results indicated that the follicular cells cannot directly influence oocytes but must have some type of cell-to-cell contact. Follicular cell supplementation to the medium has not only added variability into the system but conflicting results as well. Abeydeera et al. (1998) reported that culturing

with follicular cells did not significantly change fertilization parameters or cleavage rates but did increase the percent of embryos reaching the blastocyst stage of development.

Porcine follicular fluid supplemented to the maturation medium significantly increases MPN formation in oocytes undergoing fertilization (Naito et al., 1988; Rath et al., 1995). Porcine follicular fluid was also found to increase the rates of nuclear maturation and cleavage in zygotes, which may be due to an acidic substance found in the follicular fluid (Yoshida et al., 1992; Rath et al., 1995). Porcine follicular fluid from 5.0-7.0 mm follicles significantly reduces the occurrence of polyspermy during IVF compared to no supplementation or supplementation with fluid from smaller follicles (Vatzias and Hagen, 1999).

In addition to follicular supplementation, much research has been done on hormonal supplementation to the IVM medium. Initially, Mattioli et al. (1991) studied the effects of LH and FSH on oocyte maturation. They observed that both LH and FSH significantly accelerated meiotic progression while LH improved cytoplasmic maturation for MPN formation. Significant cytoplasmic maturation and cumulus cell expansion was observed when gonadotropins were removed after 20 h of IVM, indicating that there is a relationship between duration of oocyte exposure to gonadotropins and incubation time (Funahashi and Day, 1993).

Other hormones supplemented to the media have made significant advances in oocyte maturation as well, such as transforming growth factor-β (Singh et al., 1993), androstenedione (Singh et al., 1993), pregnant mare serum gonadotropin (Funahashi and Day, 1993; Funahashi et al., 1994), human chorionic gonadotropin (Funahashi and Day, 1993; Funahashi et al., 1994), insulin like growth factor-I (Xia et al., 1994; Illera et al., 1998), and estradiol-17β (Funahashi and Day, 1993; Singh et al., 1993; Bing et al., 2001). The addition of epidermal growth factor (EGF) to the maturation medium has been shown to significantly increase developmental competence, cleavage rate, and blastocyst formation in porcine oocytes (Ding and Foxcroft, 1994; Abeydeera et al., 1998; Illera et al., 1998; Abeydeera et al., 2000).

More recently, researchers have been supplementing maturation medium with glycosaminoglycans in hope that IVF and IVC will be more successful. Kano et al. (1998) observed that hyaluronic acid and chondroitin sulfate A significantly increased the

incidence of blastocyst formation. Tatemoto et al. (2001) reported that the addition of ascorbic acid 2-*O*-α-glucoside increased cytoplasmic maturation and increased developmental competence after IVF.

The general IVM of porcine oocytes in the following experiments is performed using North Carolina State University (NCSU) 23 medium (Peters and Wells, 1993), which contains essential osmolytes and culture constituents (see Appendix A).

***In vitro* fertilization**

Fertilization occurs only when a spermatozoon penetrates the oocyte. In order for fertilization to occur, the spermatozoa must first undergo capacitation in the female reproductive tract, in which the protective plasma coating covering the spermatozoa surface molecules is removed to allow sperm to be able to bind to the oocyte. Then spermatozoa adhere to the oocyte's zona pellucida to induce the acrosome reaction, in which the fusion of the spermatozoal plasma membrane and the outer acrosomal membrane occurs to allow the acrosomal enzymes to be released. Only after these two critical events are completed can fusion of the two gametes occur.

Surrounding the oocyte are approximately 3,000 cumulus cells (Lin et al., 1994) embedded in a thick extracellular matrix which presents an unavoidable obstacle for spermatozoa. One of the significant components comprising the extracellular matrix is a specific disaccharide, hyaluronic acid. Spermatozoa posses an enzyme called hyaluronidase which breaks down hyaluronic acid found throughout the extracellular matrix surrounding the oocyte, thereby creating a pathway so that spermatozoa can travel towards and adhere to the zona pellucida. Drobnis and Katz (1991) observed using videomicroscopy spermatozoa that had undergone the acrosomal reaction on the zona pellucida were able to work through their acrosomal shroud and penetrate into the oocyte and those recovered from female reproductive tracts prior to reaching the oocyte had not undergone the acrosome reaction (Suarez et al., 1983; Kopf and Gerton, 1991).

A glycoprotein, PH-20, found on the head of mammalian spermatozoon is homologous to that of hyaluronidase (Lathrop et al., 1990). Observations indicate PH-20 has hyaluronidase activity and spermatozoa without the PH-20 on their membrane can not traverse the cumulus cells (Lin et al., 1994). The activation of PH-20 occurs during

spermatozoan transport and is regulated by deglycosylation (Deng et al., 1999). Seaton et al. (2000) observed that the PH-20 migrates during capacitation from the tail to the acrosomal domain and that PH-20 undergoes endoproteolytic cleavage to optimize hyaluronidase activity.

The IVF portion of the *in vitro* system is designed to closely resemble the steps that occur *in vivo* around the time of fertilization. The *in vitro* conditions are inferior to those found *in vivo* as evidenced by low rates of MPN formation and a high incidence of polyspermy found during fertilization (Hunter, 2000). Polyspermic zygotes have fewer inner cell mass numbers and abnormal cleavage patterns (Han et al., 1999). The IVF system consists of preparing the spermatozoa and the oocytes for syngamy and the co-incubation of oocytes and spermatozoa for a certain duration of time.

Successful IVF of porcine oocytes was first reported by Cheng et al. (1986). *In vitro*, spermatozoa must undergo capacitation prior to coincubation with the oocytes. This occurs by incubating the spermatozoa in media for 2 to 4 h prior to IVF (Nagai et al., 1984). The medium used is Tris-buffered medium (Berger and Horton, 1988) without antibiotics with the addition of 5 m*M* of caffeine and 5 m*M* of calcium ion which increases the rate of sperm penetration (Wang et al., 1991; Abeydeera and Day, 1997; Abeydeera and Day, 1997). The cumulus cells surrounding the oocytes are removed prior to IVF by mixing them with 0.1% hyaluronidase. This is done to allow the spermatozoa to reach the egg faster and more efficiently, and reduce the number of spermatozoa needed for IVF. The main focus of recent research is optimizing the IVF environment, spermatozoa number, and coincubation time.

Coy et al. (1993) reported that IVF using oocytes surrounded by cumulus cells significantly increased the rate of fertilization using fresh boar semen and percent of monospermic penetrated oocytes compared to IVF of oocytes with removed cumulus cells. Wang et al. (1991) reported that frozen-thawed boar spermatozoa have significantly reduced motility and viability. Removal of the cumulus cells increased the penetration rate and was significantly highest between 25 to 50 x 10^6 spermatozoa/mL (Wang et al., 1995). An increase in spermatozoa number correlated to an increase in penetration rate and incidence of polyspermy in the oocyte (Wang et al., 1991).

Abeydeera et al. (1997) reported maximum penetration and MPN formation using 1 x 10^6 spermatozoa/mL during IVF of porcine oocytes after cumulus cell removal. Maximum penetration and the minimum incidence of polyspermy occurred after 6 h of coincubation (Abeydeera et al., 1997) in comparison to 3 h and 12 h.

The inadequacies that reduce the block of polyspermic penetration *in vitro* are unknown. Cran and Cheng (1986) observed that the cortical granules in the perivitelline space were not dispersed and remained circular in structure after IVF, contrary to *in vivo* fertilized oocytes. After the spermatozoon has penetrated the oocyte, the cortical granules release their contents exocytotically into the perivitelline space surrounding the oocyte. The cortical granule contents alter the properties of the zona pellucida, creating a block against polyspermy (Hunter, 1991; Dandekar et al., 1992). The thickness of the zona pellucida and perivitelline space is significantly smaller in *in vitro* derived oocytes than *in vivo* derived (Wang et al., 1998) and may be a part of the reason behind the insufficient block of polyspermy of IVF oocytes. The insufficient modification of the zona pellucida and cortical granule may be due to the lack of a secreted oviducal factor *in vitro* (Kim et al., 1996; Kano et al., 1994). One method to significantly reduce the incidence of polyspermy is by coculturing the spermatozoa prior to IVF with oviducal cells (Dubuc and Sirard, 1995) or coculturing the spermatozoa and oocytes with oviducal cells during IVF (Nagai and Moor, 1990).

***In vitro* culture**

The final step of the *in vitro* production system is IVC. The IVC step begins after IVF, consisting of sustaining the porcine embryos in a suitable environment. This begins at the ootid stage of development and continues through the zygote stage and cleavage processes up to a desired point of termination. *In vitro* derived embryos at the two to four cell stages are able to be implanted into recipients and carried to term (Mattioli et al., 1989). However, porcine embryos are able to develop *in vitro* all the way to the blastocyst stage and hatch from the zona pellucida (Mattioli et al., 1989; Yoshida et al., 1990). The developmental competence of *in vitro* derived offspring is significantly lower than natural offspring with respect to growth rate and weight (Kazuhiro et al., 1999).

As in IVM and IVF, the *in vitro* system is not as successful in producing viable embryos as is the *in vivo* system (Prather and Day, 1998). *In vitro* derived embryos have lower and delayed cleavage rates and asynchronous pronucleus development compared to *in vivo* derived embryos (Laurincik et al., 1994). Further along in development, the number of nuclei of *in vitro* derived blastocyts is significantly lower than *in vivo* blastocysts (Machaty et al., 1999) and the failure to induce significant rates of pregnancy in live recipients using *in vitro* derived embryos is significantly lower than *in vivo* derived embryos (Rath et al., 1995).

The optimum incubation parameters for IVC of porcine embryos is at 5% carbon dioxide and 5% oxygen (Berthelot and Terqui, 1996; Kikuchi et al., 2002). The greatest variable in optimizing the IVC of embryos is the medium used. All media used contains various salts in low concentrations (Beckmann and Day, 1993), energy sources (Kikuchi et al., 2002), and macromolecules (Petters and Wells, 1993). The medium most often used, NCSU 23, was first reported by Petters and Wells (1993) to sustain viable porcine embryos. Bovine serum albumin has been shown to support the development from one-cell porcine embryos (Menino and Wright, 1982) through the blastocyst stage (Dobrinski et al., 1996). A combination of NCSU 23 medium supplemented with BSA has been shown to yield significantly higher percentages of blastocyts than other media (Rath et al., 1995; Long et al., 1999).

Similar to IVM, much experimentation has been done adding various supplements to the media. The most reported supplementation is that of oviducal fluid. Fluid from oviducts on day one of the estrous cycle has been demonstrated to improve the development of porcine embryos (Archibong et al., 1989; White et al., 1989; Reed et al. 1992).

Glutathione

L-γ-glutamyl-L-cysteinyl-glycine, generally known as GSH, was first isolated in its crude form from yeast by De Rey-Pailhade in 1888 (Isherwood, 1959) and its crystalline structure was determined by Hopkins (1921). The exact structure and formula was determined from a physiochemical study by Pirie and Pinhey (1929). Glutathione has two structural characteristics, a sulfhydryl group and a γ-glutamyl

linkage making it the most abundant intracellular thiol and γ-glutamyl compound (Meister and Tate, 1976) (Figure 3).

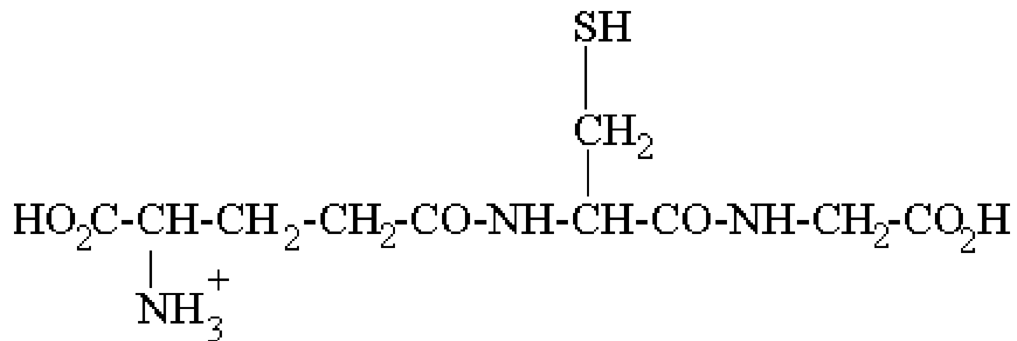


Figure 3. The biochemical structure of the tripeptide, non-protein thiol, glutathione (L-γ-glutamyl-L-cysteinyl-glycine). The two two structural characteristics are the sulfhydryl group and the γ-glutamyl linkage.

Glutathione is a tripeptide, non-protein thiol, and is found in all living cells (Isherwoord, 1959). Glutathione is found in two main forms, as a sulfhydryl group (GSH) and as a disulphide (GSSG) with GSH as the predominant form, accounting for 99.5% of the total GSH detected (Anderson, 1985). The conversion of GSH to GSSG is readily catalyzed by the enzyme glutathione reducatase allowing GSH to act as a major intracellular reductant (Jocelyn, 1959; Meister, 1982).

Glutathione is also considered to be a major amino acid transporter, especially for cysteine (Meister, 1982) and a cofactor for such enzymes as formaldehyde dehydrogenase, glyoxalase, maleylacetoacetate and glyceraldehyde 3-phosphate dehydrogenase (Jocelyn, 1959). The levels of circulating GH have been shown to influence the synthesis of GSH (Schacter and Law, 1956). An increase in the concentration of circulating GH results in the increased synthesis of GSH.

A crucial function of GSH is to protect cell membranes (Kosower and Kosower, 1973). High levels of circulating oxidants can damage cell membranes. 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 also provides a reducing environment for oocytes and embryos against oxidative damage. Elevated levels of GSH are reported to have beneficial effects on the oocytes and promotion of the oocyte-spermatozoon complex to develop a MPN after IVF (Yoshida, 1993; Sawai et al., 1997). Yamauchi and Nagai (1999) further demonstrated that GSH functions in DNA and protein synthesis and amino acid transport inside maturing oocytes. A lack of GSH in oocytes prior to fertilization results in the inability to form a MPN after fertilization. Glutathione promotes the breaking of the disulfide bond in protamine found in spermatozoa heads by making the oocyte cytoplasm into a oxidation-reduction state (Nagai, 2001). Adequate concentrations of intracellular GSH have beneficial effects on subsequent porcine embryo development and the percent of IVF oocytes reaching the blastocyst stage (Abeydeera et al., 1998; Abeydeera et al., 1999). However, GSH additions to the fertilization medium do not affect the IVF or IVC parameters and must be supplemented during IVM or as the precursors to GSH (Boquest et al., 1999). In the bovine, high intracellular GSH levels are also important in cytoplasmic maturation and embryo developmental rates, which may also hold true in porcine (Matos and Furnos, 2000).

γ-Glutamyl cycle

The synthesis and metabolism of GSH in a living cell are linked by the γ-glutamyl cycle that includes a series of six enzyme-catalyzed reactions. The γ-glutamyl cycle has two main functions: first, to synthesize GSH for the cell, and secondly, to act as an amino acid transporter across cell membranes (Meister et al., 1973). In order to function properly, one amino acid and three ATP molecules are required for one complete turn of the cycle.

Within the cell membrane, γ-glutamyl transpeptidase transports an amino acid from outside the cell to the inside by attaching it to a GSH derived carrier, γ-glutamyl. The remaining peptide from GSH, cysteinyl-glycine is cleaved by a peptidase and the amino acids are recruited back into the cycle. The amino acid/GSH carrier complex is broken down by γ-glutamyl cyclotransferase and the amino acid is released into the cell. The remaining carrier, 5-oxoproline, is converted to glutamic acid via 5-oxoprolinase,

and will eventually be converted to GSH through a series of 3 ATP requiring enzymatic steps (Meister, 1982) (Figure 4).

Glutathione reductase is an enzyme involved in the cycle that catalyzes the reduction of GSSG to GSH using NADH phosphate or NADH as the hydrogen donor (Beutler, 1973). This acts as a regulatory step in the γ-glutamyl cycle by regulating the concentration of active GSH.

Glutathione S-transferase is another enzyme involved in the cycle and GSH function. Glutathione S-transferase functions in neutralizing toxic foreign compounds and/or excreting toxic compounds from the inside to the outside of a cell (Chasseaud, 1973). Control of GSH synthesis and utilization is regulated at any of the enzymatic reaction steps that occur during the cycle, making the cycle very responsive to toxic elements and changes in the surrounding environment of the cell, indicating that the γ-glutamyl cycle is crucial in protecting the cell.

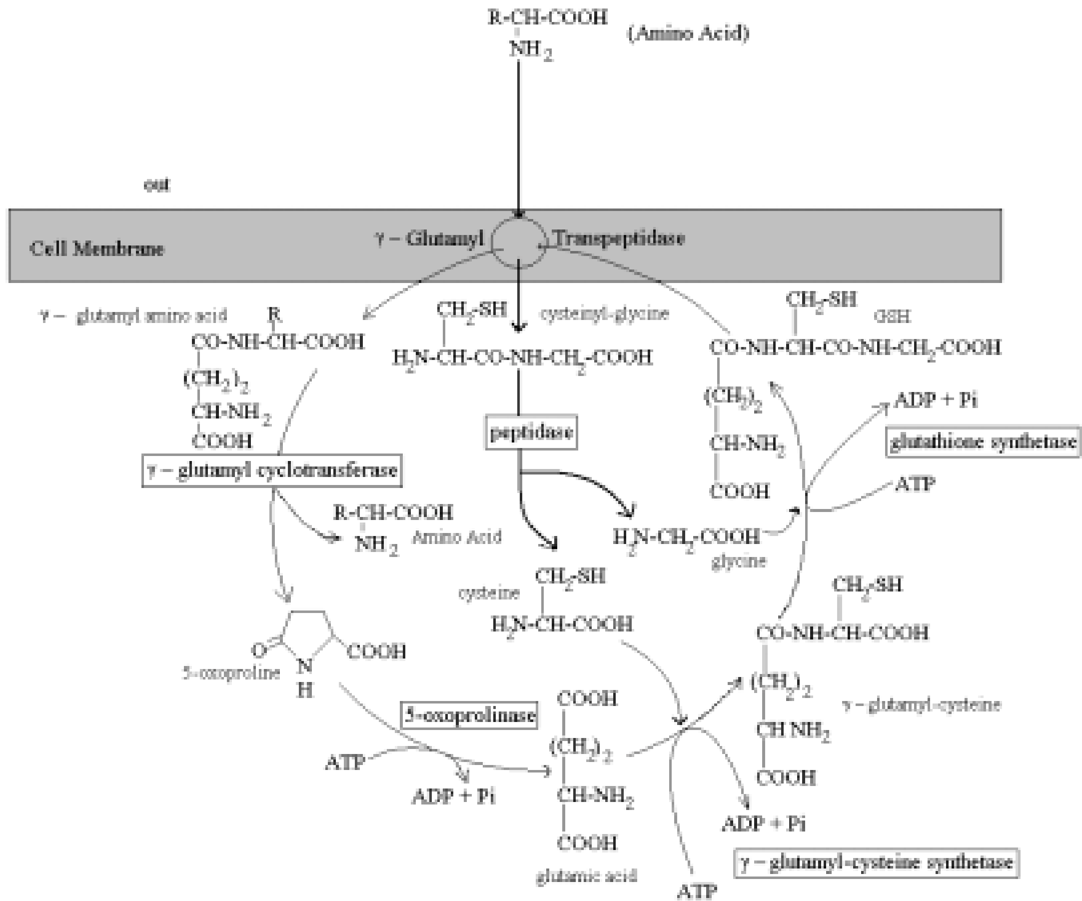


Figure 4. The γ-glutamyl cycle. Embedded in the membrane, γ-glutamyl transpeptidase transports an amino acid inside by attaching it to a glutathione (GSH) derived carrier, γ-glutamyl. The amino acid linked carrier is broken down by γ-glutamyl cyclotransferase and the amino acid is released into the cell. The carrier portion, 5-oxoproline, is converted to glutamic acid via 5-oxoprolinase, and is converted to GSH through a series of 3 ATP requiring enzymatic steps.

Cell death

Mammalian cell death results from one of two distinct mechanisms, apoptosis or necrosis (Schwartzman and Cidlowski, 1993; Vermes and Haanan, 1994). Necrosis is the pathological process that occurs when a cell encounters extremely unfavorable conditions which the cell can not control or overcome. Apoptosis is the process whereby a cell that is no longer wanted or needed, is eliminated via an intricate and elaborate series of cascades and signals.

Morphologically, cells undergoing necrosis first lose membrane integrity, then increase in cytoplasm and mitochondrial size due to swelling, and end with total cell lysis, organelle disintegration, and no observable vesicle formation. Cells undergoing apoptosis do not lose membrane integrity but rather experience membrane blebbing, encounter aggregation of chromatin around the nuclear membrane, cytoplasmic shrinking, nuclear condensation, leaky mitochondria, and finally the cell fragments into small membrane bound bodies, known as apoptotic bodies.

Biochemically, cells undergoing necrosis lose all regulation of ion homeostasis, do not require energy to die, and random digestion of DNA is observed along with post-lytic DNA fragmentation. Cells undergoing apoptosis, have an energy dependant, tightly regulated pathway they follow, involving numerous caspases and factors. Additionally, DNA fragmentation is pre-lytic and not random. The membrane of the cell is altered asymmetrically (Cohen, 1993).

Physiologically, necrosis affects groups of cells, that are ultimately phagocytosed by macrophages which are significant in the inflammatory response of the system (Van Furth and Van Zwet, 1988). Apoptosis affects individual cells, which are also phagocytosed by macrophages, but does not induce an inflammatory response (Savill et al., 1989) (Figure 5).

The concept of apoptosis was first introduced by Kerr et al. (1972) and is evolutionarily conserved across all species. Apoptosis is comprised of three main components: 1) Bcl-2 proteins, 2) interleukin-1β-converting enzyme-like proteases (caspases), and 3) Apaf-1/CED-4 proteins (Budihardjo et al., 1999).

The Bcl-2 proteins are located on the membranes of many organelles including, mitochondria, endoplasmic reticulum, and the nucleus. Mitochondrial damage has been

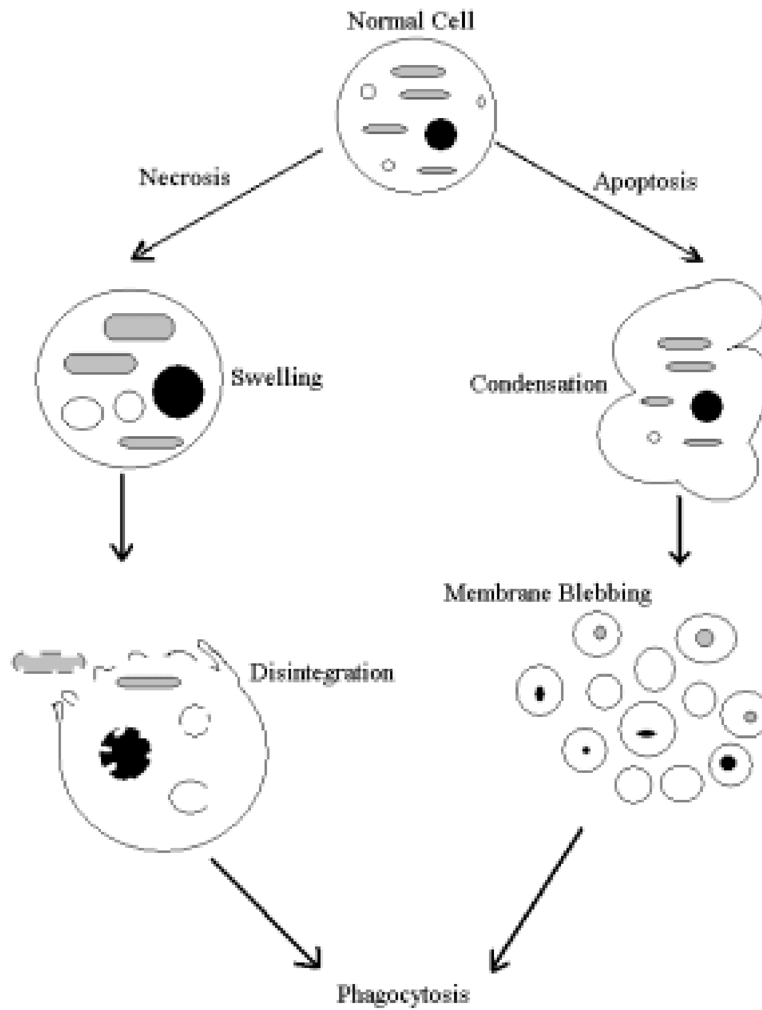


Figure 5. Cell death by either apoptosis or necrosis. Necrotic cells lose membrane integrity and swell, and end with total cell lysis and organelle disintegration. Apoptotic cells experience membrane condensation and blebbing into small apoptotic bodies. Both pathways result in phagocytosis by macrophages.

shown to be an indicator of the onset of apoptosis (Zamzami et al., 1996). The loss of membrane potential and subsequent membrane collapse is thought to be regulated by the Bcl-2 proteins (Kroemer et al., 1997). The Bcl-2 proteins act as ion channels, creating pores in the inner membrane of the mitochondria. The disruption of homeostasis causes the membrane to swell and rupture, causing the sudden release of cytochrome c (Vander Heiden et al., 1997).

When the cell surface death receptors are activated, intracellular signals are translated which eventually lead to the activation of caspases (Budihardjo et al., 1999). Inactive procaspases are circulating in the cytoplasm until this signaling cascade occurs (Chang and Yang, 2000) and the procaspases are cleaved to become cysteine proteases (caspases) that cleave in locations after aspartic acid residues found on a protein (Budihardjo et al., 1999). After one caspase is activated, it cleaves a different procaspase to its active caspase counterpart downstream, and the cascade is set in motion (Cohen, 1997).

When cytochrome c is released from the mitochondria, it causes the activation of Apaf-1 (Susin et al., 2000) which converts procaspase-9 to caspase-9. Caspase-9 converts procaspase-3 to caspase 3 which then targets the nucleus. Caspase-3 is the main enzyme responsible for nuclear DNA fragmentation in the apoptotic cell (Porter and Janicke, 1999; Wolf et al., 1999; Susin et al., 2000) (Figure 6).

Cell death occurs in developing embryos *in vitro* and *in vivo* to select for viable cells and eliminate cells without function (Hardy, 1997). Research in the area of cell death in developing embryos is recent with the focus towards the bovine embryo (Matawee et al., 2000; Watson et al., 2000; Betts and King, 2001; Makarevich and Markkula, 2002) and little emphasis on the porcine embryo.

Necrosis occurs in developing embryos due to drastic changes in the environment that disrupt the metabolic pathways and homeostasis. Apoptosis is thought to occur throughout development and is believed to be the major cause of embryo mortality (Jurisicova et al, 1998). Apoptosis in bovine embryos is thought to be dependant on the developmental stage of the embryo and could not be detected during cleavage stages, only in the advanced stage (Matawee et al., 2000).

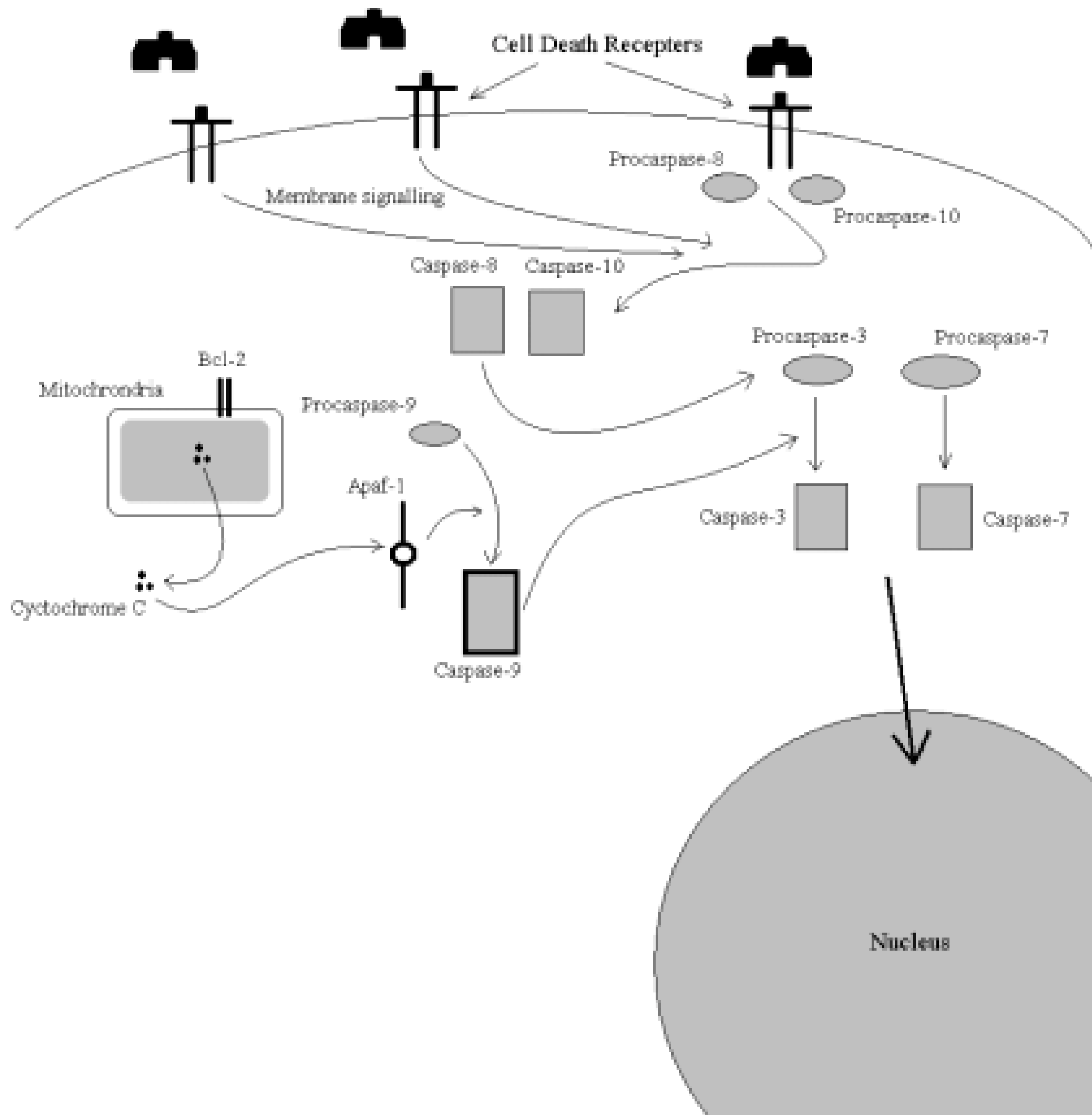


Figure 6. Pathway of Apoptosis. When the death receptors are activated, inactive procaspases are cleaved to caspases. The Bcl-2 proteins of mitochondria act as ion channels, disrupting homeostasis, causing the release of cytochrome c. Cytochrome c activates Apaf-1 which converts procaspase-9 to caspase-9. Caspase-9 converts procaspase-3 to caspase-3, the main enzyme causes nuclear DNA fragmentation.

Cell death is thought to be correlated to cell numbers: blastocysts with low cell numbers have a higher rate of cell death and on the contrary, blastocysts with large cell numbers have a lower rate of cell death (Hardy, 1997).

Research has indicated that supplementation to the IVM medium has no affect on the incidence of apoptosis in developing bovine embryos (Watson et al., 2000) but supplementation to the IVC medium does have and affect on the rate of apoptosis during embryo development (Makarevich and Markkula, 2002). This may indicate that apoptosis is preprogrammed and changes to the surrounding environment have no affect on the onset of apoptosis.

Summary

The *in vitro* production system of porcine embryos consists of three stages, IVM, IVF, and IVC. Many questions on the mechanisms of oocyte maturation, fertilization, and embryo development still remain despite extensive research in the past 15 yr (Sato, 1997). Systems implementing the addition of serum and/or components from the pig itself, have become very successful in obtaining large percentages of *in vitro* derived offspring. However, in order to have a highly repeatable and invariant system, the conditions need to be defined. This is the focus of recent research and it has been difficult to obtain comparable results to a non-defined system or *in vivo* derived offspring.

Glutathione, a major reductant inside cells, has been shown to aid in IVF by creating a reducing environment to protect the spermatozoa and the oocytes. An increase in GSH concentration has been shown to increase IVF success and subsequent IVC success as well. Researchers are currently attempting to increase levels of intracellular GSH in order to increase IVF efficiency and success.

A substantial portion of developing embryos spontaneously die in culture. Embryo mortality occurs either by necrosis or apoptosis. In order to reduce the occurrence of necrosis within the IVC system, researchers are attempting to isolate the causative factors and determine the time of death during development. Although apoptosis is accepted as a predetermined event in embryo development, the ability to

“turn off” the event may lead to a higher success rate of IVC blastocysts and *in vitro* derived porcine offspring.

CHAPTER III

EXOGENOUS γ-GLUTAMYL CYCLE COMPOUNDS INFLUENCE LEVELS OF INTRACELLULAR GLUATHIONE IN PORCINE OOCYTES

Introduction

Supplementation of a single or combination of GSH precursors found in the different branches of the γ-glutamyl cycle may increase the intracellular concentration of GSH during IVM. The readily available precursors that are suitable to add to the IVM medium are cysteine, cysteamine, glycine, L-glutamate, and L-α-aminobutyrate.

Cysteine is an integral part of the γ-glutamyl cycle. When an L-amino acid enters the cell, γ-glutamyl transpeptidase may act on it to form L-cysteinyl-glycine. L-cysteinyl-glycine is cleaved by a peptidase and cysteine is one of the remaining products. Cysteine is then attached to glutamate via γ-glutamyl-cysteine synthetase and ATP to form γ-glutamyl-cysteine. γ-glutamyl-cysteine then has glycine attached via ATP and GSH synthetase to form GSH (Meister and Tate, 1976). Cysteine supplemented in the media at 3.3 m*M* has been shown to significantly increase the intracellular concentrations of GSH (Abeydeera et al., 1998).

Cysteine in media is easily oxidized to form cystine. Cystine is able to enter the oocyte through the cumulus cells only using the Xc^- system (Yoshida and Takahashi, 1998). The Xc^- system is a sodium independent anionic amino acid transport system. If too much cysteine is oxidized to cystine, the Xc^- system will be overloaded and GSH production will not be efficient. Cysteine is transported into the oocyte using the ASC system, a sodium dependant neutral amino acid transport system (Yoshida and Takashi, 1998).

L-α-aminobutyrate is not an endogenous component in the γ-glutamyl cycle. When L-α-aminobutyrate replaces cysteine in the cycle, γ-glutamyl-cysteine synthetase remains active (Meister and Tate, 1976). L-α-aminobutyrate does not oxidize spontaneously like cysteine, therefore supplementing L-α-aminobutyrate into the

maturation media could eliminate the spontaneous oxidation problems with cysteine and increase the efficiency of GSH synthesis.

Cysteamine is a thiol compound that has a reducing function. Cysteamine is thought to reduce the high extracellular concentrations of cystine to cysteine so that the oocyte can use the cysteine to produce GSH (Nagai, 2001). Cysteamine supplemented to the medium at 150 μ*M* has been shown to significantly increase the GSH content of the oocyte (Yamauchi and Nagai, 1999).

Glycine is also an integral part of the γ-glutamyl cycle. When an L-amino acid enters the cell, γ-glutamyl transpeptidase may act on it to form L-cysteinyl-glycine. L-cysteinyl-glycine is cleaved by a peptidase and glycine is one of the remaining products. Glycine is then attached to γ-glutamyl-cysteine via ATP and GSH synthetase to form GSH (Meister and Tate, 1976).

The supplementation of 0.276 m*M* glycine to the maturation medium has shown that there is a higher rate of embryo development during IVC (Iwasaki et al., 1999). If there is no glycine available to the cell, GSH synthesis may be decreased due to the suppression of the γ-glutamyl cycle.

L-glutamate is an integral part of the γ-glutamyl cycle. When an L-amino acid enters the cell, γ-glutamyl transpeptidase may act on it to form an L-γ-glutamyl-L-amino acid. The L-γ-glutamyl-L-amino acid is cleaved by γ-glutamyl cyclotransferase and 5-oxo-L-proline and an L-amino acid is formed. 5-oxo-L-proline converts to glutamate via 5-oxoprolinase and ATP. Glutamate is then attached to cysteine via γ-glutamyl-cysteinyl synthetase and ATP to form γ-glutamyl-cysteine. The γ-glutamyl-cysteine will then have glycine attached via ATP and GSH synthetase to form GSH (Meister and Tate, 1976).

L-glutamate is transported across the cell membrane facilitated by the sodium independent amino acid transporter x^-_C. Previous work in mice has shown that the sodium independent system x^-_C is present in mature oocytes but not detectable in blastocysts (Van Winkle et al., 1992). Prather et al. (1993) demonstated that the x^-_C system was not detectable in porcine oocytes or blastocysts and exogenous glutamate could not be transported into the cell. The explanation given for this was that the zona pellucida surrounding the oocyte acted as a block for amino acid uptake. However, no L-

glutatmate uptake may have been a result of the low exogenous concentration (1μ*M*) administered (Prather et al., 1993).

β-mercaptoethanol is a thiol compound, similar to cysteamine, which is also thought to reduce extracellular cystine to cysteine (Nagai, 2001). Supplementations up to 25 μ*M* of β-mercaptoethanol were found to significantly increase the GSH content of maturing porcine oocytes and the rate of blastocyst development *in vitro* (Abeydeera et al., 1998; Abeydeera et al., 1999).

The specific objectives of these experiments were to:

1) Increase the intracellular concentrations of GSH in oocytes.
2) Determine if exogenous cysteine and cysteamine increase intracellular GSH concentrations (Exp. 1).
3) Determine the concentration of exogenous L-glycine needed to maximize intracellular GSH concentrations (Exp. 2).
4) Determine the concentration of exogenous L-glutamate needed to maximize intracellular GSH concentrations (Exp. 3).
5) Compare the intracellular concentrations of GSH as a result of exogenous cysteine and L-α-aminobutyrate supplementation (Exp. 4).
6) Determine the effect of β-mercaptoethanol supplementation in media containing cysteine and L-α-aminobutyrate with respect to GSH concentrations (Exp. 4).

Materials and Methods

Treatment Groups. The treatment groups for Exp. 1 were as follows: treatment 1 = NCSU 23 maturation media (CONTROL); treatment 2 = NCSU 23 maturation media supplemented with 3.3 m*M* cysteine (CYS); treatment 3 = NCSU 23 maturation media supplemented with 150 μ*M* cysteamine (CYSTE); treatment 4 = NCSU 23 maturation media supplemented with 3.3 m*M* CYS and 150 μ*M* CYSTE.

The treatment groups for Exp. 2 were as follows: treatment 1 = NCSU 23 maturation media (CONTROL), treatment 2 = NCSU 23 maturation media supplemented with 1.0 m*M* glycine (GLY); treatment 3 = NCSU 23 maturation media supplemented with 2.5 m*M* GLY; treatment 4 = NCSU 23 maturation media supplemented with 5.0 m*M* GLY.

The treatment groups for Exp. 3 were as follows: treatment 1 = NCSU 23 maturation media (CONTROL), treatment 2 = NCSU 23 maturation media supplemented with 1.0 m*M* L-glutamate (GLU); treatment 3 = NCSU 23 maturation media supplemented with 2.5 m*M* GLU; treatment 4 = NCSU 23 maturation media supplemented with 5.0 m*M* GLU.

The treatment groups for Exp. 4 were as follows: treatment 1 = NCSU 23 maturation media (CONTROL), treatment 2 = NCSU 23 maturation media supplemented with 3.3 m*M* CYS; treatment 3 = NCSU 23 maturation media supplemented with 3.3 m*M* L-α-aminobutyrate (aAB); treatment 4 = NCSU 23 maturation media supplemented with 25 μ*M* β-mercaptoethanol (BME); treatment 5 = NCSU 23 maturation media supplemented with 3.3 m*M* CYS and 25 μM BME; treatment 6 = NCSU 23 maturation media supplemented with 3.3 m*M* aAB and 25 μ*M* BME. Each treatment and experiment was repeated three times to obtain 10 to 40 oocytes per treatment per replicate.

General. Experiments were conducted in the summer (Exp. 1), fall (Exp. 2 and 3), and winter (Exp. 4) of 2001. All ovaries were obtained from sows and gilts slaughtered at a local abattoir in Goode, Virginia. The ovaries used were determined to be normal using visual observation and contained no cysts, blood clots, or discoloration. Ovaries were initially rinsed twice in 21°C 8.75% physiological saline and once in modified-Dulbecco's-phosphate buffered saline (m-D-PBS) containing 10,000 units/mL Penicillin G sodium and 10,000 μg/mL streptomycin sulfate. Ovaries were then stored in 21°C m-D-PBS and transported to the laboratory in an insulated cooler. The elapsed time between ovary collection and follicular aspiration ranged between 2 to 3 h.

Oocyte Recovery. Once at the laboratory, the ovaries were washed three times in m-D-PBS and medium sized follicles (3 to 6 mm in diameter) were aspirated using an 18-guage needle fixed to a 10-mL disposable syringe. The aspirated fluid was placed into a 50 mL polypropylene conical tube (Fisher Scientific, Pittsburgh, PA). After the oocytes settled for 15 min, the supernatant was discarded so that the solid contents remained in the tube and 25 mL of mTL-HEPES–PVA medium at 39°C was added to wash the pellet. The wash procedure was repeated for a total of three times and then collected into 100 x 35 mm^2 polystyrene sterile culture dishes (Fisher Scientific, Pittsburgh, PA).

IVM. Only oocytes surrounded by a compact cumulus cell mass and uniform ooplasm were utilized. The selected oocytes were washed three times in a 50 x $9mm^2$ Falcon polystyrene dish (Fischer Scientific, Pittsburgh, PA) using 100 µL drops NCSU 23 maturation medium. Then oocytes were randomly and equally assigned to treatment groups and placed into separate wells of a Nunclon 6-well multidish (Fisher Scientific, Pittsburgh, PA) previously equilibrated containing 500 µL of maturation medium and supplements under mineral oil.

After the oocytes were placed into the assigned wells, 0.0005 units/mL FSH, 0.000625 units/mL LH, and 10 ng/mL EGF were added to each well and mixed. The oocytes were incubated at 39°C in an atmosphere of 5% CO_2 for 40 to 48 h.

Assay preparation. After incubation, cumulus cells were removed by mixing oocytes with 0.1% hyaluronidase in NCSU 23 solution for 15 to 30 s. Oocytes were then washed three times in a 50 x $9mm^2$ Falcon polystyrene dish in 100 µL drops of 0.2 *M* sodium phosphate buffer containing 10 m*M* EDTA (pH 7.2). Within each treatment, the oocytes (approximately 20 to 30) were transferred with 5 µL 0.2 *M* sodium phosphate buffer containing 10 m*M* EDTA (pH 7.2) to a 1.5 mL microcentrifuge tube (Fischer Scientific, Pittsburgh, PA) and stored at –80°C until the day of the assay. Figure 7 shows a timeline of events for Exp. 1, 2, 3, and 4.

5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) – GSSG reductase recycling assay. On the day of the assay, each tube had 5 µL of 1.25 *M* phosphoric acid added to it and the oocytes were ruptured using a blunt glass rod. The contents of each tube was added to an individual well in a Nunclon 42-well multidish (Fisher Scientific, Pittsburgh, PA). The assay was performed as described in Appendix B. The absorbency of the samples was continuously read using a µQuant Universal Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT) at 412 nm for a total of 10 min. The amount of GSH was then determined using a standard curve of concentration GSH versus rate of change in absorbency (Appendix C).

Statistical Analyses. Data analyses were conducted using the general linear model (GLM) procedure in Statistical Analysis System (SAS, 2001). Data were analyzed as a complete randomized design with pmol GSH per oocyte per well as the dependant variable and treatment and replicate as the independent variables. Significance between

treatment groups was analyzed using the least-squares means (LSMEANS) statement with the standard error (STDERR) and possible probability values (PDIFF) options.

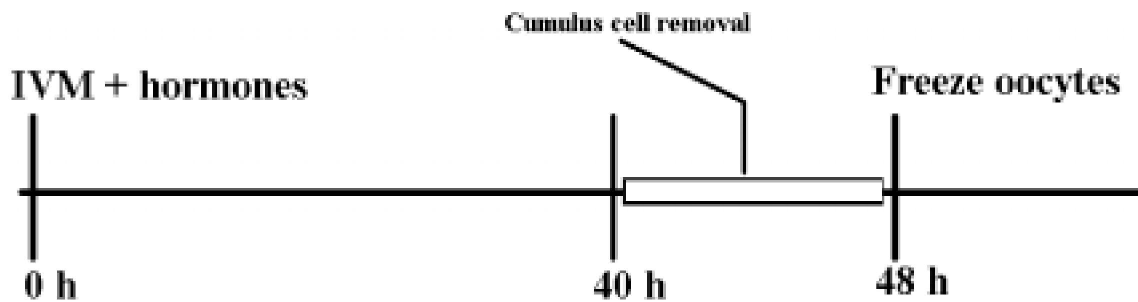


Figure 7. Timeline of Events for Exp. 1, 2, 3, and 4. After being randomly assigned to different treatment groups, all oocytes were subjected to identical hormonal supplementation of 0.0005 units/mL FSH, 0.000625 units/mL LH, and 10 ng/mL epidermal growth factor and identical *in vitro* maturation (IVM) environments of 39°C in an atmosphere of 5% CO_2 at 0 h . Between 40 to 48 h post-hormone administration, oocytes were mixed with 0.1% hyaluronidase for 15 to 30 s to remove the cumulus cells and then washed in 0.2 *M* sodium phosphate buffer containing 10 m*M* EDTA (pH 7.2). The denuded oocytes were frozen in the 0.2 *M* sodium phosphate buffer containing 10 m*M* EDTA (pH 7.2) at -80° C until the day of the assay.

Experimental Results

The control value in all experiments was adjusted to 0 and treatment values are reported in respect to the control value as pmol of GSH per oocyte per well.

Exp. 1. This experiment utilized 139 oocytes over 4 treatment groups. There was no significant effect of replication on pmol of GSH per oocyte per well (Table 1). The CYS and CYSTE treatment groups significantly increased ($P < 0.05$) the pmol GSH per oocyte per well when compared to the CONTROL (0.8521 ± 0.008 and 1.2174 ± 0.008 pmol GSH per oocyte per well, respectively) (Table 2).

Exp. 2. This experiment utilized 296 oocytes over 4 treatment groups. There were no significant effects of replication or treatment on pmol of GSH per oocyte per well (Table 3). None of the GLY treatment groups significantly increased the pmol GSH per oocyte per well when compared to the CONTROL (Table 4).

Exp. 3. This experiment utilized 434 oocytes over 4 treatment groups. There was no significant effect of replication on pmol of GSH per oocyte per well (Table 5). The 1.0 m*M* GLU treatment group significantly increased ($P < 0.05$) the pmol GSH per oocyte per well when compared to the CONTROL (3.9907m $\pm$ 0.03 pmol GSH per oocyte per well) (Table 6).

Exp. 4. This experiment utilized 298 oocytes over 6 treatment groups. There was no significant effect of replication on pmol of GSH per oocyte per well (Table 7). All treatment groups were significantly different ($P < 0.05$) when compared to the CONTROL but the aAB, BME, and CYS + BME treatment groups were not significantly different between each other (Table 8).

Table 1. The overall analysis of variance for replication and treatment effects on pmol glutathione per oocyte per well (n=139).

Source	df	Mean Square	P < 0.05
Treatment	3	1.1285	*
Replicate	2	0.0002	
Error	6	0.0002	
Total	11	1.1289	

Using type III means squared.

Table 2. Relative intracellular glutathione (GSH) concentrations expressed in pmol GSH per oocyte per well when compared to NCSU 23 maturation medium (CONTROL). Oocytes were matured for 40 to 48 h in CONTROL supplemented with 3.3 m*M* cysteine (CYS), 150 µ*M* cysteamine (CYSTE), or 3.3 m*M* CYS + 150 µ*M* CYSTE. The amount of GSH was then determined from the 5,5'-dithio-bis(2-nitrobenzoic acid) – GSH disulphide reductase recycling assay using a standard curve of concentration GSH versus rate of change in absorbency.

Treatment	Number of Oocytes	pmol GSH per oocyte per well ± SE	P < 0.05
CONTROL	31	0 ± 0.008	
CYS	32	0.8521 ± 0.008	*
CYSTE	42	1.2174 ± 0.008	*
CYS + CYSTE	34	0 ± 0.008	

Table 3. The overall analysis of variance for replication and treatment effects on pmol glutathione per oocyte per well (n=296).

Source	df	Mean Square	P < 0.05
Treatment	3	0.0001	
Replicate	2	0.0001	
Error	6	0.0002	
Total	11	0.0004	

Using type III means squared.

Table 4. Relative intracellular glutatione (GSH) concentrations expressed in pmol GSH per oocyte per well when compared to NCSU 23 maturation medium (CONTROL). Oocytes were matured for 40 to 48 h in CONTROL supplemented with 1.0 m*M* glycine (GLY), 2.5 m*M* GLY, or 5.0 m*M* GLY. The amount of GSH was then determined from the 5,5'-dithio-bis(2-nitrobenzoic acid) – GSH disulphide reductase recycling assay using a standard curve of concentration GSH versus rate of change in absorbency.

Treatment	Number of Oocytes	pmol GSH per oocyte per well ± SE	P < 0.05
Control	79	0 ± 0.009	
1.0 m*M* GLY	82	0 ± 0.009	
2.5 m*M* GLY	69	0 ± 0.009	
5.0 m*M* GLY	66	0 ± 0.009	

Table 5. The overall analysis of variance for replication and treatment effects on pmol glutathione per oocyte per well (n=434).

Source	df	Mean Square	P < 0.05
Treatment	3	11.913	*
Replicate	2	0.002	
Error	6	0.003	
Total	11	11.918	

Using type III means squared.

Table 6. Relative intracellular glutathione (GSH) concentrations expressed in pmol GSH per oocyte per well when compared to NCSU 23 maturation medium (CONTROL). Oocytes were matured for 40 to 48 h in CONTROL supplemented with 1.0 m*M* L-glutamate (GLU), 2.5 m*M* GLU, or 5.0 m*M* GLU. The amount of GSH was then determined from the 5,5'-dithio-bis(2-nitrobenzoic acid) – GSH disulphide reductase recycling assay using a standard curve of concentration GSH versus rate of change in absorbency.

Treatment	Number of Oocytes	pmol GSH per oocyte per well ± SE	P < 0.05
Control	104	0 ± 0.03	
1.0 m*M* GLU	109	3.9907 ± 0.03	*
2.5 m*M* GLU	112	0 ± 0.03	
5.0 m*M* GLU	109	0 ± 0.03	

Table 7. The overall analysis of variance for replication and treatment effects on pmol glutathione per oocyte per well (n=298).

Source	df	Mean Square	$P < 0.05$
Treatment	5	90.948	*
Replicate	2	0.014	
Error	10	0.007	
Total	17	90.969	

Using type III means squared.

Table 8 Relative intracellular glutathione (GSH) concentrations expressed in pmol GSH per oocyte per well when compared to NCSU 23 maturation medium (CONTROL). Oocytes were matured for 40 to 48 h in CONTROL supplemented with 3.3 m*M* cysteine (CYS), 3.3 m*M* L-α-aminobutyrate (aAB), 25 μ*M* β-mercaptoethanol (BME), 3.3 m*M* CYS and 25 μ*M* BME, or 3.3 m*M* aAB and 25 μ*M* BME. The amount of GSH was then determined from the 5,5'-dithio-bis(2-nitrobenzoic acid) – GSH disulphide reductase recycling assay using a standard curve of concentration GSH versus rate of change in absorbency.

Treatment	Number of Oocytes	pmol GSH per oocyte per well ± SE
CONTROL	57	0 ± 0.05^{a}
CYS	42	2.2795 ± 0.05^{bc}
aAB	57	1.4823 ± 0.05^{b}
BME	45	1.5073 ± 0.05^{b}
CYS + BME	50	1.7125 ± 0.05^{b}
aAB + BME	47	14.757 ± 0.05^{bd}

$^{a, b, c, d}$ Values in the same column with different superscripts are different ($P<0.05$).

Discussion

Glutathione protects cell membranes by providing a reducing environment to prevent cell membrane damage from circulating oxidants(Kosower and Kosower, 1973). Elevated levels of intracellular GSH are reported to have beneficial effects on the oocytes and promotion of the oocyte-sperm complex to develop a MPN after IVF (Yoshida, 1993; Sawai et al., 1997). Additionally, GSH is thought to have a function in DNA and protein synthesis and amino acid transport inside maturing oocytes (Yamauchi and Nagai, 1999). Adequate concentrations of intracellular GSH have beneficial effects on subsequent porcine embryo development and the percent of IVF oocytes reaching the blastocyst stage (Abeydeera et al., 1998; Abeydeera et al., 1999). Exogenous GSH supplementation to the fertilization medium does not affect the IVF or IVC parameters and must be supplemented during IVM or as the precursors to GSH (Boquest et al., 1999). In the bovine, high intracellular GSH levels are also important in cytoplasmic maturation and embryo developmental rates, which may also hold true in porcine (Matos and Furnos, 2000).

Using the γ-glutamyl cycle (Figure 4) pathway as a basis for supplementation of a GSH precursor, cysteine, glycine, and L-glutamate were used. Including the supplements cysteamine and β-mercaptoethanol were thought to provide a reducing environent for cysteine based compounds. The supplementation of L-α-aminobutyrate was based on findings that demonstrated similar kinetics between cysteine and L-α-aminobutyrate (Meister and Tate, 1976) except theat L-α-aminobutyrate does not spontaneously oxidize as does cysteine. By increasing the intracellular concentrations of γ-glutamyl cycle compounds, it was thought that this may increase the intracellular concentration of GSH during IVM by increasing the rate of GSH production.

In the first experiment, the addition of 3.3 m*M* cysteine significantly increased ($P < 0.05$) the pmol of intracellular GSH per oocyte per well by 48 h of IVM compared to the control (0.8521 ± 0.008). The increase of GSH from cysteine supplementation is in agreement with results from previous studies (Yoshida and Takahashi, 1998; Abeydeera et al., 1999). The addition of 150μ*M* cysteamine significantly increased ($P < 0.05$) the pmol of intracellular GSH per oocyte per well by 48 h of IVM than the control ($+1.2174 \pm 0.008$). The increase of GSH following cysteamine supplementation is in agreement

with results from previous studies (Yamauchi and Nagai, 1999). Supplementing the IVM medium with both 3.3 m*M* cysteine and 150µ*M* cysteamine was hypothesized to further increase the intracellular GSH levels; however, there was no significant increase of intracellular GSH compared to the other treatment groups.

No significant additional increase from the 3.3 m*M* cysteine and 150µ*M* cysteamine treatment may be due to an excessively high concentration of thiol-based compounds in the media. Since cysteamine is thought to stop the spontaneous oxidation of cysteine to cystine, the only way for cysteine to enter the oocyte would be through the sodium dependant ASC transporter. The amino acid transport carriers may have been overloaded or experiencing competitive inhibition. If this is true, the levels of sodium may need to be adjusted in the medium to handle the increased need for amino acid transportation. Finally, this experiment was the first time that the DTNB-GSSG reductase recycling assay was performed in our laboratory and the results may not be as accurate as those obtained after becoming more proficient at performing the assay.

In the second experiment, none of the glycine treatment groups significantly increased the pmol of intracellular GSH per oocyte per well by 48 h of IVM compared to the control. There were no previous studies examining the effects of glycine supplementation to the IVM medium, however supplementation of glycine to the IVC medium increased the percent of blastocysts formed (Iwasaki et al., 1999). This may indicate that the amino acid transporter for L-glycine is not fully operational until the stages of embryo development that are attained during IVC. This also indicates that even though L-glycine is an integral part of the γ-glutamyl cycle, it is not a component of the rate limiting step in GSH synthesis.

In the third experiment, the addition of 1.0 m*M* L-glutamate significantly increased ($P < 0.05$) the pmol of intracellular GSH per oocyte per well by 48 h of IVM compared to the control (3.9907 ± 0.03). Previous research reports that the amino acid transporter is not present in the porcine during IVM because the zona pellucida blocks it, but is present during IVC (Prather et al., 1993). This reasoning does not explain how other amino acids are able to pass through the zona pellucida and enter the maturing oocyte.

The 2.5 and 5.0 m*M* L-glutamate supplements may have been too concentrated and the amino acid transport carriers may have been overloaded or experiencing competitive inhibition. If this is true, the levels of sodium may need to be adjusted in the medium to handle the increased demand for amino acid transportation. Finally, the L-glutamate transporter may not be active in the porcine oocyte and the concentrations of intracellular L-glutamate are adequate to synthesize GSH throughout maturation. The increase in pmol GSH found may be due to experimental error from the assay.

In the fourth experiment, all of the treatment groups significantly increased ($P < 0.05$) the pmol of intracellular GSH per oocyte per well by 48 h of IVM compared to the control. The increase of GSH from all of the treatments is in agreement with results from previous studies (Yoshida and Takahashi ,1998; Abeydeera et al., 1998; Abeydeera et al., 1999; Nagai, 2001).

Supplementation of 3.3 m*M* L-α-aminobutyrate, 25µ*M* β-mercaptoethanol, and 3.3 m*M* cysteine and 25µ*M* β-mercaptoethanol were not significantly different between the treatment groups. The supplementation of 3.3 m*M* cysteine and 3.3 m*M* L-α-aminobutyrate and 25µ*M* β-mercaptoethanol elicited the greatest increase of pmol GSH per oocyte per well (2.2795 ± 0.05 and 14.757 ± 0.05, respectively).

The combination of L-α-aminobutyrate and β-mercaptoethanol elicited the greatest increase of intracellular GSH because L-α-aminobutyrate was able to replace cysteine in the γ-glutamyl cycle, but did not oxidize spontaneously and β-mercaptoethanol further inhibited oxidation of L-α-aminobutyrate. This combination may be optimal because it does not overload the amino acid transporter system and creates a reducing environment surrounding the oocyte.

Additional studies were needed to examine the effects that these compounds and elevated GSH intracellular concentrations have on IVF and IVC. Therefore, Exp. 5 was performed using only significant treatments that elicited high pmol of GSH per oocyte per well. The treatment groups selected were 3.3 m*M* cysteine, 1.0 m*M* L-glutatate, 3.3 m*M* L-α-aminobutyrate, and 3.3 m*M* L-α-aminobutyrate and 25µ*M* β-mercaptoethanol. Although not significantly eliciting the highest increase in GSH per oocyte per well, the 3.3 m*M* cysteine treatment group was selected for two reasons: 1) the values considered

were those obtained from the fourth experiment to reduce the chance of experimental error, and 2) the comparison between cysteine and L-α-aminobutyrate needs to be analyzed throughout the *in vitro* production system. This was the reasoning behind selecting only L-α-aminobutyrate as a treatment group instead of other significant treatments.

In conclusion, many γ-glutamyl compounds when supplemented to IVM medium increase the intracellular concentrations of GSH with exogenous supplementation of L-α-aminobutyrate and β-mercaptoethanol eliciting the largest response (14.757 ± 0.05) compared to all other treatments. It has been shown that the higher the levels of intracellular GSH produced, the greater the success of IVF in respect to a low incidence of polyspermy (Sawai et al., 1997), and the greater the success of IVC in respect to the 2-cell and blastocyst developmental stage (Abeydeera et al., 1999). The effects of these treatment groups on IVF and IVC need to be analyzed as well.

CHAPTER IV

EXOGENOUS γ-GLUTAMYL CYCLE COMPOUNDS INFLUENCE *IN VITRO* FERTILIZATION AND CULTURE PARAMETERS IN PORCINE OOCYTES

Introduction

In order to obtain large quantities of embryos, oocytes must not only successfully complete IVM, but also IVF and IVC as well.

Treatments from the previous experiments were selected based on levels of intracellular GSH observed. These treatments were then applied to IVM, IVF, and IVC to determine if the treatments had any significant effects on IVF and IVC parameters.

Treatments demonstrating high levels of intracellular GSH content were used because high levels of GSH help promote a MPN (Yoshida et al., 1993), and have beneficial effects on porcine embryo development (Abeydeera et al., 1998).

Yoshida et al. (1993) concluded that sufficient GSH within the oocyte is crucial in aiding in spermatozoa decondensation and transformation from the spermatozoan head into the MPN. A lack of GSH causes the spermatozoan head not to decondense in synchrony with the oocyte and fertilization fails to occur.

In addition to the problems with IVF, a block in the development of embryos has been reported (Sato, 1997). This block occurs after cleavage into the 2-cell embryo and may be a result of the surrounding environments. A rise in GSH during IVM may reduce this developmental block during IVC.

Using specific treatments shown to increase GSH based upon the previous four experiments, the specific objectives of this experiment were to:

1) Determine the effects of the treatments on IVF parameters:
   a. % of oocytes that were penetrated by spermatozoa
   b. % of oocytes that were polyspermic
   c. % of oocytes that were undergoing MPN formation
2) Determine the effects of the treatments on IVC parameters:
   a. % of embryos that were cleaved after 48 h post-IVF.

b. % of embryos that were cleaved/hatched after 144 h post-IVF.

Materials and Methods

Treatment Groups. The treatment groups were as follows: treatment 1 = NCSU 23 maturation medium (CONTROL), treatment 2 = NCSU 23 maturation medium supplemented with 3.3 m*M* cysteine (CYS); treatment 3 = NCSU 23 maturation medium supplemented with 1.0 m*M* L-glutamate (GLU); treatment 4 = NCSU 23 maturation medium supplemented with 3.3 m*M* L-α-aminobutyrate (aAB); treatment 5 = NCSU 23 maturation medium supplemented with 3.3 m*M* L-α-aminobutyrate and 25 μ*M* β-mercaptoethanol (aAB+BME). Each treatment was repeated three times in order to obtain 70 to 75 oocytes total per treatment.

General. Experimentation was conducted during the winter of 2002. All ovaries were obtained from sows and gilts slaughtered at a local abbatoir in Goode, Virginia. The ovaries used were determined to be normal using visual observation and contained no cysts, blood clots, or discoloration. Ovaries were initially rinsed twice in 21°C 8.75% physiological saline and once in m-D-PBS containing 10,000 units/mL Penicillin G sodium and 10,000 μg/mL streptomycin sulfate. Ovaries were then stored in 21°C m-D-PBS and transported to the laboratory in an insulated cooler. The elapsed time between ovary collection and follicular aspiration ranged between 2 to 3 h.

Oocyte Recovery. Once at the laboratory, the ovaries were washed three times in m-D-PBS and medium sized follicles (3 to 6 mm in diameter) were aspirated using an 18-guage needle fixed to a 10-mL disposable syringe. The aspirated fluid was placed into a 50 mL polypropylene conical tube (Fisher Scientific, Pittsburgh, PA). After the oocytes settled for 15 min, the supernatant was discarded so that the solid contents remained in the tube and 25 mL of mTL-HEPES–PVA medium at 39°C was added to wash the pellet. The wash procedure was repeated for a total of three times and then collected into 100 x 35 mm^2 polystyrene sterile culture dishes (Fisher Scientific, Pittsburgh, PA).

IVM. Only oocytes surrounded by a compact cumulus cell mass and uniform ooplasm were utilized. The selected oocytes were washed three times in a 50 x 9mm^2 Falcon polystyrene dish (Fischer Scientific, Pittsburgh, PA) using 100 μL drops of NCSU 23 maturation medium. Then oocytes were randomly and equally assigned to treatment

groups and placed into separate wells of a Nunclon 6-well multidish (Fisher Scientific, Pittsburgh, PA) previously equilibrated containing 500 µL of maturation medium and supplements under mineral oil.

After the oocytes were placed into the assigned wells, 0.0005 units/mL FSH, 0.000625 units/mL LH, and 10 ng/mL EGF were added to each well and mixed. The oocytes were incubated at 39°C in an atmosphere of 5% CO_2 for 40-48 h.

IVF. After incubation, cumulus cells were removed by mixing oocytes with 0.1% hyaluronidase in NCSU 23 solution for 15 to 30 s. Oocytes were then washed three times in a 50 x 9 mm^2 Falcon polystyrene dish in 100 µL drops of Tris-fertilization medium and stored in 50 µL drops of Tris-fertilization medium under mineral oil.

A frozen semen straw (Swine Genetics, International, Ltd., Cambridge, IA) was then thawed in 8 mL of fertilization D-PBS at 40°C in a 50 mL polypropylene conical tube and centrifuged at 73 X *g* for 5 min to remove dead spermatozoa. The supernatant was poured into a new tube and fertilization D-PBS was added to bring the volume to 15 mL and centrifuged at 1052 X *g* for 5 min to collect viable spermatozoa. The supernatant was then discarded and the pellet was washed once more as describe above. Following the wash, the supernatant was discarded and 1 mL of Tris-fertilization medium was added to the pellet. The number of spermatozoa were counted using a Bright-Line hemocytometer (Fisher Scientific, Pittsburgh, PA). The spermatozoa were diluted with Tris-fertilization medium so that the final concentration was 1 x 10^6 spermatozoa/mL. Then 50 µL of spermatozoa were added to each well, mixed, and the oocytes and spermamtozoa were coincubated at 39°C in an atmosphere of 5% CO_2 for 6 to 10 h.

IVF Parameter Evaluation. At the end of IVF, 5 to 15 ootids from each treatment group were washed three times in 100 µL drops of D-PBS then placed in 110 µL of D-PBS containing 10 µL of 1 mg/mL BisBenzimide H 33342 (Hoechst 33342) stain. After 10 min of staining, the oocytes were destained in D-PBS for 5 min and examined under a fluorescent microscope. Hoechst 33342 is a membrane permeable compound that intercalates in the adenosine – tyrosine regions of DNA and is visible using fluorescence microscopy (excitation maximum wavelength = 346 nm and emission maximum wavelength = 460 nm) so nuclear material can be quantified (nuclei) (Latt and Stetten,

1976; Crissmann and Steinkamp, 1987). Oocytes were characterized as whether or not they were penetrated, polyspermic, or undergoing male pronuclear formation.

IVC. The remaining zygotes were placed in separate wells of a Nunclon 6-well multidish after being washed three times in 100 μL of NCSU 23 culture medium and incubated at 39°C in an atmosphere of 5% CO_2 in 500 μL of culture medium under mineral oil. After 48 h post-IVF, embryos were placed in fresh NCSU 23 culture media in the same manner as described above. Cleavage was evaluated and recorded after 48 h and 144 h post-fertilization. Figure 8 shows a timeline of events for Exp. 5.

Statistical Analyses. Data analyses were conducted using the general linear model (GLM) procedure in Statistical Analysis System (SAS, 2001). *In vitro* fertilization data were analyzed as a complete randomized design with spermatozoa penetration (+/-) (PENET), polyspermic fertilization (+/-) (POLY), and male pronuclear formation (+/-) (MPN) as the dependant variables and treatment, replicate, and the interaction between treatment and replicate as the independent variables. Significance between treatment groups was analyzed using the least-square means (LSMEANS) statement with the possible probability values (PDIFF) options.

The IVC data were analyzed as a complete randomized design with the observation of the 2-cell embryo after 48 h (+/-) (2-CELL) and formation of a blastocyst after 144 h (+/-) (BLAST) as the dependant variables and treatment, replicate, and the interaction between treatment and replicate as the independent variables. Significance between treatment groups was analyzed using the least-square means (LSMEANS) statement with the possible probability values (PDIFF) options.

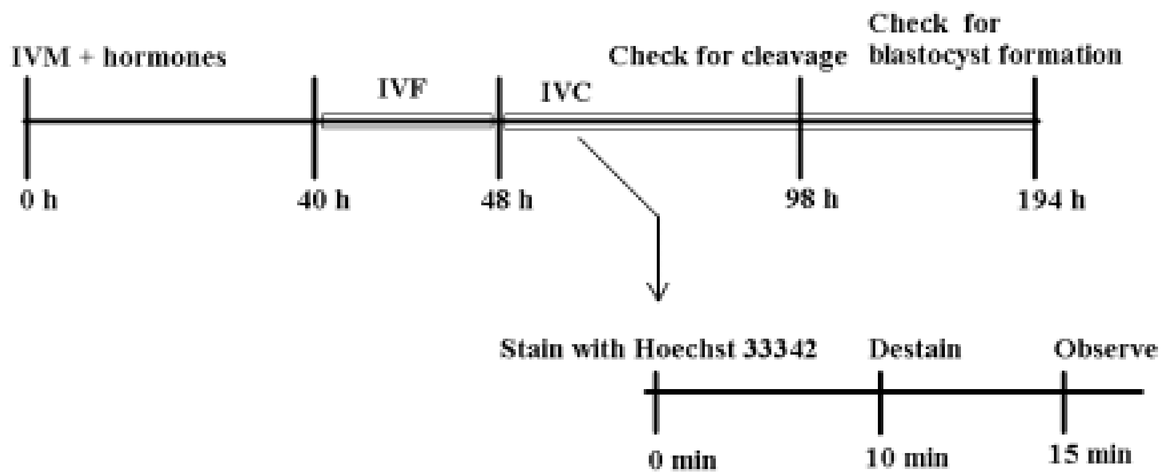


Figure 8. Timeline of Events for Exp. 5. After being randomly assigned to different treatment groups, all oocytes were subjected to identical hormonal supplementation of 0.0005 units/mL FSH, 0.000625 units/mL LH, and 10 ng/mL epidermal growth factor and identical *in vitro* maturation (IVM) environments of 39°C in an atmosphere of 5% CO_2 at 0 h. Between 40 to 48 h post-hormone administration, oocytes were mixed with 0.1% hyaluronidase for 15 to 30 s to remove the cumulus cells and fertilized with 1 x 10^6 spermatozoa/mL in Tris-fertilization medium for 6 to 10 h. After *in vitro* fertilization (IVF), 5 to 15 ootids were stained with Hoechst 33342 and examined for spermatozoa penetration, polyspermy, and male pronuclear formation under a fluorescent microscope. The remaining zygotes were subject to identical *in vitro* culture (IVC) for 144 h post-IVF and examined for cleavage at 48 h and blastocyst formation at 144 h post-IVF.

Experimental Results

This experiment utilized a total 371 oocytes split between 5 treatment groups and the IVF and IVC parameter evaluations. The IVF analyses used 125 oocytes over 5 treatment groups. There was no significant effect of replication or treatment on the IVF parameter PENE (Table 9).

There was no significant effect of replication on the IVF parameter POLY (Table 10). The aAB + BME treatment group had a significantly lower ($P < 0.05$) incidence of polyspermy (31.82%) compared to all other treatment groups except for the aAB treatment group (Figure 9).

There was no significant effect of replication on the IVF parameter MPN (Table 11). The aAB + BME treatment group had a significantly higher ($P < 0.05$) incidence of MPN formation (81.82%) compared to all other treatment groups (Figure 10).

The IVC analyses used 246 embryos over 5 treatment groups. There was no significant effect of replication on the IVC parameter 2CELL (Table 12). The aAB + BME treatment group had a significantly higher ($P < 0.05$) percentage of embryos reaching the 2-cell developmental stage by 48 h post-IVF (48.98%) compared to all other treatment groups (Figure 11).

There was no significant effect of replication on the IVC parameter BLAST (Table 13). The aAB + BME treatment group had a significantly higher ($P < 0.05$) percentage of embryos reaching the blastocyst stage of development by 144 h post-IVF (69.39%) compared to all other treatment groups (Figure 12).

Table 9. The overall analysis of variance for replication and treatment effects on *in vitro* fertilization parameter spermatozoa penetration (n=125).

Source	df	Mean Square	P < 0.05
Treatment	4	0.025	
Replicate	2	0.139	
Treatment*Replicate	8	0.063	
Error	110	0.058	
Total	124	0.285	

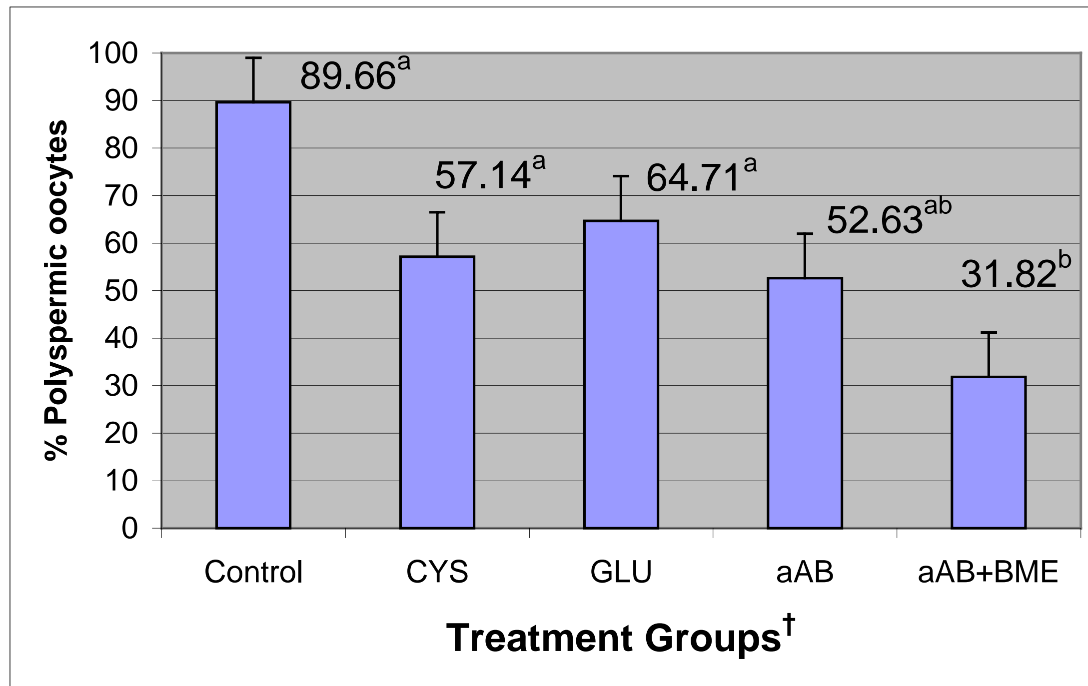
Using type III means squared.

Table 10. The overall analysis of variance for replication and treatment effects on *in vitro* fertilization parameter polyspermy (n=125).

Source	df	Mean Square	P < 0.05
Treatment	4	1.138	*
Replicate	2	0.293	
Treatment*Replicate	8	0.361	
Error	110	0.198	
Total	124	1.99	

Using type III means squared.

Figure 9. Percent of polyspermic oocytes after *in vitro* fertilization determined by Hoechst 33342 staining, comparing between treatment groups (n=125).



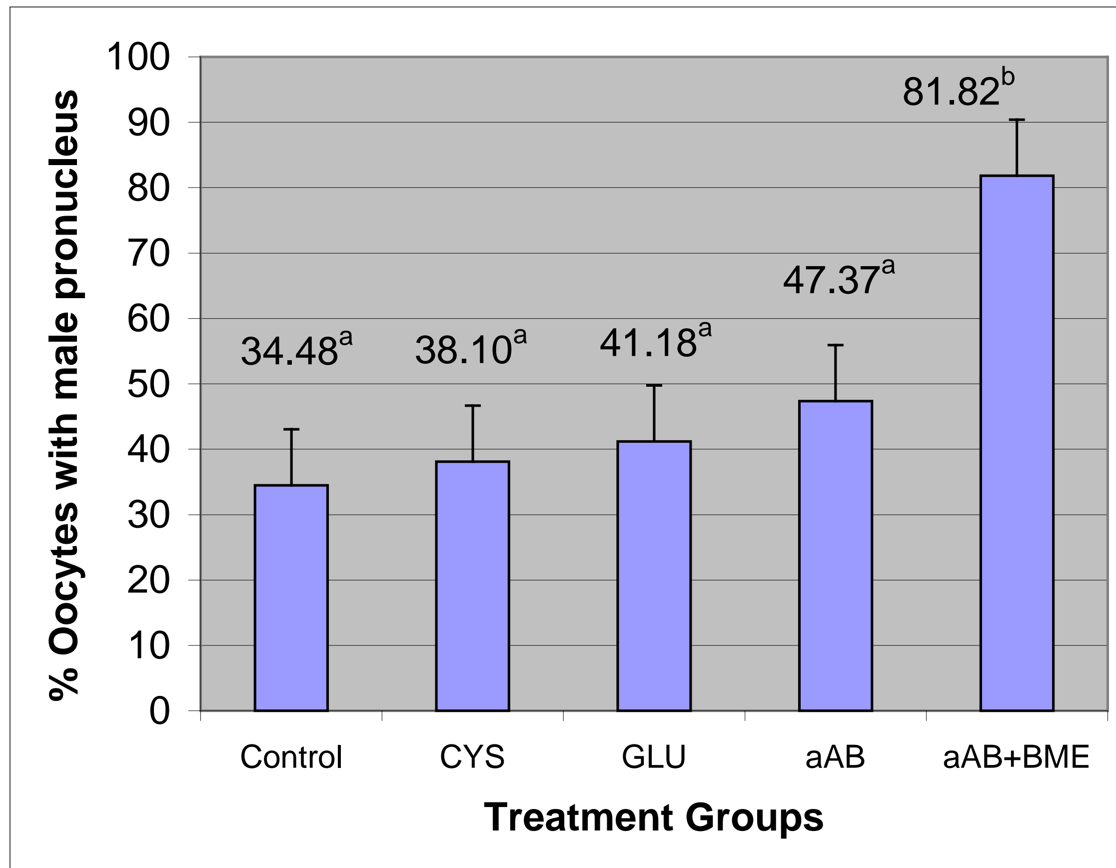
† Treatments include NCSU 23 maturation media (Control) (n = 29) supplemented with: 3.3 mM cysteine (CYS) (n = 21), 1.0 mM L-glutamate (GLU) (n = 34), 3.3 mM L-α-aminobutyrate (aAB) (n = 19), and aAB with 25 µM β-mercaptoethanol (BME) (n = 22).
[a,b] Columns and values with different superscripts are different ($P<0.05$)

Table 11. The overall analysis of variance for replication and treatment effects on *in vitro* fertilization parameter male pronuclear formation (n=125).

Source	df	Mean Square	P < 0.05
Treatment	4	0.878	*
Replicate	2	0.115	
Treatment*Replicate	8	0.235	
Error	110	0.234	
Total	124	1.462	

Using type III means squared.

Figure 10. Percent of oocytes with male pronucleus after *in vitro* fertilization determined by Hoechst 33342 staining, comparing between treatment groups (n=125).



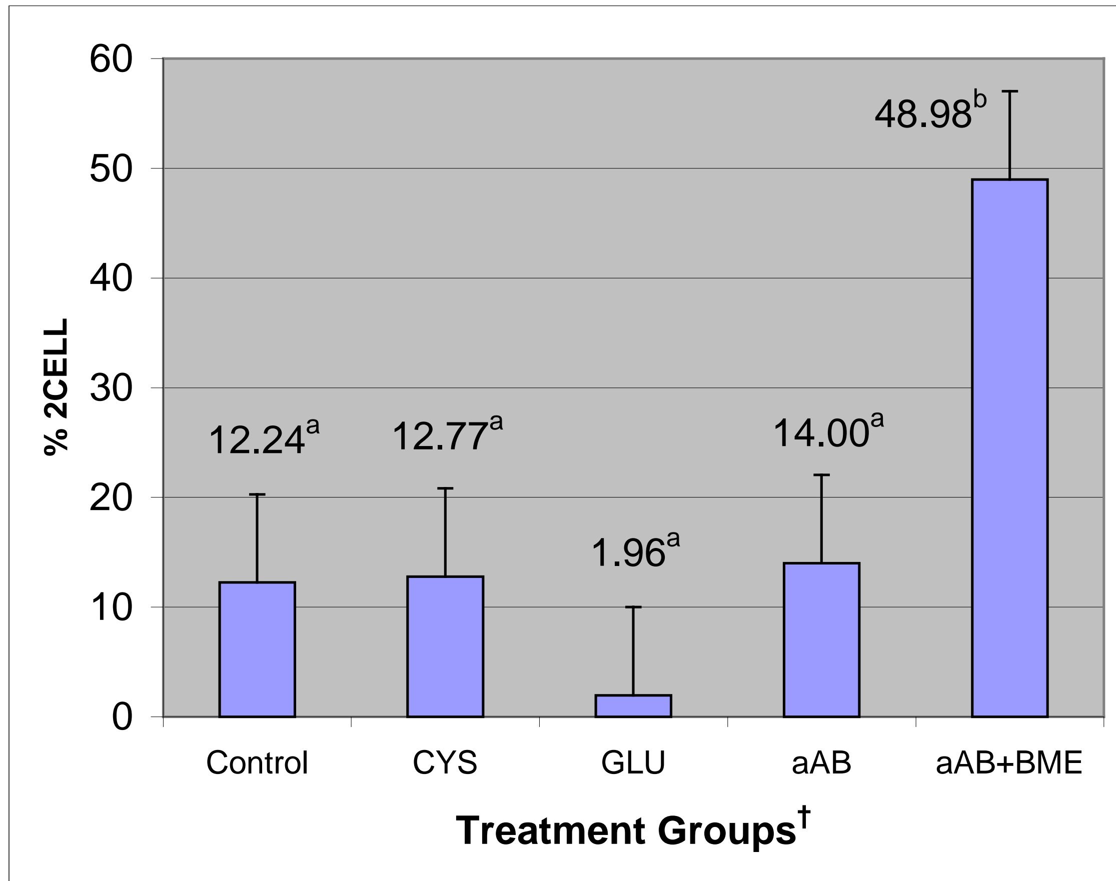
† Treatments include NCSU 23 maturation media (Control) (n = 29) supplemented with: 3.3 mM cysteine (CYS) (n = 21), 1.0 mM L-glutamate (GLU) (n = 34), 3.3 mM L-α-aminobutyrate (aAB) (n = 19), and aAB with 25 μM β-mercaptoethanol (BME) (n = 22).
[a,b] Columns and values with different superscripts are different (P<0.05)

Table 12. The overall analysis of variance for replication and treatment effects on *in vitro* culture parameter 2-cell stage of development (n=246).

Source	df	Mean Square	P < 0.05
Treatment	4	1.493	*
Replicate	2	0.100	
Treatment*Replicate	8	0.146	
Error	231	0.123	
Total	245	1.862	

Using type III means squared.

Figure 11. Percent of embryos reaching the 2-cell developmental stage (2CELL) 48 h post-*in vitro* fertilization (IVF) after *in vitro* maturation in different treatment groups and identical IVF (n=246).



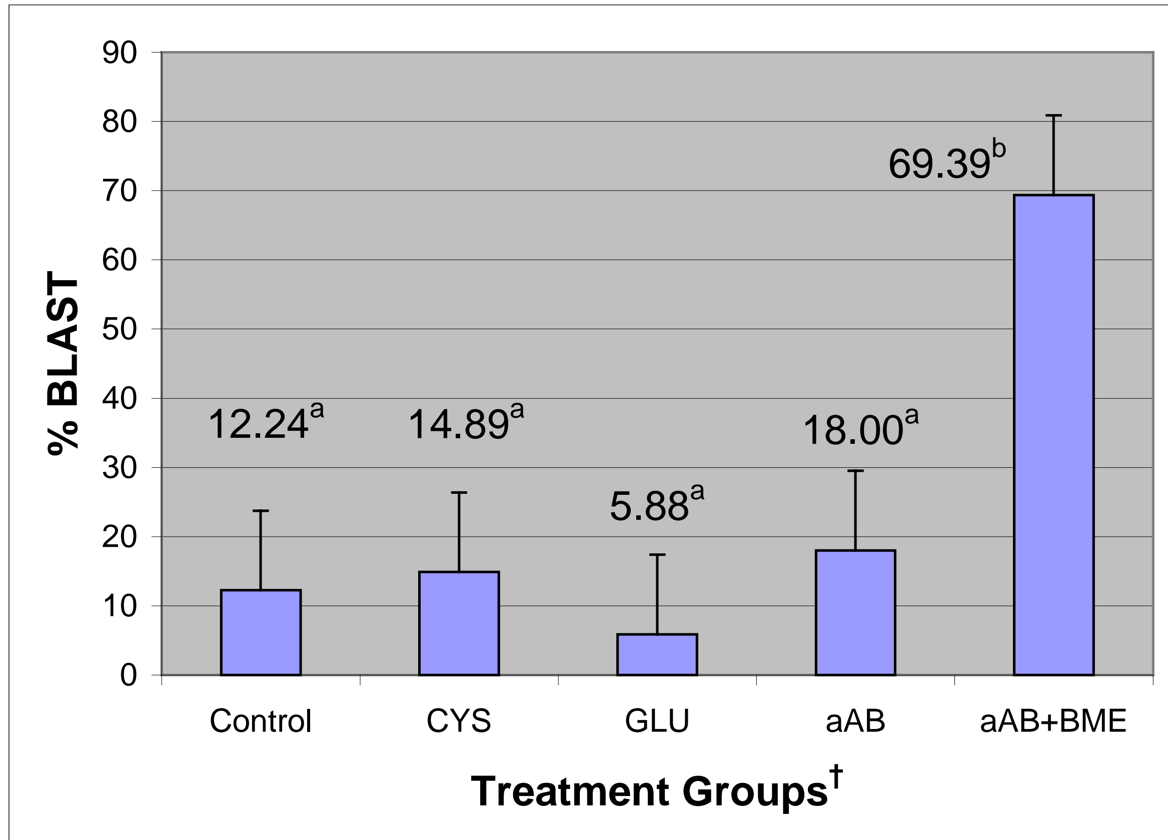
† Treatments include NCSU 23 maturation media (Control) (n = 49) supplemented with: 3.3 mM cysteine (CYS) (n = 49), 1.0 mM L-glutamate (GLU) (n = 50) , 3.3 mM L-α-aminobutyrate (aAB) (n = 49), and aAB with 25 μM β-mercaptoethanol (BME) (n = 49).
[a,b] Columns and values with different superscripts are different (P<0.05)

Table 13. The overall analysis of variance for replication and treatment effects on *in vitro* culture parameter blastocyst stage of development (n=246).

Source	df	Mean Square	P < 0.05
Treatment	4	2.958	*
Replicate	2	0.085	
Treatment*Replicate	8	0.116	
Error	231	0.133	
Total	245	3.292	

Using type III means squared.

Figure 12. Percent of embryos reaching the blastocyst stage (BLAST) by 144 h post-*in vitro* fertilization (IVF) after *in vitro* maturation in different treatment groups and identical IVF (n=246).



† Treatments include NCSU 23 maturation media (Control) (n = 49) supplemented with: 3.3 mM cysteine (CYS) (n = 49), 1.0 mM L-glutamate (GLU) (n = 50), 3.3 mM L-α-aminobutyrate (aAB) (n = 49), and aAB with 25 μM β-mercaptoethanol (BME) (n = 49).
[a,b] Columns and values with different superscripts are different ($P<0.05$)

Discussion

Oocytes demonstrating significantly high concentrations of intracellular GSH significantly reduced the incidence of polyspermy and increased the formation of a MPN (Yoshida et al., 1993). Sufficient GSH within the oocyte is crucial in aiding spermatozoa decondensation and transformation of the spermatozoan head into the MPN (Yoshida et al., 1993). In subsequent IVC, these oocytes undergo embryo development at a significantly faster rate and have a higher success rate (Abeydeera et al., 1998). The 2-cell embryo block is a reoccuring problem in porcine IVC (Sato, 1997) and may be reduced with an increase in intracellular GSH. In order to increase the intracellular levels of GSH during IVM, the following treatment groups were selected: 3.3 m*M* cysteine, 1.0 m*M* L-glutatate, 3.3 m*M* L-α-aminobutyrate, and 3.3 m*M* L-α-aminobutyrate and 25μ*M* β-mercaptoethanol. These supplements were based on previous results (see Exp. 1, 2, 3, and 4).

For IVF, IVM medium supplemented with 3.3 m*M* L-α-aminobutyrate and 25μ*M* β-mercaptoethanol for 40 to 48 h had no significant effect on the penetration rate of spermatozoa into the oocyte during IVF. The supplementation of 3.3 m*M* L-α-aminobutyrate and 25μ*M* β-mercaptoethanol for 40 to 48 h during IVM did significantly ($P < 0.05$) reduce the incidence of polyspermic penetration of the oocytes (31.85%) compared to the CONTROL (89.66%), CYS (57.14%), and GLU (64.71%). In addition, the 3.3 m*M* L-α-aminobutyrate and 25μ*M* β-mercaptoethanol supplementation for 40 to 48 h during IVM significantly increased ($P < 0.05$) the MPN formation after IVF (81.82%) compared to the CONTROL (34.48%), CYS (38.10%), GLU (41.18%), and aAB (47.37%). These results are in agreement with studies that show higher levels of GSH are beneficial for IVF of porcine oocytes (Yoshida et al., 1993).

For IVC, IVM medium supplemented with 3.3 m*M* L-α-aminobutyrate and 25μ*M* β-mercaptoethanol for 40 to 48 h had a significant ($P < 0.05$) effect on the percent of embryos reaching the 2 cell stage of development by 48 h post –IVF (48.98%) compared to the CONTROL (12.24%), CYS (12.77%), GLU (1.96%), and aAB (14.00%). Later on during IVC, the IVM medium supplemented with 3.3 m*M* L-α-aminobutyrate and 25μ*M* β-mercaptoethanol for 40 to 48 h had a significant ($P < 0.05$) effect on the percent of

embryos reaching the blastocyst developmental stage by 144 h post –IVF (69.39%) compared to the CONTROL (12.24%), CYS (14.89%), GLU (5.88%), and aAB (18.00%) treatments. These results are in agreement with studies that show higher levels of GSH are beneficial for embryo development of porcine oocytes (Abeydeera et al., 1998).

The combination of the IVF and IVC results strengthen the idea that a high level of intracellular GSH during IVM causes a significantly greater ($P < 0.05$) success rate during IVF and IVC of porcine embryos. Results also indicate that suppementation of 3.3 m*M* L-α-aminobutyrate and 25μ*M* β-mercaptoethanol to the IVM medium is a viable means of increasing the success rate of porcine oocytes undergoing IVF and IVC.

CHAPTER V

EXOGENOUS L-α-AMINOBUTYRATE SUPPLEMENTED WITH β-MERCAPTOETHANOL INFLUENCE PORCINE EMBRYO VIABILITY

Introduction

When L-α-aminobutyrate (3.3mM) and β-mercaptoethanol (25 μM) are supplemented to the IVM medium, significantly ($P < 0.05$) higher levels of intracellular GSH per oocyte per well are observed compared to the control (Exp. 4). In addition to high levels of GSH, this supplementation significantly ($P < 0.05$) decreases the rate of polyspermy and increases the MPN formation, and significantly ($P < 0.05$) increases the number of embryos reaching the 2-cell stage by 48 h and blastocysts by 144 h when compared to the NCSU 23 control medium. To obtain further refinements of the *in vitro* system, a better understanding of embryo viability must be researched.

The cells comprising a porcine embryo or the embryo itself may die during development via two distinctly different mechanisms, apoptosis or necrosis (Schwartzman and Cidlowski, 1994; Vermes and Haanan, 1994). Necrosis is the pathological process that occurs when a cell encounters extremely unfavorable conditions to which it can not adapt. Apoptosis is the process in which a cell that is no longer essential or functional in its system is eliminated via an intricate and elaborate series of cascades and signals. Currently, it is thought that cell death occurs in developing embryos *in vitro* to select for viable cells and eliminate cells without function (Hardy, 1997).

Experimentation in porcine embryo cell death is just beginning to become an area of research and very little is known. One of the dilemmas encountered is differentiating between necrosis and apoptosis of an embryo. Many of the detection methods are not able to distinguish between the two. Those methods that claim to be able to distinguish between necrosis and apoptosis, are still not able to differentiate between the two in the later stages of apoptosis or necrosis. In order to implement these strategies, a narrower range of time for the onset of cell death in porcine embryos is needed.

One method observes DNA fragmentation using terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling (TUNEL) to detect apoptotic nuclei (Byrne et al., 1999). Another method is to observe general DNA damage within a cell using single cell gel electrophoresis (comet assay) (Singh et al., 1988), using the correlation that the DNA damage is related to cell death (Takahashi et al., 1999; Takahashi et al., 2000).

The annexin V assay allows identification of cell surface changes during early cell death, before necrosis and apoptosis are distinguishable (Martin et al., 1995). Phosphatidylserine (PS), a membrane bound phospholipid, flips from the inner to the outer membrane during the onset of cell death (Tait and Gibson, 1992) for macrophage recognition and removal (Fadok et al., 1992; Verhoven et al., 1995). Annexin V binds tightly (K_d 5×10^{-10} M) to the PS (Meers and Mealy, 1993) and can be easily detected and quantified using *in situ* fluorescence detection methods.

Using L-α-aminobutyrate (3.3mM) and with β-mercaptoethanol (25 μM) supplemented to the IVM medium and analysis of cell death using the AnnexinV-FITC assay, the specific objectives of this experiment were to:

1) Determine the effects of the treatment on IVC parameters
    a. % of embryos that were cleaved after 48 h post-IVF.
    b. % of embryos that were cleaved/hatched after 144 h post-IVF.
2) Determine the approximate time of cell death prior to the 2-cell embryo.
3) Determine the effects of exogenous L-α-aminobutyrate and β-mercaptoethanol on embryo viability.
4) Determine the effects of exogenous L-α-aminobutyrate and β-mercaptoethanol on the onset of cell death during IVC.

Materials and Methods

Treatment Group. The treatment groups were as follows: treatment 1 = NCSU 23 maturation medium (CONTROL), treatment 2 = NCSU 23 maturation medium supplemented with 3.3 mM L-α-aminobutyrate and 25 μM β-mercaptoethanol (aAB+BME). The treatments were repeated three times in order to obtain 150 to 175 oocytes per treatment.

General. All experimentation was conducted in the spring of 2002. All ovaries were obtained from sows and gilts slaughtered at a local abattoir in Goode, Virginia. The ovaries used were determined to be normal using visual observation and contained no cysts, blood clots, or discoloration. Ovaries were initially rinsed twice in 21°C 8.75% physiological saline and once in m-D-PBS containing 10,000 units/mL Penicillin G sodium and 10,000 µg/mL streptomycin sulfate. Ovaries were then stored in 21°C m-D-PBS and transported to the laboratory in an insulated cooler. The elapsed time between ovary collection and follicular aspiration ranged between 2 to 3 h.

Oocyte Recovery. Once at the laboratory, the ovaries were washed three times in m-D-PBS and medium sized follicles (3 to 6 mm in diameter) were aspirated using an 18-guage needle fixed to a 10-mL disposable syringe. The aspirated fluid was placed into a 50 mL polypropylene conical tube (Fisher Scientific, Pittsburgh, PA). After the oocytes settled for 15 min, the supernatant was discarded so that the solid contents remained in the tube and 25 mL of mTL-HEPES–PVA medium at 39°C was added to wash the pellet. The wash procedure was repeated for a total of three times and then collected into 100 x 35 mm^2 polystyrene sterile culture dishes (Fisher Scientific, Pittsburgh, PA).

IVM. Only oocytes surrounded by a compact cumulus cell mass and uniform ooplasm were utilized. The selected oocytes were washed three times in a 50 x 9mm^2 Falcon polystyrene dish (Fischer Scientific, Pittsburgh, PA) using 100 µL drops NCSU 23 maturation medium. Then oocytes were randomly and equally assigned to treatment groups and placed into separate wells of a Nunclon 6-well multidish (Fisher Scientific, Pittsburgh, PA) previously equilibrated containing 500 µL of maturation medium and supplements under mineral oil.

After the oocytes were placed into the assigned wells, 0.0005 units/mL FSH, 0.000625 units/mL LH, and 10 ng/mL EGF were added to each well and mixed. The oocytes were incubated at 39°C in an atmosphere of 5% CO_2 for 40 to 48 h.

IVF. After incubation, cumulus cells were removed by mixing oocytes with 0.1% hyaluronidase in NCSU 23 solution for 15 to 30 s. Oocytes were then washed three times in a 50 x 9 mm^2 Falcon polystyrene dish in 100 µL drops of Tris-fertilization medium and stored in 50 µL drops of Tris-fertilization medium under mineral oil.

A frozen semen straw (Swine Genetics, International, Ltd., Cambridge, IA) was then thawed in 8 mL of fertilization D-PBS at 40°C in a 50 mL polypropylene conical tube and centrifuged at 73 X *g* for 5 min to remove dead spermatozoa. The supernatant was poured into a new tube and fertilization D-PBS was added to bring the volume to 15 mL and centrifuged at 1052 X *g* for 5 min to collect viable spermatozoa. The supernatant was then discarded and the pellet was washed once more as describe above. Following the wash, the supernatant was discarded and 1 mL of Tris-fertilization medium was added to the pellet. The number of spermatozoa was counted using a Bright-Line hemocytometer (Fisher Scientific, Pittsburgh, PA). The spermatozoa were diluted with Tris-fertilization medium so that the final concentration was $1 x 10^6$ spermatozoa/mL. Then 50 µL of spermatozoa were added to each well, mixed, and the oocytes and spermamtozoa were coincubated at 39°C in an atmosphere of 5% CO_2 for 6 to 10 h.

IVC. The zygotes were placed in separate wells of a Nunclon 6-well multidish after being washed three times in 100 µL of NCSU 23 culture medium and incubated at 39°C in an atmosphere of 5% CO_2 in 500 µL of culture medium under mineral oil. After 48 h post-IVF, embryos were placed in fresh 500 µL drops of NCSU 23 culture media under mineral oil until 144 h post-IVF. Figure 13 shows a timeline of events for Exp. 6.

Assay Preparation. Random samples of 3 to 6 embryos from each treatment group were collected at the termination of IVF and subjected to the Annexin V-FITC assay as described in Appendix D to determine cell viability status. Prior to the assay, embryos were incubated in 0.5% Triton-X100 medium for 20 min to disrupt the zona pellucida surrounding the embryo in order for the Annexin V conjugate to reach the cell membranes. The random sampling of 3 to 6 embryos and assay were subsequently repeated every 6 h for 60 h post-IVF.

Assay Validation. Prior to performing the experiment, the Annexin V-FITC assay was validated using porcine embryos as the experimental model. A group of 50 oocytes was subject to IVM in NCSU 23, identical IVF parameters, and IVC. After 12 h of IVC, ten embryos were removed and left at 21°C for 12 h to induce cell death. After 24 h post-IVF, the ten sacrificed embryos and an additional ten embryos still in culture, were subject to the Annexin V-FITC assay as described in Appendix D. The ten sacrificed

embryos were observed to be close to 100% fluorescent, whereas the ten cultured embryos were barely or not fluorescent at all.

Statistical Analyses. Data analyses were conducted using general linear model (GLM) procedure in Statistical Analysis System (SAS, 1985). The embryo viability data for the Annexin V-FITC assays were analyzed as a complete randomized design with embryo viability score as the dependant variable and time, treatment, and the interaction between time and treatment as the independent variables. Significance between times were analyzed using the least-square means (LSMEANS) statement with the possible probability values (PDIFF) options.

Throughout the experiment, embryo viability was scored on a 5 point system determined from the observations from the assay: 0 = viable embryo/no embryonic cells fluorescing, 1 = initiation of embryo death/10% or less of embryonic cells fluorescing, 2 = up to 50% of the embryonic cells dead/up to 50% of embryonic cells fluorescing, 3 = more than half of the embryonic cells dead/50 to 80% of embryonic cells fluorescing, 4 = dead embryo/close to 100% of embryonic cells fluorescing (Figure 14).

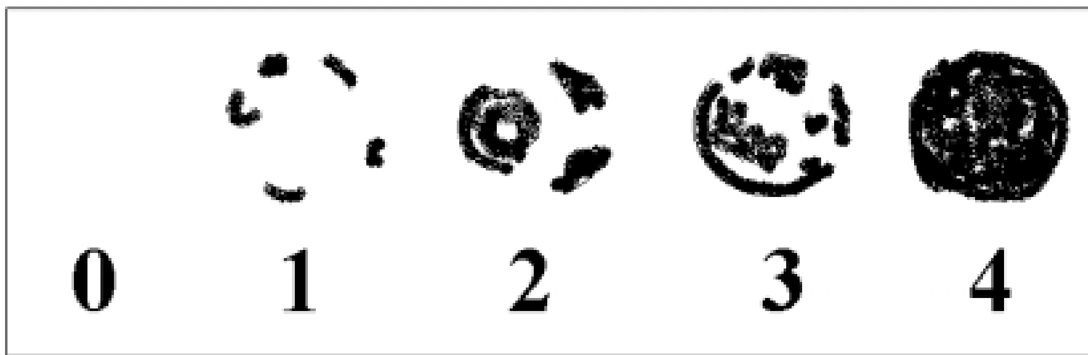


Figure 14. Embryo viability point system. Embryo viability was scored on a 5 point system determined from the observations of fluorescence from the assay: 0 = viable embryo/no embryonic cells fluorescing, 1 = initiation of embryo death/10% or less of embryonic cells fluorescing, , 2 = up to 50% of the embryonic cells dead/up to 50% of embryonic cells fluorescing, 3 = more than half of the embryonic cells dead/50 to 80% of embryonic cells fluorescing, 4 = dead embryo/close to 100% of embryonic cells fluorescing.

The IVC data were analyzed as a complete randomized design with the observation of the 2-cell embryo after 48 h (+/-) (2-CELL) and formation of a blastocyst after 144 h (+/-) (BLAST) as the dependant variables and treatment, replicate, and the interaction between treatment and replicate as the independent variable. Significance between treatment groups was analyzed using the least-square means (LSMEANS) statement with the possibility values (PDIFF) options.

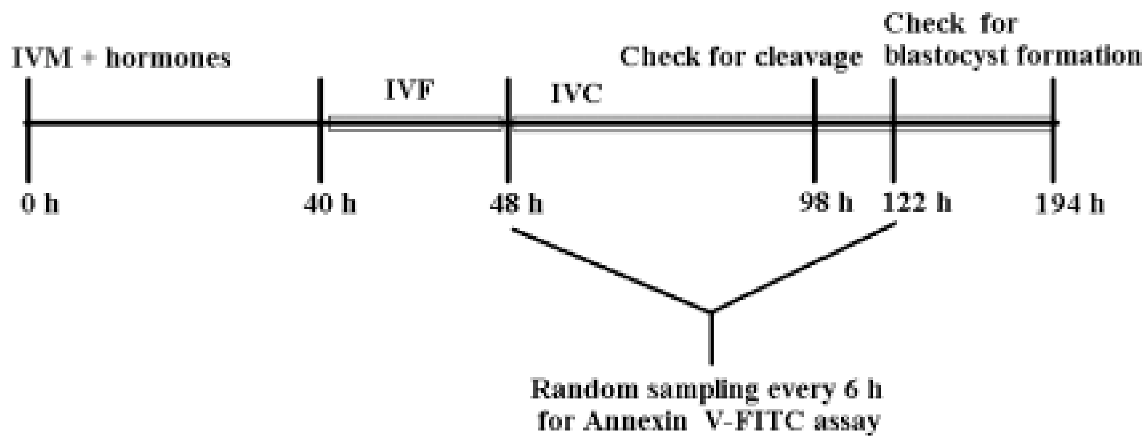


Figure 13. Timeline of Events for Exp. 6. After randomly being assigned to different treatment groups, all oocytes were subjected to identical hormonal supplementation of 0.0005 units/mL FSH, 0.000625 units/mL LH, and 10 ng/mL epidermal growth factor and identical *in vitro* maturation (IVM) environments of 39°C in an atmosphere of 5% CO_2 at 0 h. Between 40 to 48 h post-hormone administration, oocytes were mixed with 0.1% hyaluronidase for 15 to 30 s to remove the cumulus cells and fertilized with 1 x 10^6 spermatozoa/mL in Tris-fertilization medium for 6 to 10 h. After *in vitro* fertilization (IVF), embryos were subject to identical *in vitro* culture (IVC) for 144 h post-IVF and examined for cleavage at 48 h and blastocyst formation at 144 h post-IVF. Random samples of 3 to 6 embryos were taken every 6 h during IVC and examined for embryo viability using the fluorescent Annexin V-FITC assay.

Experimental Results

This experiment utilized a total of 330 embryos over 2 treatment groups and embryo viability and IVC parameter evaluation. The embryo viability analyses used 235 embryos over 2 treatment groups. There was a significant effect of time ($P<0.05$), but no significant effect of treatment on the viability score (Table 14).

The LSMEANS and STDERR of the viability score within the different sampling times are reported in Table 15 and significance ($P < 0.05$) between times in Table 16. The overall viability score between times is shown in Figure 15.

The IVC analyses used 95 embryos over 2 treatment groups. There was no significant effect of replication on the IVC parameter 2CELL (Table 17). The aAB + BME treatment groups had a significantly higher ($P < 0.05$) percentage of embryos reaching the 2 cell developmental stage by 48 h post-IVF (81.25%) compared to the CONTROL (59.57%) (Figure 16).

There was no significant effect of replication on the IVC parameter BLAST (Table 18). The aAB + BME treatment group had a significantly higher ($P < 0.05$) percentage of embryos reaching the blastocyst stage of development by 144 h post-IVF (31.25%) compared to the CONTROL (12.77%) (Figure 17).

Table 14. The overall analysis of variance for time and treatment effects on embryo viability score (n=235).

Source	df	Mean Square	P < 0.05
Time	10	7.656	*
Treatment	1	4.98	
Time*Treatment	10	0.893	
Error	213	2.146	
Total	234	15.675	

Using type III means squared.

Table 15. Least-square means (LSMEANS) ± standard error (SE) for overall viability score (0 = viable embryo, 1 = initiation of embryo death, 2 = up to 50% of the embryonic cells dead, 3 = more than half of the embryonic cells dead, 4 = dead embryo) at the different observational times post-*in vitro* fertilization (n=235).

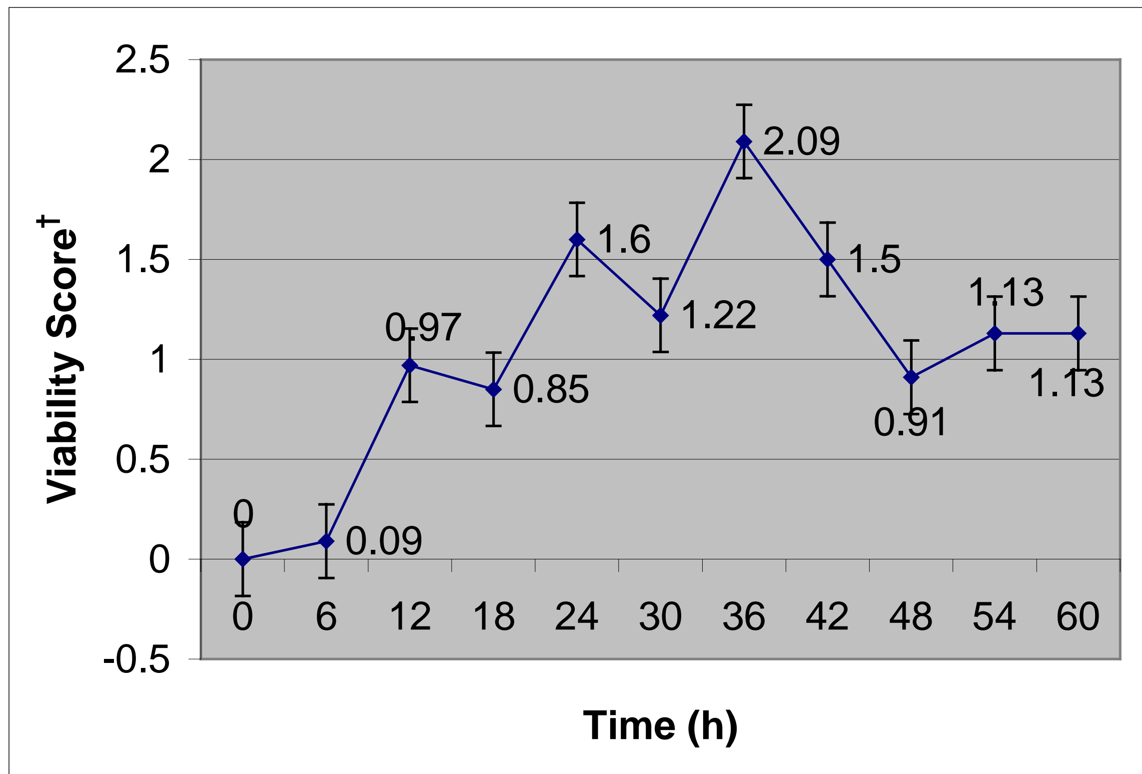
Time (h)	n	LSMEAN ± SE
0	20	0 ± 0.33
6	20	0.09 ± 0.32
12	28	0.97 ± 0.28
18	19	0.85 ± 0.33
24	16	1.60 ± 0.36
30	20	1.22 ± 0.32
36	21	2.09 ± 0.31
42	19	1.50 ± 0.33
48	21	0.91 ± 0.31
54	19	1.13 ± 0.33
60	22	1.13 ± 0.31

Table 16. Significant differences of embryo viability scores between observational times (h) post-*in vitro* fertilization (n=235).

Time (h)	0	6	12	18	24	30	36	42	48	54
6										
12		*								
18										
24	*	*								
30	*	*								
36	*	*	*	*						
42	*	*								
48	*						*			
54	*	*					*			
60	*	*					*			

* Indicates significantly different ($P < 0.05$) times.

Figure 15. Viability score of porcine embryos every 6 h post-*in vitro* fertilization up to 60 h, determined by the Annexin V-FITC assay (n= 235).



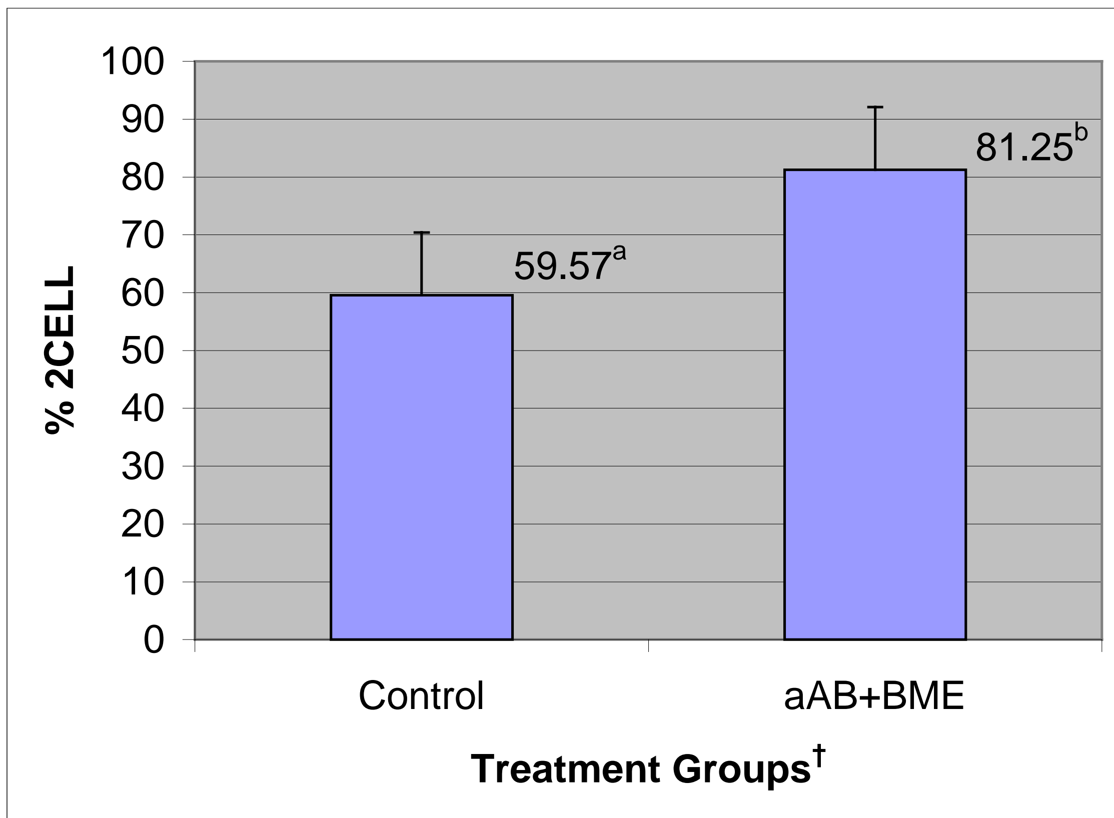
† Viability score was based on the following scale: 0 = viable embryo, 1 = initiation of embryo death, 2 = up to 50% of the embryonic cells dead, 3 = more than half of the embryonic cells dead, 4 = dead embryo.

Table 17. The overall analysis of variance for replication and treatment effects on *in vitro* culture parameter 2-cell stage of development (n=95).

Source	df	Mean Square	P < 0.05
Treatment	1	1.134	*
Replicate	2	0.560	
Treatment*Replicate	2	0.059	
Error	89	0.195	
Total	94	1.948	

Using type III means squared.

Figure 16. Percent of embryos reaching the 2-cell developmental stage (2CELL) 48 h post-*in vitro* fertilization (IVF) after *in vitro* maturation in different treatment groups and identical IVF(n=95).



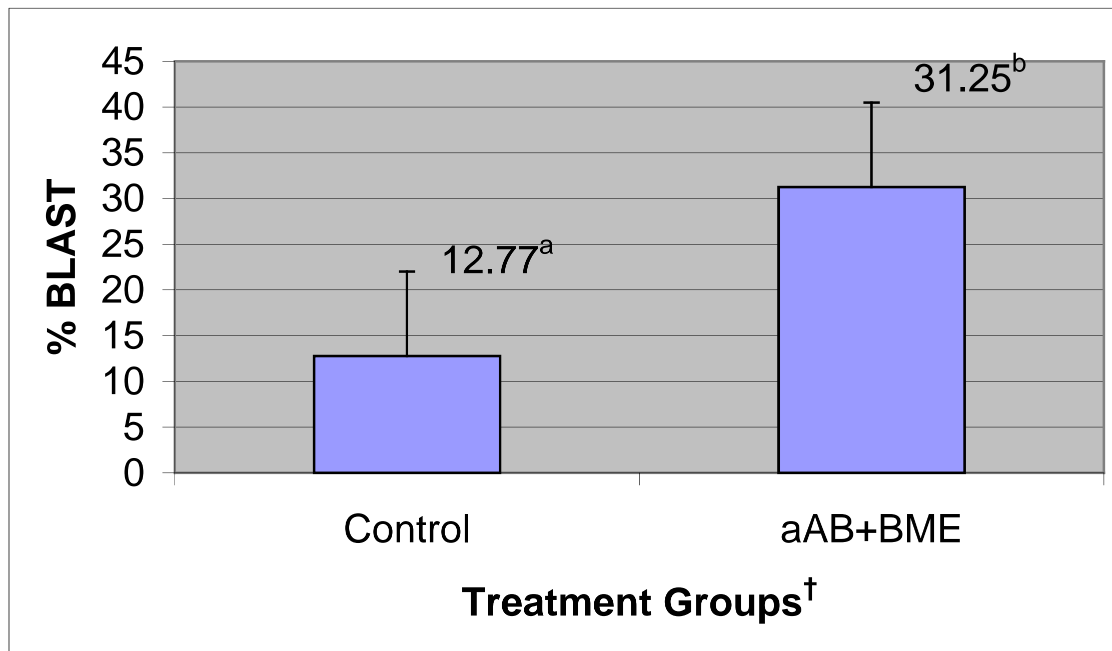
† Treatments include NCSU 23 maturation media (Control) and Control supplemented with 3.3 m*M* L-α-aminobutyrate and 25 μ*M* β-mercaptoethanol (aAB + BME).
[a,b] Columns and values with different superscripts are different (P<0.05)

Table 18. The overall analysis of variance for replication and treatment effects on *in vitro* culture parameter blastocyst stage of development (n=95).

Source	df	Mean Square	P < 0.05
Treatment	1	1.485	*
Replicate	2	1.097	
Treatment*Replicate	2	0.409	
Error	89	0.141	
Total	94	3.132	

Using type III means squared.

Figure 17. Percent of embryos reaching the blastocyst stage (BLAST) by 144 h post-*in vitro* fertilization (IVF) after *in vitro* maturation in different treatment groups and identical IVF (n=95).



† Treatments include NCSU 23 maturation media (Control) and Control supplemented with 3.3 m*M* L-α-aminobutyrate and 25 µ*M* β-mercaptoethanol (aAB + BME).
[a,b] Columns and values with different superscripts are different (P < 0.05)

Discussion

A specific problem associated with IVC is the unknown cause of a high percentage of embryo mortality. It is not uncommon for some of the embryonic cells to die during IVC development; however, if too large a portion of them die, the embryo itself may die. Embryo death can occur by either apoptosis or necrosis (Schwartzman and Cidlowski, 1994; Vermes and Haanan, 1994). Necrosis is the pathological process that occurs when a cell encounters extremely unfavorable conditions to which it can not adapt. Apoptosis is the process in which a cell that is no longer essential or functional in its system is eliminated via an intricate and elaborate series of cascades and signals. Currently, it is thought that cell death occurs in developing embryos *in vitro* to select for viable cells and eliminate cells without function (Hardy, 1997).

If embryonic cell death can be retarded or inhibited, there may be an increase in viable embryos at the end of IVC. Using compounds that have increased the success rate of IVM, IVF, and IVC might have beneficial effects on reducing embryonic cell mortality.

IVM medium supplemented with 3.3 m*M* L-α-aminobutyrate and 25μ*M* β-mercaptoethanol has been shown to have beneficial effects on the embryo during IVF and IVC (Exp. 5). However, the IVM medium supplemented with 3.3 m*M* L-α-aminobutyrate and 25μ*M* β-mercaptoethanol had no significant effect on embryo viability during IVC. This indicates that supplementation to the IVM medium has no effect on subsequent embryo viability during IVC. This supports the idea that if an embryo was apoptotic, it was selected to undergo apoptosis prior to embryonic development (Hardy, 1997). Once an embryo is destined to undergo apoptosis, there is nothing that can be done to reverse the process.

Death of an embryo will occur prior to the first cleavage into the 2-cell stage of development (Sato, 1997). At 36 h post-IVF, the viability score peaked at 2.09 ± 0.31 indicating that over 50% of the embryonic cells were dead. The only other times that were not significantly less than an embryo viability score of 2.09 ± 0.31 were at 24 h (1.6 ± 0.36), 30 h (1.2 ± 0.32), and 42 h (1.5 ± 0.33) post-IVF. These experimental results indicate that the time of embryonic cell death occurs most often during 24 to 42 h post-IVF with the greatest occurrence around 36 h.

The IVC analysis was performed again to ensure repeatability of results from previous experiments (Exp. 5). Embryos supplemented during IVM with 3.3 m*M* L-α-aminobutyrate and 25μ*M* β-mercaptoethanol had a significantly greater ($P < 0.05$) percentage reaching the 2-cell developmental stage by 48 h post-IVF (81.25% vs. 59.57% for the controls) and significantly greater ($P < 0.05$) percentage reaching the blastocyst developmental stage by 144 h post-IVF (31.25% vs. 12.77% for the controls) when compared to the NCSU 23 control medium. This is in agreement with previous results (Exp. 5).

The embryo viability results indicate that supplementation of L-α-aminobutyrate and β-mercaptoethanol, or perhaps any substance to the IVM medium does not change embryo viability during IVC. However, the time at which embryo cell death occurs prior to the 2-cell developmental stage is between 24 to 42 h post-IVF with the maximum incidence occurring at 36 h. The IVC results strengthen the idea that supplementation of 3.3 m*M* L-α-aminobutyrate and 25μ*M* β-mercaptoethanol to the IVM medium is one viable solution to increase the success rate of porcine oocytes undergoing IVC.

CHAPTER VI

SUMMARY

The ability to produce large quantities of viable *in vitro* derived porcine embryos has been the focus of intense research for the past 20 yr. The availability of large quantities of embryos would aid research progress in numerous areas including human IVF, embryology, and transgenic animal production. Currently the production system has many shortcomings including poor IVM, polyspermic IVF, and a low rate of blastocyst formation during IVC. When *in vitro* derived embryos are transplanted into recipient sows/gilts the offspring, though viable, have a slow rate of development and growth (Abeydeera, 2002).

Many γ-glutamyl compounds when supplemented to IVM medium increase the intracellular concentrations of GSH. The supplementation of exogenous 3.3 m*M* L-α-aminobutyrate and 25 μ*M* β-mercaptoethanol to NCSU 23 elicited the most significant increase ($P < 0.05$) of pmol GSH per oocyte per well (Exp. 4). The higher the levels of intracellular GSH produced, the greater the success of IVF in respect to a significantly ($P< 0.05$) lower incidence of polyspermy and higher incidence of MPN formation, and the greater the success of IVC in respect to the 2-cell and blastocyst developmental stages (Exp. 5). Exogenous supplementation of L-α-aminobutyrate and β-mercaptoethanol, or perhaps any substance to the IVM medium does not significantly change embryo viability during IVC (Exp. 6). However, the time at which embryonic cell death occurs prior to the 2-cell developmental stage is between 24 to 42 h post-IVF with the greatest incidence occurring at 36 h post-IVF (Exp. 6).

IMPLICATIONS

In order to increase the success of *in vitro* derived porcine embryos and offspring, the basic fundamentals of *in vitro* procedures need to be completely understood. The precise roles of GSH within the developing oocyte needs to be determined as well as its role during IVF and IVC. The kinetics of the amino acid transporters needs to be understood to maximize nutrient transport into the developing oocyte and embryo. The precise time of embryo death needs to be isolated in order to differentiate between necrosis and apoptosis before research can be done to prevent both. When methods are found to reduce necrosis and apoptosis, the success of obtaining viable embryos will increase dramatically. However, this needs to be done in a systematic manner so that the reasons for and understanding behind why the system is successful is fully elucidated.

CHAPTER VII

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APPENDIX A

The following reagents and media used throughout this thesis are as follows:

Water

All water was Type I reagent grade from a Megapure system (Corning, Corning, NY).

Filter

All filtering was done using a 0.2 µm pore HT Tuffryn Membrane Acrodisc disposable syringe filter (Fisher Scientific, Pittsburgh, PA).

Mineral Oil

All mineral oil used was light mineral oil, embryo stem cell qualified (Specialty Media, Phillipsburg, NJ) and filtered.

Dulbecco's-Phosphate Buffered Saline Solution (D-PBS)

Constituent	m*M*
KCl	2.68
KH_2PO_4	1.47
$MgCl_2 \bullet 6H_2O$	0.49
NaCl	136.89
Na_2HPO_4	9.53

Adjust the pH between 7.3 and 7.4 and store at 4°C.

Modified TL-HEPES-PVA Medium (mTL-HEPES-PVA)

Constituent	m*M*
NaCl	114
KCl	3.2
NaH_2PO_4	0.34
Na Lactate (60% syrup, w/w)	10
$MgCl_2 \bullet 6H_2O$	0.5
HEPES	10
Na pyruvate	0.2
Sorbitol	12
$NaHCO_3$	2
Gentamicin	0.0250 g/L
Penicillin G	0.0650 g/L
PVA	0.1000 g/L

Add 2.0 m*M* $CaCl \bullet 2H_2O$, adjust the pH between 7.3 and 7.4, filter, and store at 4°C.

BSA-free NCSU 23 Medium – IVM medium

Constituent	m*M*
NaCl	108.73
KCl	4.78
KH_2PO_4	1.19
$MgSO_4 \bullet 7H_2O$	1.19
D-glucose	5.55
Glutamine	1.00
Taurine	7.00
Hypotaurine	5.00
$NaHCO_3$	25.07
$CaCl_2 \bullet 2H_2O$	1.70
Penicillin G	75 µg/mL
Streptomycin	50 µg/mL

Adjust the pH between 7.3 and 7.4, filter, and store at 4°C.

NCSU 23 Medium – IVC medium

Add 4 mg/mL of BSA to BSA-free NCSU 23 maturation medium. After the BSA dissolves, filter and place in the incubator at 5% CO_2 and 39°C to equilibrate.

Tris-Fertilization Medium

Constituent	mM
NaCl	113.1
KCl	3.0
$CaCl_2 \bullet 2H_2O$	7.5
Tris	20.0
D(+)-glucose	11.0
Na pyruvate	5.0

Add BSA at a concentration of 1 mg/mL and caffeine at a concentration of 0.38 mg/mL. Filter and place in the incubator at 5% CO_2 and 39°C to equilibrate.

Fertilization D-PBS

Add 0.10 g/L $CaCl_2$ to D-PBS. Adjust the pH between 7.2 and 7.3, filter, then add BSA at a concentration of 1mg/mL and place in the incubator at 5% CO_2 and 39°C to equilibrate.

0.2 M Sodium Phosphate Buffer, 10 mM EDTA, pH 7.2

Constituent	Molarity
$NaH_2PO_4 \bullet H_2O$	0.1 M
Na_2HPO_4	0.1 M
Na4 – EDTA	10 mM

Adjust the pH to 7.2, filter, and store at 4°C.

APPENDIX B

Procedure for DTNB – GSSG reductase recycling assay.

The following reagents were used in this assay:

A. Stock Buffer (0.2 *M* sodium phosphate, 10 m*M* Na_4-EDTA, pH 7.2)

1. Place 28.392 g Na_2HPO_4 in 1000 mL beaker.
2. Add 27.5980 g $NaH_2PO_4 \bullet H_2O$.
3. Add 3.7220 g Na_4-EDTA.
4. Dissolve in 1000 mL water.
5. Adjust pH to 7.2.
6. Store at RT.

B. Daily Buffer

1. Dissolve in stock buffer, NADPH at a concentration of 0.300 mg/mL.
2. Prepare daily and store at 4°C.

C. DTNB solution (6 mM 5,5'-dithio-bis(2-nitrobenzoic acid))

1. Place 2.3778 g DTNB in 1000 mL beaker.
2. Add 1000 mL water.
3. Aliquot in 10 ml tubes.
4. Store frozen at 0°C.

D. Glutathione reductase solution (250 units/mL)

1. Dissolve in stock buffer, GSSG reductase at a concentration of 250 units/mL.
2. Store at 4°C.

E. GSH stock standards (0.100 *M* GSH)

1. Dissolve 0.3073 g GSH in 10 mL water.
2. Prepare weekly and store frozen.

The assay was preformed in the following manner:

A. In a cuvette place 700 μL of daily buffer.
B. Add 100 μL of DTNB solution.
C. Add water in the amount of [200 μL – sample volume].
D. Add the sample with mixing.
E. Add 10 μL of glutathione reductase solution to cuvettes and mix quickly.
F. Follow formation of TNB (5-thio-2-nitrobenzoic acid) at 412 nm and record the spectrophotometer reading every 10 s for a total of 10 min.
G. Determine amount of GSH from standard curves obtained from each GSH standard curve plotted against the rate of change in absorbance.

APPENDIX C

The standard curve was determined in the following manner:

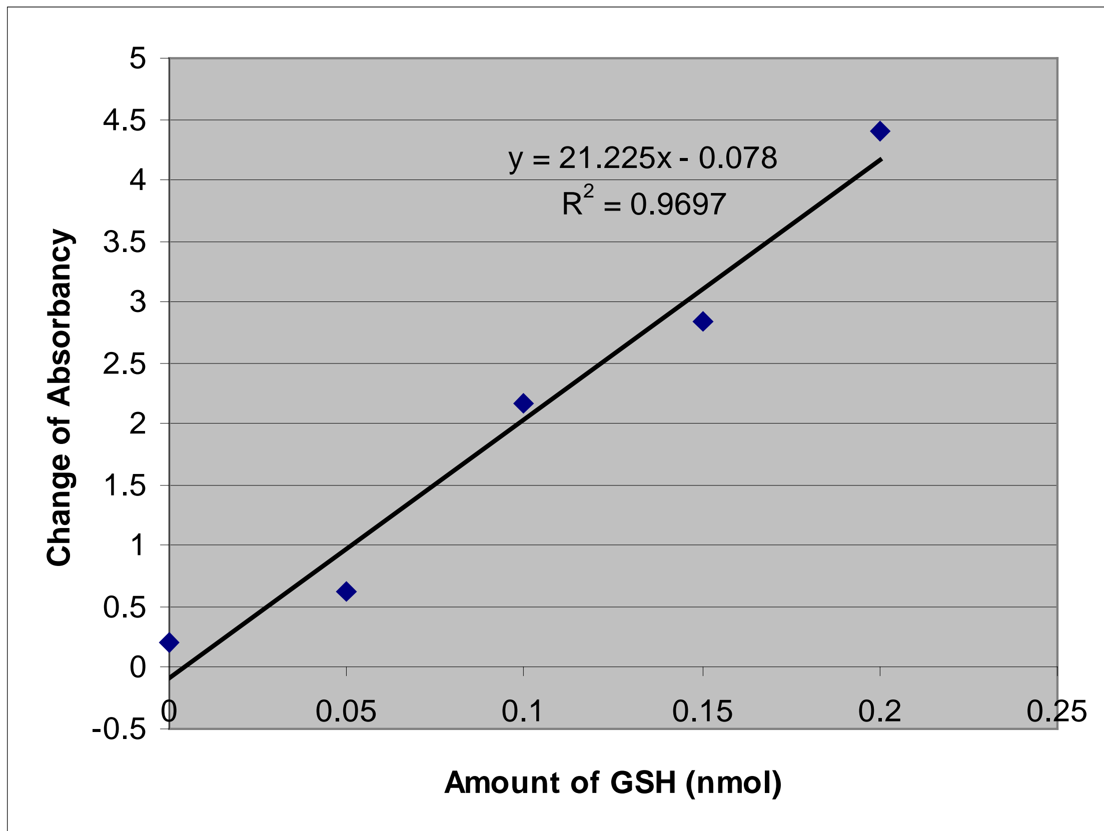
A. Prepare standards using GSH stock standard solution and dilute with water.

A = GSH stock solution (.1 *M*)
B = dilute A 1:100 (1 m*M*)
C = dilute B 1:25 (0.04 m*M*)
D = mix one part C:E (0.035 m*M*)
E = mix one part C:G (0.03 m*M*)
F = mix one part E:G (0.025 m*M*)
G = dilute C 1:1 (0.02 m*M*)
H = mix one part G:I (0.015 m*M*)
I = dilute G 1:1 (0.01 m*M*)
J = dilute I 1:1 (0.005 m*M*)

1. Blank = 5 µL water + 5 µL phosphoric acid.
2. 0.025 nmol GSH = 5 µL J + 5 µL phosphoric acid.
3. 0.05 nmol GSH = 5 µL I + 5 µL phosphoric acid.
4. 0.075 nmol GSH = 5 µL H + 5 µL phosphoric acid.
5. 0.1 nmol GSH = 5 µL G + 5 µL phosphoric acid.
6. 0.125 nmol GSH = 5 µL F + 5 µL phosphoric acid.
7. 0.15 nmol GSH = 5 µL E + 5 µL phosphoric acid.
8. 0.175 nmol GSH = 5 µL D + 5 µL phosphoric acid.
9. 0.2 nmol GSH = 5 µL C + 5 µL phosphoric acid.

B. Prepare cuvettes and perform the assay as described in Appendix B. For sample addition, use 10 µL of standard solution.
C. Determine standard curve in which the GSH equivalents present are plotted against the rate of change of absorbance at 412 nm.
D. Sample blank without GSH is a measure of background rate.
E. Report values in GSH equivalents.

Figure 18. Standard curve for intracellular glutathione (GSH) concentrations using the 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) – disulfide GSH (GSSG) reductase recycling assay.



APPENDIX D

Procedure for TACS Annexin V-FITC assay. All supplies were from Trevigen, Gaithersburg, MD.

The following reagent was used in this assay:

Annexin V Incubation Reagent

10 µL 10X Binding Buffer
10 µL Propidium Iodide
1 µL Annexin V Conjugate
79 µL Deionized, Distilled water

The assay was preformed in the following manner:

1. Wash embryos in 4° C 1X Phosphate Buffered Saline (PBS) for 10 min.
2. Place embryos in 100 µL of Annexin V Incubation Reagent and incubate in dark for 15 min at RT.
3. Wash embryos in RT 1X Binding Buffer for 10 min.
4. Place embryos on glass microscope slide and dry slightly.
5. Add a drop of fluorescent mounting media and cover-slip.
6. Determine stage of apoptosis from relative fluorescence of the embryos through a fluorescein compatible filter.

VITA

Brian Daniel Whitaker was born on April 29, 1977 in Schenectady, New York. In 1981 he moved to Westminster, Massachusetts where he attended the Westminster public schools. He graduated from Oakmont Regional high school in June of 1995. Brian attended the University of Massachusetts at Amherst in the fall of 1995. In May of 1999, Brian graduated from the University of Massachusetts at Amherst with a Bachelor of Science in Veterinary and Animal Science. Brian accepted a Master of Science position at the Virginia Polytechnic Institute and State University in Blacksburg, Virginia under the direction of Dr. James W. Knight in the summer of 1999. Brian graduated in the summer of 2002 with a M.S. degree in Animal Science with a concentration in reproductive physiology.