Influence of Growth Factors on Bovine Embryo Development

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ABSTRACT

Many attempts have been made to improve the in vitro production of cattle embryos by refining in vitro maturation (IVM) and culture systems. Cysteine supplementation to IVM media of bovine oocytes increases cellular glutathione production, which reduces reactive oxygen species (ROS). Similarly, beneficial effects of growth factors for improving the rate of blastocyst development have been reported, but combined effects are unknown. This study was conducted to determine the additive effect of the antioxidant cysteine with epidermal growth factor (EGF) and/or insulin-like growth factor-I (IGF-I) on subsequent embryo development. Bovine oocytes from slaughterhouse ovaries were matured in TCM-199 (control), with or without the addition of 0.6 mM cysteine (C) at 0 or 12 h of maturation. After in vitro fertilization, embryos were allocated to culture treatments containing synthetic oviductal fluid medium. Culture treatments included fetal calf serum (FCS, 4%) alone; IGF-I (100 ng/mL); EGF (10 ng/mL); and IGF-I+EGF (100 ng/mL+10 ng/mL) for all IVM treatments. Although rates for blastocysts development were not different among treatments, an increased proportion of embryos attaining morula formation was achieved when cysteine was added to the IVM media (12 h C IGF-I+EGF, 41.4%; 0 h C EGF, 40.0%) as compared to control (FCS: 34.6%). When cysteine treatments were combined, percent cleavage was greater for IGF-I+EGF (70.8%) compared to FCS (61.2%). The abundance of mRNA from the

apoptotic genes, Bax and Bcl-2, and the oxidative stress genes, copper (Cu)-zinc (Zn) superoxide dismutase (SOD; SOD1) and manganese (Mn) SOD (SOD2) in embryos was assessed. No significant treatment effect was observed on the expression of apoptotic and oxidative stress genes. Bax was expressed strongly (4-fold) in morulae with the addition of IGF-I, but was less prevalent in all other morula and blastocyst groups relative to FCS. There was slightly less expression of both SOD1 and SOD2 with treatments compared to FCS in morulae and blastocysts, indicative of low mitochondrial activity and/or a low level of oxidative stress in treatments. There was no significant treatment effect on total cell number, apoptotic nuclei, or apoptotic index. In conclusion, supplementation of cysteine during IVM of oocytes, in conjunction with growth factors could effectively be used as a replacement for FCS.

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CHAPTER ONE

REVIEW OF LITERATURE

1.1 Overview of in vitro Embryo Production

Following the first report of the birth of live calves from the transfer of completely in vitro produced embryos (Brackett et al., 1982), many attempts have been made to improve in vitro production (IVP) of cattle embryos by refining maturation, fertilization, and culture conditions. However, the efficiency of the in vitro procedure to produce morulae and blastocysts remains lower than in vivo (Merton et al., 2003) and low quality of in vitro produced embryos can not be disregarded. A number of factors influence the survival of embryos produced with in vitro systems. These include medium composition (Thompson, 2000), oocyte quality (Blondin and Sirrard, 1995), environment, and embryo genotype. The developmental competence of oocytes produced by in vitro maturation (IVM) is far inferior to those undergoing maturation in vivo. It is thought that many of the essential biochemical pathways to complete oocyte maturation prior to fertilization are incomplete or inadequate during IVM of oocytes.

1.1.1 Supplementing in vitro maturation and culture media

Supplements such as serum, growth factors, and amino acids have been added to IVM and in vitro culture (IVC) media to improve oocyte maturation and early embryo development. Serum, the most common supplement used, is an effective but highly variable and undefined adjunct, containing energy substrates, amino acids, vitamins, antioxidants, and growth factors. Bavister (1995) reported a biphasic effect of fetal calf serum (FCS) on cattle embryos. Serum inhibited the first cleavage divisions (two-cell to the morula stage; Figure 1-1), but FCS accelerated subsequent development of cultured

embryos to blastocyst stage and/or hatching from zona pellucida. Also, amino acid utilization by early embryo is stage dependent (Steeves and Gardner, 1999) suggesting that there are temporal and differential effects of various substrates which affect development of bovine embryos from the zygote to the blastocyst stage (Figure 1-1).

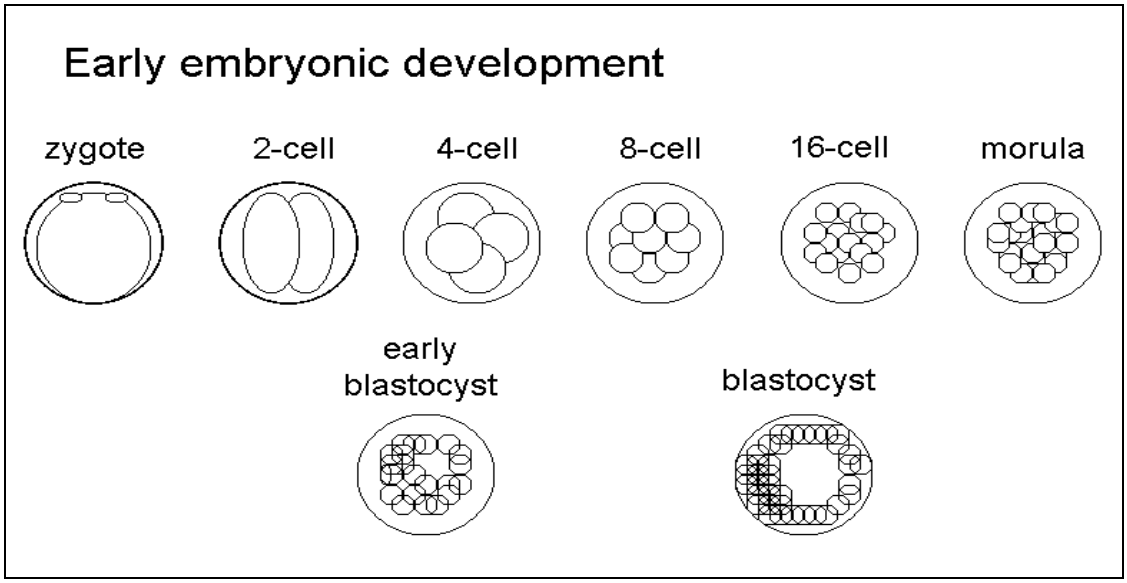

Figure 1- 1. Stages of early embryonic development.

Many experiments have investigated the effects of growth factors on bovine embryonic development. Growth factors for in vitro bovine embryo production improved embryo development, but did not yield results comparable to FCS. Previous researchers reported that the supplementation of growth factors, such as epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I), to maturation and culture media improved the rate of blastocyst formation of bovine embryos (Lonergan et al, 1996; Byrne et al, 2002; Sirisathien et al., 2003).

Cattle embryos cultured in vitro are prone to oxidative stress (Lequarre et al., 1997). Although serum provides nutrients for oocytes and embryos, it does not protect against oxidative stress and reactive oxygen species (ROS).Cysteine, an amino acid, is

supplemented for its antioxidant properties to oocytes and embryos, and other cultured mammalian cells. Cysteine is also the precursor for glutathione (GSH), a natural antioxidant present in both sperm and oocytes. Cysteine supplementation causes increased glutathione production, which regulates and protects oocytes and embryos from ROS (Guerin et al., 2001; Harvey et al., 2002). Furthermore, Ali et al. (2003) reported that medium supplementation with cysteine during IVM and IVC enhanced bovine embryo development. As oocytes progress from germinal vesicle to Metaphase II stage oocytes, an increase in GSH was found, but then GSH was decreased in fertilized oocytes (Calvin et al, 1986; Miyamura et al., 1995).

1.2 Oxidative stress and reactive oxygen species

Oxidative stress is an elevation in steady state levels of various ROS that exceeds cellular antioxidant defenses (Agarwaal et al., 2003). Reactive oxygen species are molecules or ions formed by the incomplete one-electron reduction of oxygen (Genetics Home Reference, 2008), and as by-products of energy production. Reactive oxygen species are toxic to oocyte maturation and embryo development. Three main elements of ROS in tissue culture include: type of ROS- i) superoxide anion ($O_2^- \cdot$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2); ii) the degree of ROS production during culture; and iii) the amount of each ROS produced varies depending on the stage of development. Higher ROS production has been attributed to environmental factors including, but not limited to: light exposure, high oxygen tension, presences of heavy metals in culture media (Johnson and Nasr-Esfahani, 1994), and disorders in embryo metabolism (Rieger et al, 1992). The source of ROS may be the embryos or their environment. Main endogenous sources of ROS in oocytes and embryos include: oxidative phosphorylation,

high glucose concentrations in vitro, NADPH, and xanthine oxidases. Exogenous factors that can enhance the production of ROS by embryos include: oxygen tension (hyperoxic conditions may cause an increase in the level of O_2^- · within cells), metallic cations (such as Fe and Cu, which are inducers of ROS formation through the Fenton and Haber-Weiss reactions), visible light (oxidation of bases and DNA strand break; Beehler et al., 1999), amine oxidase (catalyzes spermine and spermidine into H_2O_2; Parchment et al., 1990), and spermatozoa (sperm produce ROS during in vitro oocyte fertilization; Alvarez et al., 1996; Guerin et al., 2001).

Oxygen (O_2) metabolism is important in embryos (Magnusson et al., 1986; Houghton et al., 1996; Thompson et al., 1996) and the average O_2 consumption rate of bovine morulae and blastocysts is ~2 nL/h per embryo (Overstrom et al., 1992). Oviductal oxygen concentration is approximately one-quarter to one-third of atmospheric tension (Mastroianni and Jones, 1965; Mass et al., 1976). Culture exposes embryos directly to air, which has a higher O_2 concentration (5% CO_2) than that of the in vivo environment (Bavister, 1995), and this higher O_2 concentration increases production of ROS (Luvoni et al., 1996). Increased ROS causes DNA damage, lipid peroxidation, and oxidative modification of proteins (Johnson and Nasr-Esfahani, 1994).

Oxygen is a necessary element for the conversion of ADP to ATP in oxidative phosphorylation. However, energy production results in the formation of $O_2^{-\bullet}$, $OH^{\bullet}$, and H_2O_2 (Harvey et al., 2002). The production of these ROS changed the majority of cellular molecules, and induced mitochondrial alterations, embryo cell block, ATP depletion, retardation of development, and apoptosis (programmed cell death) in embryos (Guerin et al., 2001).

Even though high levels of ROS are harmful to gametes and embryos, ROS do play a physiological role in gamete and embryo development. For example, ROS have been linked to capacitation, the acrosomal reaction (de Lamirande et al., 1998), and fertilization (Miesel et al., 1993). Embryos may have different sensitivities to ROS depending on stages of development. Morales et al. (1999) observed that 9- to 16-cell bovine embryos were more resistant to exogenous H_2O_2 than zygotes and blastocysts.

1.3 Antioxidants

Several defense mechanisms against ROS are present in both embryos and their surroundings, including antioxidants. An antioxidant is any substance, that when present at low concentrations compared to that of an oxidizable substrate can significantly delay or inhibit the oxidation of that substrate (Halliwell and Gutteridge, 1989). These include non-enzymatic antioxidants and enzymatic defense mechanisms within cells. Compounds that function as antioxidants include vitamins A, C, and E, pyruvate, and sulfur compounds, including hypotaurine, taurine, reduced glutathione (GSH) and cysteamine (CSH)

1.3.1 Metabolizable non-enzymatic antioxidant: Cysteine

As mentioned earlier, GSH acts as the main non-enzymatic defense system against ROS (Takahashi et al., 1993; Gardiner and Reed, 1994) because it provides oocytes and embryos with a reducing environment to protect against oxidative damage. Reduced glutathione is the substrate of glutathione peroxidase (GPX), the main antioxidant enzyme of embryos. Yoshida et al. (1993) measured GSH synthesis during oocyte maturation in oocytes of the mouse, hamster, pig, and cow. Pig oocytes contained high concentrations of GSH, and the concentration of GSH was highly correlated with

successful early development and viability after freezing of bovine embryos (Takahashi et al., 1993). Furthermore, de Matos and Furnus (2000) demonstrated that supplementation of IVM medium with β-mercaptoethanol, cysteine, or cystine produced approximately 3 pmol intracellular GSH in oocytes during IVM that were maintained after IVF. This corresponded with improved rates of development to the blastocyst stage. In contrast, Takahashi et al. (1993) reported reduced embryo development to the blastocyst stage, as well as a reduction in intracellular GSH of embryos upon the addition of the GSH synthesis inhibitor buthionine sulfoximine to medium containing thiol compounds. Guyader-Joly et al. (1998) found high amounts of CSH in the follicular fluid of the cow, sow, goat, and dog, suggesting the addition of cysteine may contribute to the maintenance of endogenous pools of GSH and CSH in cultured oocytes.

The availability of precursor amino acids impacts GSH synthesis. For example, cysteine supplementation to IVM and IVC media increased cellular GSH production (de Matos and Furnus, 2000). Sawai et al. (1997) suggested that supplementation of cysteine to maturing porcine oocytes was critical only between 42 and 48 h of culture, when the oocytes were in the late Metaphase-I to Metaphase-II stage of development, and when cysteine is utilized for GSH synthesis. Additionally, supplementation of culture media with 1.71 mM cysteine improved fertilization rates (56%) compared to lower concentrations (0.57 and 0 mM) of cysteine (48 and 35%, respectively) with porcine zygotes. Exposure to cysteine beyond 3 h had no additional benefits on fertilization rates. When cultured with 1.71 mM cysteine for 3 h after intracytoplasmic sperm injection (ICSI), blastocyst formation was higher (31%) than controls (18%; Katayama et al., 2007).

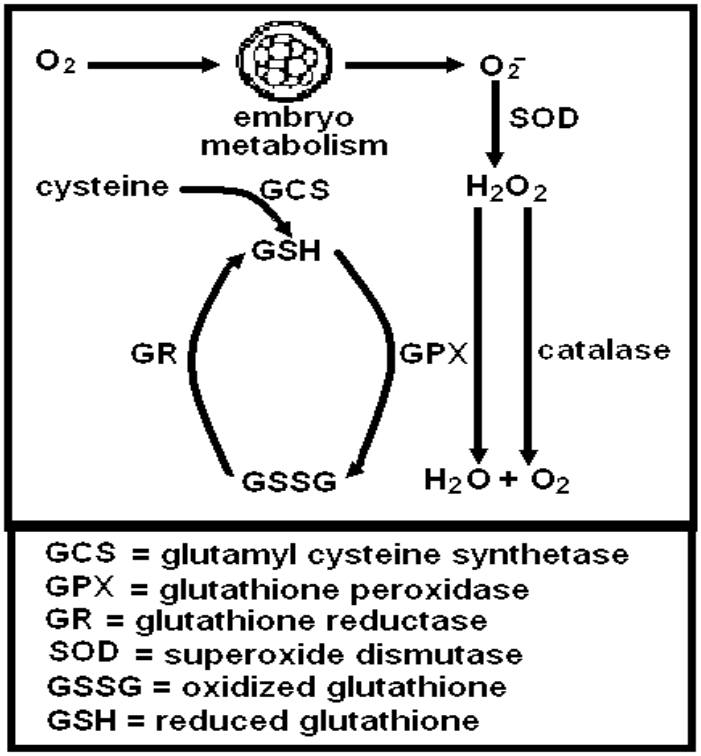

Figure 1- 2. Enzymatic and non-enzymatic pathways for defense mechanisms against oxidative stress.

1.3.2 Enzymatic antioxidants: Cu-Zn and Mn superoxide dismutases

There are several antioxidant enzymes that protect oocytes and embryos against peroxidative damage (Figure 1-2). These are superoxide dismutase (SOD), catalase, and GPX (Li et al., 1993). As indicated in Figure 1-2, the enzymes copper (Cu), zinc (Zn)-SOD (SOD 1; located in the cytosol), and manganese (Mn)-SOD (SOD 2; located in the mitochondria) allow superoxide radicals to be scavenged. These SOD are a first enzymatic step to protect cells against toxic oxygen radicals (Michiels et al., 1994). Catalase or GPX eliminates H_2O_2, the by-product of SOD action. Glutathione peroxidase reduces lipid hydroperoxides and H_2O_2, suggesting that GPX has a critical role in cell antioxidant protection (Guerin et al., 2001). El Mouatassim et al. (1999) found that transcripts coding for SOD 1 were present in human oocytes at all stages of maturation,

and were highly expressed in the mouse blastocyst. This suggests that the SOD 1 enzyme plays an essential role in protecting embryos against O_2 toxicity in vivo and in vitro.

Lequarre et al. (2001) reported that transcripts encoding for SOD 1 were detectable in immature and in vitro matured bovine oocytes, and were expressed in bovine embryos. This supports the results of Harvey et al. (1995) who found expression of SOD 1 transcripts at all preimplantation embryo stages in both the mouse (in vivo and in vitro) and cow (in vitro). Furthermore, presence of SOD 1 transcripts in in vivo derived bovine morulae and blastocysts indicated that transcription was not unique to in vitro cultured bovine embryos (Harvey et al. 1995).

In 2002, Rizos et al. reported that SOD 2 expression was highest in in vivo-produced blastocysts and those cultured in the ewe oviduct, while lower levels of SOD 2 were expressed in in vitro-cultured embryos. These observations were consistent with those of Lequarre et al. (2001), who found a culture environment-dependent expression of SOD 2. In support, Farin et al. (2001) reported that the mitochondrial population of IVP embryos was compromised and the lower expression of SOD 2 in IVP blastocysts may be indicative of low mitochondrial activity.

1.4 Growth Factors

Growth factors such as EGF and IGF are known to positively effect preimplantation development by stimulating metabolism and growth of embryos. Growth factors: i) increase proliferation of cells forming the intracellular mass (ICM) and trophectoderm (TE); ii) are involved in the compaction and formation of the blastocyst; iii) activate transport systems responsible for the uptake of glucose; iv) enhance endocytosis; and v) influence the processes of replication, translation, and degradation of

proteins (Goldman and Gonen, 1998; Herrler et al., 1998; Pantaleon and Kaye 1996; Pantaleon et al., 2003). Furthermore, Kurzawa et al. (2004) demonstrated that mouse embryos cultured in EGF and IGF-I, at a concentration of 100 ng/mL, developed faster, had increased cell counts, decreased apoptotic cells, and were protected against oxidative stress, when compared to mouse embryos to cultured in 0.1 mM H_2O_2 alone,.

1.4.1 Epidermal growth factor

Epidermal growth factor added to IVC media, promotes nuclear maturation of oocytes in multiple species including rat, mouse, bovine, porcine, and human. Morrish et al. (1987) concluded that EGF causes morphological differentiation of human trophoblasts. According to Lonergan et al. (1996), addition of EGF, irrespective of concentration, to M-199 media stimulated cumulus cell expansion and significantly increased the proportion of oocytes attaining metaphase II. Maturation in the presence of EGF significantly increased the proportion of embryos at the 5 to 8-cell stage (52%) and increased the proportion of d 8 blastocysts by 30% when compared to the controls (44% and 22%, respectively). Likewise, in the presence of 10 to 50 ng/mL EGF, the proportion of embryos developing to the blastocyst stage was significantly higher (16.5 ± 2.0 to 20.8 ± 4.9%) than in control medium (3.4 ± 2.1%; Park et al., 1997). Similarly, Sirisathien et al. (2003) demonstrated that addition of 5 ng/mL of EGF significantly increased the percentage of blastocysts (50%) developing from 4-cell embryos when compared to the control medium (36.5%).

1.4.2 Insulin-like growth factor-I

Insulin-like growth factor-I promotes oocyte maturation and embryo development in culture systems of multiple species. Previous research has shown that IGF-I has

mitogenic effects on preimplantation mammalian embryos. Culture medium supplemented with IGF-I decreased apoptosis and increased cell proliferation in human, rabbit and mouse blastocysts, improving preimplantation embryo development (Makarevich et al., 2002). Insulin-like growth factor-I provided antiapoptotic effects due to an IGF-I receptor pathway. This caused IGF-I receptor over-expression, protecting cells in vivo (Le Roith et al., 1997; Peruzzi et al., 2001). Insulin-like growth factor-I (13 nM) increased the percentage of embryos that formed blastocysts (28% compared to 19% in the control group), increased the total cell number (125 cells compared to 90 cells in the control group), and decreased the apoptotic dead cell index (3% compared to 4.5% in the control group; Byrne et al., 2002). Furthermore, Sirisathien et al. (2003) showed that IGF-I treatments at 10 and 50 ng/mL improved proportions of 4-cell stage embryos that reached blastocysts (54.4 and 64.2%, respectively) when compared to controls (39.3%). They also demonstrated that after culture with 50 ng/mL of IGF-I, numbers of inner cell mass (ICM) cells of d 8 blastocysts were higher (78 ± 3) when compared to controls (56 ± 4).

1.5 Quality of in vitro produced embryos

Although the addition of growth factors and amino acids to chemically defined embryo culture media improves IVP of embryos, quality of these embryos needs to be evaluated. Increased blastocyst yields may not correspond with higher development.

The quality of in vitro produced embryos can be assessed by many measures such as morphology, total cell number, ICM number, gene expression, and apoptosis. High implantation rates are related to high cell number and good morphological embryos. Moreover, minor differences in embryo culture systems can result in changes in apoptotic

and oxidative stress gene expression that relate to embryo quality (Wrenzycki et al., 2001; Rizos et al., 2002).

1.5.1 Apoptosis as a measure of embryo quality

Programmed cell death (PCD), also know as apoptosis, is an important mechanism in both development and homeostasis in adult tissues for the removal of excess or damaged cells. Programmed cell death occurs in a wide variety of tissues to eliminate unnecessary cells during the development process and embryogenesis (Garcia-Martinez et al., 1993). For both in vivo and in vitro derived embryos the occurrence of apoptosis is a normal event. Occurrence of apoptosis during the pre-implantation development of embryos is well established (Hardy 1997; Betts and King, 2001). Apoptosis first occurs in the preimplantation bovine embryo at the 9- to16-cell stage and is influenced by in vitro culture conditions (Byrne et al., 1999). The effect of apoptosis on embryo quality has great relevance for in vitro technologies. As described in Figure 1-3, two pathways, a receptor dependant and a receptor independent, can induce apoptosis.

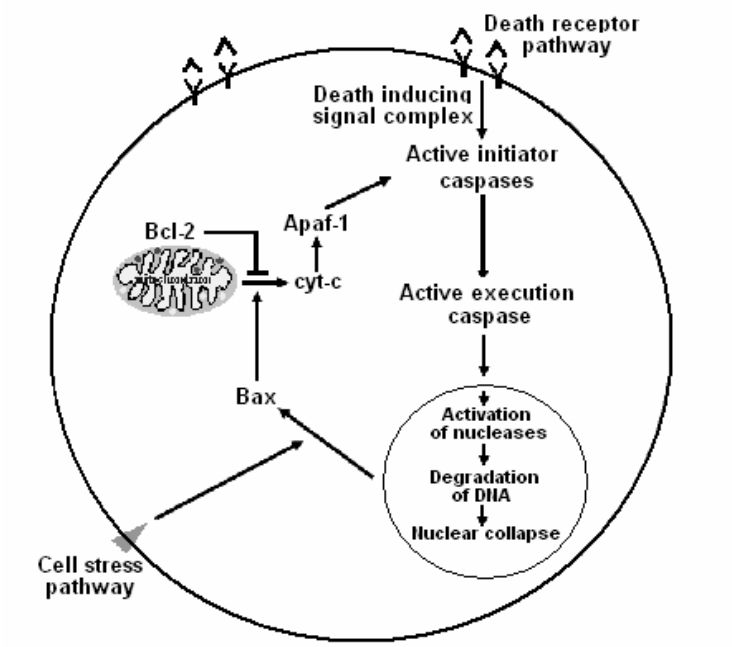

Figure 1- 3. Simplified apoptosis pathway showing receptor dependent and receptor independent means for initiation of programmed cell death (Adapted from Anchamparuthy, 2007).

1.5.2 Bcl-2 family

The receptor-dependent pathway is activated by specific ligand-receptor interactions that lead to the formation of an intracellular signaling complex (Figure 1-3). A receptor-independent pathway can be activated by cellular stress to release pro-apoptotic Bcl-2 family proteins (eg., Bax) for translocation from the cytosol to the mitochondria, where they induce the release of cytochrome c that activates apoptotic protease activating factor (APAF) (Zimmermann et al., 2001). Other anti-apoptotic Bcl-2 family proteins (eg., Bcl-2) prevent cytochrome c release from the mitochondria. Ratios of Bax and Bcl-2 within the cell determine cell death or survival. Over-expression of the Bax gene expedites cell death by producing Bax protein which heterodimerizes with Bcl-2, preventing cellular survival (Oltvai et al., 1993). In contrast, the Bcl-2 gene via

synthesis of the Bcl-2 protein acts as an anti-apoptotic gene protecting the mitochondrial membrane from Bax and the release of cytochrome c (Yang et al., 1997).

1.6 Total cell number and DNA fragmentation

A blastocyst is composed of two populations, inner cell mass (ICM) and trophectoderm (TE). Iwasaki et al. (1990) reported that the proportion of ICM cells in blastocysts is crucial for implantation, and increased cell death in ICM will compromise subsequent development. This was further supported by Fouladi-Nashta (2005) who demonstrated that counting the total number of blastomeres and the percentage of apoptotic nuclei provides more detailed information on embryo quality and the developmental potential of embryos after transfer to recipients. The incidence of DNA-fragmented nuclei detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) as a quality assessment in IVP bovine blastocysts in response to various stress stimuli was reported by several laboratories (Spanos et al., 2000; Paula-Lopes and Hansen, 2002; Walters et al., 2005). Hardy (1999) reported no evidence of DNA fragmentation prior to compaction of embryos indicating apoptosis is a consequence rather than a cause of embryonic arrest during pre-implantation development. Hao et al. (2003) observed fragmented embryos prior to d 5 in culture, but did not detect any TUNEL-positive cells in embryos prior to d 5 of development.

Interestingly, in an earlier study Byrne et al. (1999) reported that apoptosis in embryos was an indicator of poor maturation, fertilization and/or culture conditions and can be used as a screening mechanism for embryo quality. Changes in IVM media may reduce embryo death thus increasing the success rate of obtaining viable embryos. Since GSH content in IVM oocytes promotes their in vitro development after IVF, a number of

laboratories have studied the effect of cysteine on oocyte competence in different species. Specifically, high concentrations of cysteine during IVM of porcine oocytes were beneficial between 42-48 h after initiation of maturation for sufficient accumulation of GSH (Sawai et al., 1997). However, it is unknown whether cysteine is required throughout maturation or at the specific stage of meiotic maturation in bovine oocytes. To our knowledge, no previous studies have evaluated the combinatorial effect of cysteine and growth factors in IVP systems at specific time points in the 7 d window of IVC.

1.7 OBJECTIVES

Our hypothesis is that antioxidants and growth factors are as effective as FCS for early bovine embryo development. The first objective of this study is to determine the effect of the antioxidant cysteine when added at 2 time points, 0 and 12 h of oocyte maturation to IVM medium, on blastocyst formation. The second objective is to test the effect of growth factors (EGF or IGF-I) in culture, after IVF, by comparing subsequent embryo development to that of FCS. The quality of the IVP embryos will be assessed by analysis of apoptotic index using TUNEL assay. The abundance of apoptotic genes, Bax and Bcl-2, and oxidative stress genes, SOD1 and SOD2, relative to FCS in IGF-I, EGF and IGF-I+EGF treatments will also be assessed using RT-PCR.

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CHAPTER TWO

INFLUENCE OF CYSTEINE IN CONJUNCTION WITH GROWTH FACTORS ON THE DEVELOPMENT OF IN VITRO-PRODUCED BOVINE EMBRYOS

2.1 ABSTRACT

Many attempts have been made to improve the in vitro production of cattle embryos by refining in vitro maturation (IVM) and culture systems. Cysteine supplementation to IVM media of bovine oocytes increases cellular glutathione production, which reduces reactive oxygen species (ROS). Similarly, beneficial effects of growth factors for improving the rate of blastocyst development have been reported, but combined effects are unknown. This study was conducted to determine the additive effect of the antioxidant cysteine with epidermal growth factor (EGF) and/or insulin-like growth factor-I (IGF-I) on subsequent embryo development. Bovine oocytes from slaughterhouse ovaries were matured in TCM-199 (control), with or without the addition of 0.6 mM cysteine (C) at 0 or 12 h of maturation. After in vitro fertilization, embryos were allocated to culture treatments containing synthetic oviductal fluid medium. Culture treatments included fetal calf serum (FCS, 4%) alone; IGF-I (100 ng/mL); EGF (10 ng/mL); and IGF-I+EGF (100 ng/mL+10 ng/mL) for all maturation treatments. Although rates for blastocysts development were not different among treatments, an increased proportion of embryos attaining morula formation was achieved when cysteine was added to the maturation media (12 h C IGF-I+EGF, 41.4%; 0 h C EGF, 40.0%) as compared to control (FCS: 34.6%). When cysteine treatments were combined, percent cleavage was greater for IGF-I+EGF (70.8%) compared to FCS (61.2%). The abundance of mRNA from the apoptotic genes, Bax and Bcl-2, and the oxidative stress genes,

copper (Cu)-zinc (Zn) superoxide dismutase (SOD; SOD1) and manganese (Mn) SOD (SOD2) in embryos was assessed. No significant treatment effect was observed on the expression of apoptotic and oxidative stress genes. Bax was expressed strongly (4-fold) in morulae with the addition of IGF-I, but was less prevalent in all other morula and blastocyst groups relative to FCS. There was slightly less expression of both SOD1 and SOD2 with treatments compared to FCS in morulae and blastocysts, indicative of low mitochondrial activity and/or a low level of oxidative stress in treatments. There was no significant treatment effect on total cell number, apoptotic nuclei, or apoptotic index. In conclusion, supplementation of cysteine during IVM of oocytes, in conjunction with growth factors could effectively be used as a replacement for FCS.

2.2 INTRODUCTION

Many attempts have been made to improve the in vitro production of cattle embryos by refining in vitro maturation, fertilization, and culture systems after the first report (Brackett et al., 1982) of the birth of calves from the transfer of completely in vitro produced embryos. The developmental competence of oocytes produced during in vitro maturation (IVM) is inferior compared with in vivo maturation

There are various effects of amino acids on the culture of cattle embryos (Steeves and Gardner, 1999). Ali et al. (2003) reported that supplementation of media with cysteine during IVM and in vitro culture (IVC) resulted in faster and improved bovine embryo development. Reactive oxygen species have been positively linked to fertilization success (Miesel et al., 1993). Furthermore, de Matos and Furnus (2000) demonstrated that supplementation of IVM medium with cysteine or cystine increased intracellular

GSH concentrations during IVM that were maintained after IVF. This corresponded with improved rate of bovine embryo development to the blastocyst stage.

Antioxidant enzymes, such as copper (Cu), zinc (Zn)-SOD (SOD 1; located in the cytosol), and manganese (Mn)-SOD (SOD 2; located in the mitochondria), catalase, and GPX, protect oocytes and embryos against peroxidative damage, (Li et al., 1993). The enzymes SOD 1 and SOD 2 allow superoxide radicals to be scavenged. El Mouatassim et al. (1999) found that transcripts coding for SOD 1 were present in oocytes at all stages of maturation, suggesting that this enzyme plays an essential role in protecting embryos against O_2 toxicity. Rizos et al. (2002) reported that SOD 2 expression was highest in in vivo-produced blastocysts and those cultured in the ewe oviduct, while lower levels of SOD 2 were expressed with in vitro-cultured embryos. These findings could be indicative of low mitochondrial activity in IVP embryos.

Previous research has shown that growth factors such as EGF and IGF induced a positive effect on preimplantation development by stimulating metabolism and growth of embryos. Kurzawa et al. (2004) demonstrated that EGF and IGF-I had a positive effect on mouse embryos cultured with 0.1 mM H_2O_2, and therefore, were able to protect embryos against oxidative stress. Addition of 5 ng/mL of EGF increased the percentage of blastocysts (50%) developing from 4-cell embryos when compared to the control media (36.5%; Sirisathien et al., 2003). Culture medium supplemented with IGF-I was beneficial to preimplantation embryo development because it decreased apoptosis and increased cell proliferation in human, rabbit, and mouse blastocysts (Makarevich et al., 2002).

Fouladi-Nashta (2005) found that the percentage of apoptotic nuclei provided detailed information on embryo quality and the developmental potential of embryos. The incidence of DNA-fragmented nuclei detected by TUNEL, as a quality assessment for in vitro produced bovine blastocysts in response to various stress stimuli, was reported by several laboratories (Spanos et al., 2000; Paula-Lopes and Hansen, 2002; Walters et al., 2005). Byrne et al. (1999) reported that apoptosis in embryos is an indicator of poor maturation, fertilization and/or culture conditions and can be used as a screening mechanism for embryo quality. Changes in the IVM media may cause a lower incidence of embryo death, thus causing an increase in the yield of viable embryos. Since increasing GSH content in IVM oocytes promotes their in vitro development after IVF, a number of laboratories have attempted to identify the promoting effect of cysteine, a precursor of GSH, on oocyte competence in different species (de Matos and Furnus, 2000; Guyader-Joly et al., 1998; Katayama et al., 2007). However, it is unknown whether cysteine is required throughout maturation or at meiotic maturation in bovine oocytes. To our knowledge, no previous studies have evaluated the combinatorial effect of cysteine and growth factors in IVP systems at specific time points in the 7 d window of in vitro culture.

Our hypothesis is that antioxidants and growth factors are as effective as FCS for supporting early bovine embryo development. The overall objective of this study was to determine the effect of the antioxidant cysteine when added at 2 time points, 0 and 12 h of oocyte maturation to IVM medium, on blastocyst formation. Additionally, the effect of growth factors (EGF or IGF-I) post-fertilization, in culture, was determined by comparing subsequent embryo development to those embryos cultured in FCS.

2.3 METHODS

2.3.1 Collection of cumulus oocyte complexes and in vitro maturation (IVM)

Ovaries were collected at Brown Packing Company Gaffney, SC over a 15 wk period. Depending on the week, 250-500 oocytes were aspirated with a 10 cc syringe and 20 gauge needle into 50 mL conical tubes from follicles (>3mm in diameter) of 100-300 ovaries from cows of unknown reproductive status. Oocytes surrounded by a compact cumulus mass (cumulus-oocyte complexes: COC) and homogenous ooplasm were washed three times in synthetic oviductal fluid with heparin (SOFH; Table 2-1) and twice in maturation medium (Tissue culture medium, TCM 199; Table 2-2). Maturation of oocytes was completed by incubating the oocytes in TCM-199 (Gibco Life Technologies, Inc; Grand Island, NY) supplemented with 0.02 U/mL Follicle Stimulating Hormone (FSH; Sigma-Aldrich; St. Louis, MO), 0.01 U/mL Luteinizing Hormone (LH; Sigma-Aldrich), 1 μg/mL 17 β-estradiol (E_2; Sigma-Aldrich), and 10% fetal calf serum (FCS; Gibco). The COC were distributed into maturation media with or without (control) the addition of cysteine (0.6 mM) at 0 h (0 h C) or 12 h (12 h C) of a 24 h incubation (humidified 38.5°C, 5% CO_2; Walters et al., 2005).

Table 2- 1. Washing media components: Synthetic Oviductal Fluid Heparin

Stock	Final concentration (mM unless specified)	Amount to be added
1. Water	-	8.67 mL
2. NaCl	108.45	542.30 µL
3. NaHCO3	25.07	250.70 µL
4. NEAA	-	200.00 µL
5. EAA	-	100.00 µL
6. Glutamine	1.00	100.00 µL
7. KCl	7.16	71.60 µL
8. $CaCl_2$-$2H_2O$	1.71	17.10 µL
9. KH_2PO_4	1.19	11.90 µL
10. Phenol Red	5 µg/mL	10.00 µL
11. Gentamicin	50 µg/mL	10.00 µL
12. Na-Pyruvate	0.33	10.00 µL
13. $MgCl_2$-$6H_2O$	0.49	4.90 µL
14. Na-Lactate	3.30	4.70 µL
15. Heparin	1 µg/mL	10.00 µL
16. NaOH	-	~12 µl to adjust pH 7.3 to 7.4
Total volume		10 mL
FCS	-	1000 µl/50 mL
Heparin		500µL

Table 2- 2. Maturation media components for oocyte maturation.

Component	Concentration	Amount to be added
1. M199	100% V/V	9 mL
2. LH	2.5 IU/mL	50 µL
3. FSH	5.0 IU/mL	50 µL
4. Estradiol	1 mg/mL	10 µL
5. Gentamicin	50 mg/mL	10 µL
Total volume		10 mL
FCS	10% v/v	-
NaOH		2 µL to adjust pH 7.3 to 7.4

2.3.2 Preparation of bovine spermatozoa

Frozen straws of semen were thawed and prepared in synthetic oviduct fluid (SOF) supplemented with 25 m*M* HEPES (SOF-H), fetal calf serum (FCS, 2%), and sodium pyruvate (0.1 mg/mL; Sigma-Aldrich) using the swim-up method described by Parrish et al. (1995). Straws of semen were thawed in a water bath at 37°C for 1 min and 0.5 mL thawed semen was layered under the SOF of 4 snap-cap culture tubes, capped and

incubated for 1 h. After incubation, 750 µL of the overlaying SOFH was aspirated as the recovery fraction. Synthetic oviductal fluid was added to the tube to adjust the final volume to 10 mL and centrifuged (room temperature) at 120 x g for 10 min. Supernatant was removed and 105 µL of pellet from bottom of tube was transferred to another tube. Sperm concentration of the pellet was determined using a hemocytometer (Walters et al., 2005).

At 24 h post maturation, the matured COC were washed three times in SOF-H, followed by washing two times in synthetic oviductal fluid for IVF (SOF-IVF; Table 2-3). The COC were then placed into a 47 µL SOF-IVF drop (10 oocytes per drop) supplemented with 10 µg/mL heparin. Sperm were pipetted into each fertilization droplet for a final volume of 50 µL at 1 x 10^6 spermatozoa/mL and incubated (humidified 38.5°C, 5% CO_2) for 18 h (Parrish et al., 1995; Walters et al., 2005).

Table 2- 3. Synthetic Oviductal Fluid (SOF) components for oocyte fertilization (IVF).

Stock	Final concentration (mM unless specified)	Amount to be added
1. Water	-	8.67 mL
2. NaCl	108.45	542.30 µL
3. NaHCO3	25.07	250.70 µL
4. NEAA	-	200.00 µL
5. EAA	-	100.00 µL
6. Glutamine	1.00	100.00 µL
7. KCl	7.16	71.60 µL
8. $CaCl_2$-$2H_2O$	1.71	17.10 µL
9. KH_2PO_4	1.19	11.90 µL
10. Phenol Red	5 µg/mL	10.00 µL
11. Gentamicin	50 µg/mL	10.00 µL
12. Na-Pyruvate	0.33	10.00 µL
13. $MgCl_2$-$6H_2O$	0.49	4.90 µL
14. Na-Lactate	3.30	4.70 µL
15. Heparin	1 µg/mL	10.00 µL
16. NaOH	-	~12 µL to adjust pH 7.3 to 7.4
Total volume		10 ml
BSA	8 mg/mL	0.08 g

2.3.3 In vitro culture (IVC)

After 18 h of oocyte and sperm incubation, the embryos were removed from the fertilization drops and placed into a centrifuge tube containing 2 mL SOF-H, and vortexed for 4 min. The embryos were screened and washed 3 times with SOF-IVF and then twice with SOF-IVC (Table 2-4). The embryos were transferred into SOF-IVC drops, with the addition of 2 μL FCS or 1 μL of growth factor (10 ng/mL EGF or 100 ng/mL IGF-I), or a combination of 1 μL each of the growth factors. The culture dish was placed into an incubator (humidified 38.5°C, 5% CO_2) for 7 d. On d 4 of culture, culture drops were fortified with 2 μL of FCS or growth factor (10 ng/mL EGF or 100 ng/mL IGF-I), or a combination of 1 μL each of the growth factors added to each culture drop (Figure 2-1).

Table 2- 4. Synthetic Oviductal Fluid (SOF-H) components for embryo in vitro culture (IVC).

Stock	Final concentration (mM unless specified)	Amount to be added
1. Water	-	8.66 mL
2. NaCl	107.70	538.50 µL
3. NaHCO3	25.07	250.70 µL
4. NEAA	-	200.00 µL
5. EAA	-	100.00 µL
6. Glutamine	1.00	100.00 µL
7. KCl	7.16	71.60 µL
8. $CaCl_2$-$2H_2O$	1.71	17.10 µL
9. KH_2PO_4	1.19	11.90 µL
10. Phenol Red	5 µg/mL	10.00 µL
11. Gentamicin	50 µg/mL	10.00 µL
12. Na-Pyruvate	0.33	10.00 µL
13. $MgCl_2$-$6H_2O$	0.49	4.90 µL
14. Na-Lactate	3.30	4.70 µL
15. Glucose	1.50	15.00 µL
16. NaOH	-	~10 µL
Total volume		10 mL
BSA	8 mg/mL	0.08 g

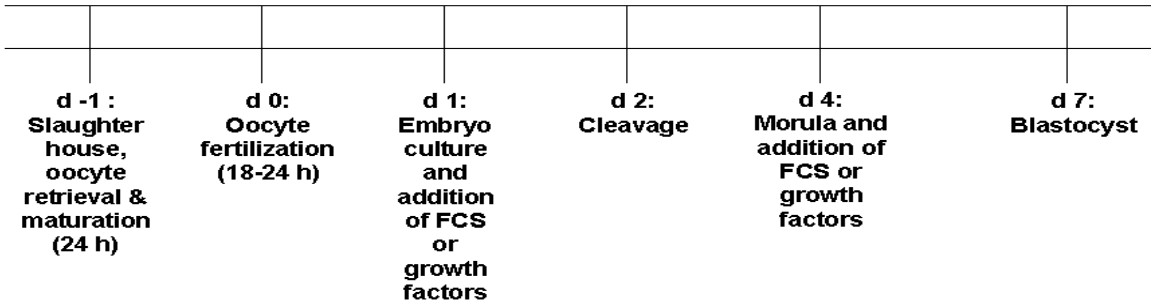

Figure 2- 1. Timeline of events and stages of development of in vitro-produced bovine embryos.

2.3.4 Embryo evaluation

Assessment of embryo development

On d 4, the embryos were evaluated at the time of adding FCS or growth factors. The number of 1-cell, 2-cell, 4-cell, 8-cell embryos and morulae in each culture drop were recorded. On d 7 of embryonic development, the embryos were evaluated for the number of early blastocysts, expanded blastocysts, and hatched blastocysts in each drop.

2.3.5 Gene Analysis

RT-PCR analysis was performed to quantify the relative abundance of apoptotic and oxidative stress genes. A combined TaqMan PreAmp-Cells-to-C_t (Ambion, Austin, TX) procedure was used for this purpose.

2.3.5.1 Preparation of cell lysate

Cell lysis was performed using the TaqMan Cells-to-C_t kit as per the manufacturer's instructions. Day 7 embryos from each treatment were washed 3 times in ice cold 0.1% polyvinyl pyrolindone (PVP; Sigma-Aldrich) in phosphate buffered saline (PBS) (PBS-PVP) and collected in separate nuclease-free Eppendorf tubes, discarding as much of the PBS as possible (Anchamparuthy, 2007). The embryos were lysed in lysis buffer (22.5 µL) containing DNase (1:100) and incubated at room temperature for 5 min. Stop solution (2.5 µL) was added to the lysate followed by room temperature incubation for 2 min. The lysate was stored for 2-3 wk at -20°C until PCR analysis.

2.3.5.2 Reverse Transcription (RT)

A 50 µL RT reaction cocktail was set as follows: 2X RT Buffer (25 µL), 20 X RT Enzyme Mix (2.5 µL), and 22.5 µL cell lysate. MultiScribe Reverse Transcriptase and random primers (Ambion, Austin, TX) were used for the RT reaction. Using an ABI

Prism 7300 Realtime PCR System (Applied Biosystems, Foster City, CA), the cocktail was incubated at 37°C for 60 min, and then at 95°C for 5 min to inactivate the RT enzyme, followed by cooling to 4°C.

2.3.5.3 Pooling the TaqMan Assays

For pooling, 5 TaqMan gene expression assays (20 X) of interest (2 µL each) were combined in a centrifuge tube. Pooled assays were diluted using 192 µL distilled water to achieve a final concentration of 0.2 X.

2.3.5.4 Performing Preamplification PCR

A preamplification reaction of 25 µL was set up as follows. TaqMan PreAmp master mix, 12.5 µL, pooled assay mix (0.2X) 6.25 µL, and cDNA 6.25 µL. The preamplification PCR cycling condition used was as follows: enzyme activation (hold) at 95°C for 10 min, PCR (cycle) at 95°C for 15 sec (10 cycles), followed by 60°C for 4 min. The preamplified product was diluted 1:5 in distilled water. The 10 cycle preamplification produced material for twenty-five 20 µL PCR tests.

2.3.5.5 Real-Time PCR

The real time PCR for the quantification of SOD1, SOD2 and Bax-Bcl-2 transcripts in d 7 embryos was carried out in an ABI Prism 7300 Realtime PCR System (Applied Biosystems). The diluted preamplified product was immediately used for PCR. The internal control, 18S rRNA, was pre-designed and pre-made. All custom TaqMan Gene Expression Assays (Table 2-5) were designed and synthesized by an assay design service within Applied Biosystems using MGB probes. Primers were constructed over exon/exon junctions to make them specific to mRNA transcripts. A 20 µL PCR reaction was set up as follows: TaqMan Gene Expression Master Mix (2X)-10 µL, TaqMan Gene

Expression Assay (20X)-1 µL, Nuclease-free water-4 µL, and diluted preamplifed product 5 µL. Then PCR was run in an ABI Prism 7300 Realtime PCR System (Applied Biosystems). The thermal cycling condition set up was an incubation step of 50°C for 2 min , enzyme activation (hold) at 95°C for 10 min, PCR (cycle) at 95°C for 15 sec (40 cycles), followed by 60°C for 1 min.

Based on the PCR amplification results, as described by Livak and Schmittgen (2001), the relative abundance of gene transcripts was calculated using $2^{-\Delta\Delta Ct}$.

Table 2-5. Primers and probes used for real time PCR

Genes	Primer sequences	Probe sequences
18S rRNA	Pre-made (no sequence information available)	
SOD1-F*	5'CACTCTCAGGAGAATATTCCATCATTGG3'	FAM-CACGATGGTGGTCCATGA-MGB
SOD1-R*	5'TCTGCCCAAGTCATCTGGTTTT3'	
SOD2-F*	5'CGTCGCCGAGGAGAAGTAC3'	FAM-CTGTAACATCACCCTTCTCC-MGB
SOD2-R*	5'AACGCAGGCTGCAGAGC3'	
BAX-F*	5 'GCTCTGAGCAGATCATGAAGACA3'	FAM-CTTTTGCTTCAGGGTTTC-MGB
BAX-R*	5'CCATTCGCCCTGCTCGAT3'	
BCL-2-F*	5'GCACCTGCACACCTGGAT3'	FAM-ACAACGGAGGCGGGG-MGB
BCL-2-R*	5'CGCATGCTAGGGCCATACAG3'	

*F- forward; *R-reverse

2.3.6 TUNEL labeling

The TUNEL assay was performed using the In Situ Cell Death Detection Kit (Fluorescein; Roche Diagnostics Corp.; Indianapolis, IN, USA; Cat. 1684795) as described (Walters et al., 2005), with slight modification. Briefly, blastocysts at d 7 of culture were washed 3 times in 100 µL drops of PBS with 1 mg/mL PVP (PBS-PVP), and fixed in 4% (w/v) paraformaldehyde solution [4% (w/v) in PBS, pH 7.4] for 1 h at room temperature, and stored at 4°C for one month. On the day of assay, the fixed embryos were removed from cold storage, allowed to return to room temperature and then

incubated in permeabilization solution [0.5% (v/v) Triton X-100, 0.1% (w/v) sodium citrate] for 30 min at room temperature.

After washing 3 times in PBS-PVP, both the positive and negative controls were incubated in a 50 µL drop DNase (50 U/mL) at 37°C for ~20 min. Similar to treatments, positive control embryos were placed in 25 µL drops of TUNEL reaction mixture (fluorescein isothiocyanate stain; FITC) and incubated in the dark in a humidified chamber for 1 h at 37°C. The negative controls were incubated in micro droplets of fluorescent label in the absence of the enzyme terminal transferase. Following the TUNEL reaction the embryos were washed twice in PBS-PVP and incubated in 500 µL of RNase A (50 µg/mL) for 1 h at room temperature in the dark. Propidium iodide (PI) was used for counterstaining by incubation of the treatment and positive controls in 500 µL of propidium iodide (Roche Diagnostics Corp.; 0.5 µg/mL) for 30 min at room temperature in the dark. The embryos were washed in PBS-PVP to remove excess propidium iodide. The prepared embryos were placed in 2-3 µL droplets of 0.1% PBS-PVP and covered with mineral oil. The embryos were examined by using laser scanning confocal microscopy (LSCM).

An argon laser at 488 and 568 nm wavelengths and two-channel scanning was used to detect FITC and propidium iodide, respectively. The image stacks were reconstructed with the Zeiss LSM 510 Software (Version 3.2). The total number of nuclei per embryo and the number of TUNEL-positive nuclei were counted separately. The collapsed 3-D image of each embryo was copied into Microsoft® Paint, version 5.1 and the image was divided into 4 equal sections. Total cell numbers and TUNEL-positive nuclei were counted per section and totaled.

Total cell number was indicated by the red fluorescence of propidium iodide bright green/yellow fluorescence indicated TUNEL positive (apoptotic) cells. The apoptotic index was calculated by dividing the number of TUNEL-positive cells by the total number of cells per embryo. Three replicates from each treatment were selected for the TUNEL assay.

2.3.7 Statistical Analysis

For experiments on the effect of cysteine in oocyte maturation media and growth factors in embryo culture media on the developmental capacity of bovine embryos, data were analyzed by using the PROC GLIMMIX procedure for cleavage, morulae, and blastocyst development rates in SAS (9.1). To analyze the variation in the occurrence of different developmental stages (cleavage through expanded/hatched blastocyst), each embryo was assigned a score of either 1 (embryo developed to the stage of interest) or 0 (embryo did not develop to the stage of interest). In PROC GLIMMIX, when analyzing for cysteine-growth factor effects on morula and blastocyst development, the estimation method used was the residual mean pseudo-likelihood (RMPL) because there were too many comparisons across treatments for the default (residual solution pseudo-likelihood; (RSPL); for growth factors alone on cleavage and blastocyst development, the method used was RSPL. The effects included in the model were treatment (fixed effect), replicate, and the interaction between treatment and replicate. Dependent variables were percentage of embryos cleaved, percentage of morulae, and percentage of blastocysts. Cleaved embryo, morula and blastocyst data are presented as percentages of development for each treatment, and were considered significant at $P < 0.05$. For gene transcript analysis, ΔCt values were calculated for all treatments, and for each day the FCS ΔCt was

subtracted from each treatment ΔCt (making the treatment data relative to FCS when calculating the $2^{-\Delta\Delta Ct}$ fold change for treatments). This put FCS equal to 1 and excluded FCS from statistical analysis. Gene transcript data were then analyzed for normal distribution. Fold changes in gene expression for treatments relative to FCS were calculated using the $2^{-\Delta\Delta Ct}$ method, and were analyzed using the PROC MIXED procedure of SAS, and were considered significant at $P < 0.05$. Total cells and apoptotic nuclei were counted and an apoptotic index was calculated. Analysis of treatment effects on total cell number, apoptotic nuclei, and apoptotic index were analyzed by PROC GLIMMIX in SAS. The model included the independent variable treatment and the dependent variables: total cell number, apoptotic nuclei, and apoptotic index.

2.4 RESULTS

2.4.1 Effect of cysteine on embryonic development

The developmental rates for cleaved embryos and cleaved embryos that developed to morulae are in Table 2-6. The 12 h IGF-I+EGF treatment yielded the highest percentage (75.7%) of cleaved embryos and was followed by the treatments EGF (69.1%), 0 h IGF-I+EGF (68.7%), and IGF-I+EGF (67.5%). The treatments 12 h IGF-I, IGF-I, 0 h EGF, 12 h EGF, and 0 h IGF-I had 66.5, 65.9, 65.1, 64.7 and 61.9% cleaved embryos, respectively,. The FCS control had the lowest percentage (61.2%) of cleaved embryos. There was no significant difference ($P < 0.05$) in the percentage of embryos that cleaved between treatments from the total number of embryos placed in culture. However, there was a significant difference ($P = 0.03$) among some treatments in comparison to the control (FCS) for the percentage of cleaved embryos that developed to the morula stage. The 12 h IGF-I+EGF treatment yielded the highest percentage (71.2%)

of morulae, and was followed by the treatments 0 h IGF (69.0%), 0 h IGF-I+EGF (69.0%), 12 h IGF-I (67.0%), and 0 h EGF (66.3%). In contrast, the treatments 12 h EGF, FCS, IGF+EGF, EGF and IGF resulted in lower percentages of morulae (61.3, 61.0, 57.0, 55.3, and 50.0%, respectively). Further statistical analysis using the PROC GLIMMIX on cleaved-to-morula rates resulted in odds ratios of 1.6, 1.4, 1.4, 1.3, 1.3, 1.02, 0.85, 0.80, and 0.64 for 12 h IGF-EGF, 0 h IGF, 0 h IGF-EGF, 12 h IGF, 0 h EGF, 12 h EGF, IGF-EGF, EGF, and IGF, respectively, compared to FCS ($P < 0.05$). Odds ratios for the likelihood that cleaved embryos in a particular treatment would develop to the morula stage for any of the treatments were not statistically different from FCS, as the odds ratios were within their lower and upper limits.

The percentages of blastocyst development for each treatment and the control are in Figure 2-1. There was no significant difference ($P < 0.05$) in the rate of blastocyst development across treatments, nor was there a significant difference ($P < 0.05$) in the rate of blastocyst formation when the oocytes were matured with cysteine addition at 0 or 12 h. The treatment 0 h IGF-I yielded 39% blastocyst development followed by EGF, 12 h IGF-I, and 0 h IGF-I+EGF (36.1, 35, and 33.3%, respectively). The IGF-I treatment had 23.7% blastocyst development, while IGF-I+EGF, 12 h IGF-I+EGF, 12 h EGF, FCS, and 0h EGF had 32.4, 31.5, 31.4, 30.6, and 26.7% blastocysts, respectively.

Table 2- 6. Cleavage and embryo development to the morula stage for treatment with (0 [0 h] and 12 [12 h]) and without cysteine during in vitro maturation and subsequent in vitro culture with IGF-I (IGF), epidermal growth factor (EGF) or combined IGF + EGF (IGFEGF).

Treatment	Embryos per treatment	Percent Cleaved	Percent Morulae of Cleaved Embryos*
FCS	170	61.2	61.0
IGF	179	65.9	50.0
EGF	139	69.1	55.3
IGF+EGF	160	67.5	57.0
12 IGF	221	66.5	67.0
12 EGF	238	64.7	61.3
12 IGF+EGF	214	75.7	71.2
0 IGF	239	61.9	69.0
0 EGF	238	65.1	66.3
0 IGF+EGF	233	68.7	69.0
Total Embryos	2,031	1,352	1,352
P-value		0.12	0.03

*Further development from cleaved to morulae stage is based on the number of cleaved embryos.
**Significant difference across treatments for morulae development.

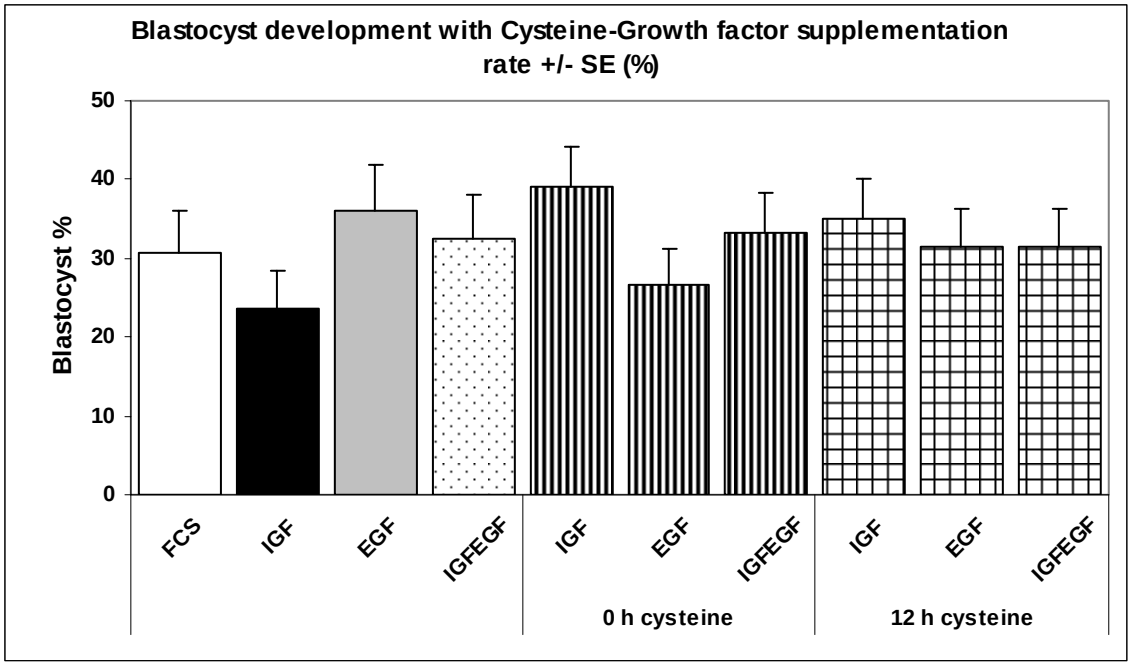

Figure 2- 2. Blastocyst development for treatment with cysteine (0 [0 h] and 12 [12 h] oocyte maturation) and without cysteine during in vitro maturation and subsequent in vitro culture with IGF-I (IGF), epidermal growth factor (EGF) or combined IGF + EGF (IGFEGF), $P < 0.05$.

2.4.2 Effect of growth factors on embryonic development

The developmental rates for cleaved embryos and cleaved embryos that developed to morulae are in Table 2-7. Data were analyzed excluding maturation treatments because there was no significant effect of cysteine supplemented during maturation at either 0 or 12 h on blastocyst development. When combining all culture treatments, there was not a significant difference in the percentage of embryos that cleaved across treatments or in the percent of cleaved embryos that developed to morulae. The control treatment (FCS) had the lowest percentage of embryos cleave (61.2%), and IGF-I+EGF had the highest percentage of embryos that cleaved (66.5%). Even though the control had the lowest percentage of embryos that cleaved, it did result in 34.6% morula development, suggesting that FCS delays embryonic cleavage in comparison to the other treatments. The IGF-I+EGF treatment resulted in 35.8% percentage of morulae, followed by FCS, IGF-I and EGF with the least embryos to develop to morulae. Further statistical analysis using PROC GLIMMIX on cleavage rates resulted in odds ratios of 1.1, 1.2, and 1.3 for IGF-I, EGF and IGF-I+EGF, respectively, compared to FCS ($P < 0.05$). Odds ratios for the likelihood that embryos in a particular treatment would cleave for any of the treatments were not statistically different from FCS, as the odds ratios were within their lower and upper limits.

Table 2-7. Cleavage and development to morulae with treatment of FCS, IGF-I (IGF), epidermal growth factor (EGF) and combined treatments of IGF+EGF after combining treatments with or without cysteine.

Treatment	Number of Embryos per Treatment	Percent Cleaved	Percent Morulae of Cleaved**
FCS	170	61.2	34.6
IGF	639	64.6	30.8
EGF	615	64.0	30.9
IGF+EGF	607	66.5	35.8
Total Embryos	2,031	1,352	1,352
P-value		0.6	0.4

PROC GLIMMIX P-values are significantly different at $P<0.05$.
**Further embryonic development to morulae stage is based on the number of cleaved embryos.

The percentage of blastocyst development for each culture treatment and the control are shown in Figure 2-2. Data were analyzed excluding maturation treatments because there was no significant effect of cysteine supplemented during maturation at either 0 or 12 h maturation on blastocyst development. There was no difference ($P < 0.05$) in the rate of blastocyst development across treatments, and the combined culture treatments of IGF-I (33.0%) and IGF-I+EGF (32.6%) developed to the blastocyst stage, while EGF and FCS had 30.6 and 30.5% blastocysts, respectfully. Additional statistical analysis using the PROC GLIMMIX resulted in similar odds ratios of 1.13, 1.0, and 1.1 for IGF-I, EGF and IGF-I+EGF, respectively, compared to FCS ($P < 0.05$).

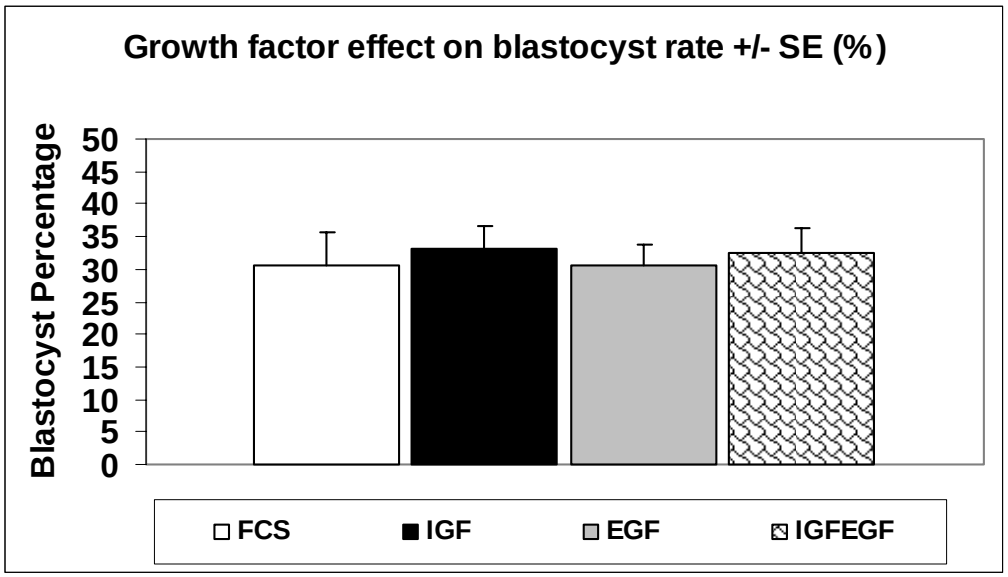

Figure 2- 3. Development to blastocyst with treatment of FCS, IGF-I (IGF), epidermal growth factor (EGF) and combined treatments of IGF+EGF after combining treatments with or without cysteine. $P < 0.05$

2.4.3 Expression of apoptotic and oxidative stress genes

The expression of two apoptotic genes, Bax and Bcl-2, and oxidative stress genes, SOD1 and SOD2, were quantified in developmentally arrested morulae and blastocysts cultured in IGF-I, EGF, and IGF-I+EGF at 7 d of culture. The embryos cultured in FCS were used as the control. The data for Bcl-2 gene expression was not analyzed because though the Bcl-2 transcript was consistently detected in the FCS embryos only one of three replicates showed expression in IGF-I, EGF and IGF-I+EGF treated embryos The same primer sequence for Bcl-2 was used for the quantification of Bcl-2 in bovine oocytes earlier (Anchamparuthy, 2007) and was consistently expressed in the tested samples. Strikingly, a 4-fold level of expression of Bax was detected in the IGF-I treated embryos in the arrested morulae (Figure 2-4), but was not statistically significant from any of the treatments or the FCS control. Similarly, Bax expression was up-regulated in the EGF and IGF+EGF treated embryos, 1.3 and 1.5-fold, respectively, compared to the FCS treated control. The oxidative stress mRNAs, SOD1 and SOD2, showed a uniform

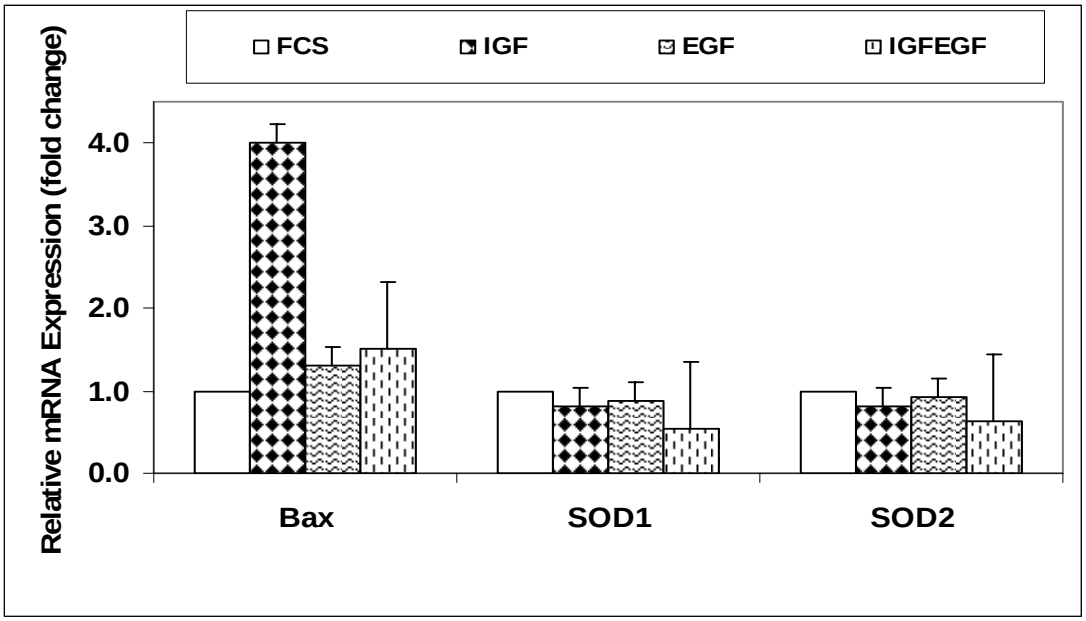

Figure 2- 4. Differential expression of apoptotic and oxidative stress genes in bovine morulae with 7 d culture treatment of FCS, IGF-I (IGF), epidermal growth factor (EGF) and combined treatments of IGF+EGF after combining treatments with or without cysteine. Data are shown as mean ± SEM, ($P < 0.05$) of 3 replicates.

decrease in the level of expression in the morulae for treatments IGF-I, EGF and IGF-I+EGF, relative to FCS. The levels of SOD1 in IGF-I, EGF, and IGF-I+EGF treated embryos were 0.81, 0.88, and 0.50, respectively, compared to FCS, but was not significantly different. A similar pattern of expression was seen for SOD2 and was 0.81 0.92 and 0.62, respectively, in the IGF-I, EGF and IGF-I+EGF treated morulae relative to the FCS control.

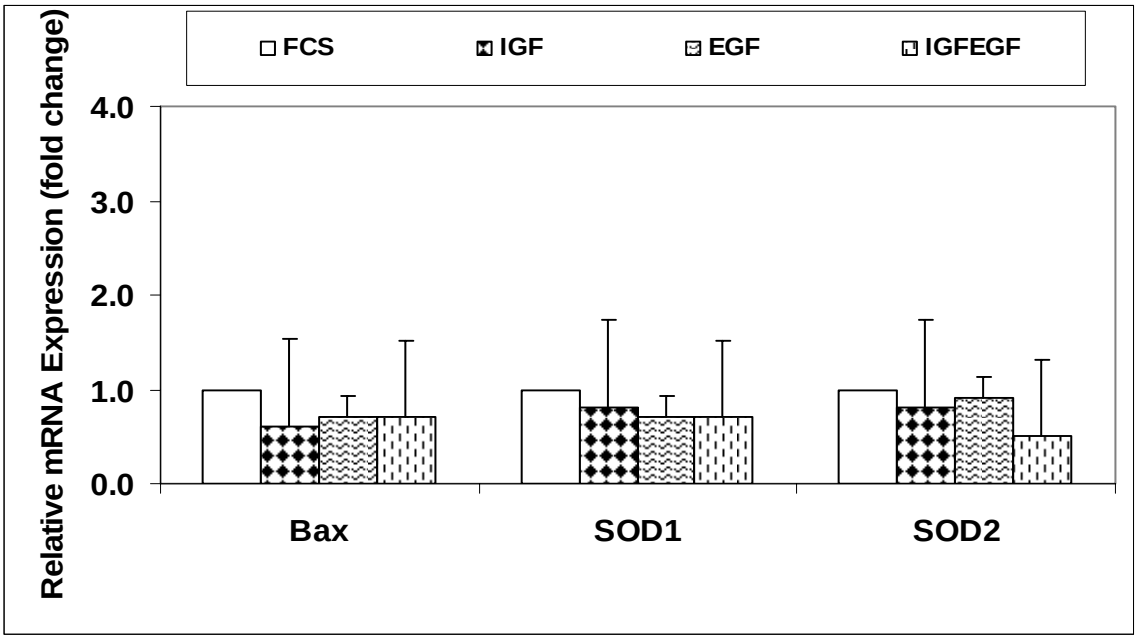

Figure 2- 5. Differential expression of apoptotic and oxidative stress genes in bovine blastocysts with 7 d culture treatment of FCS, IGF-I (IGF), epidermal growth factor (EGF) and combined treatments of IGF+EGF after combining treatments with or without cysteine. Data are shown as mean ± SEM, ($P < 0.05$) of 3 replicates.

The normal appearing blastocysts at d 7 showed a uniform pattern in the expression of both Bax and oxidative stress genes (Figure 2-4). The level of Bax mRNAs was 0.6, 0.7, and 0.7-fold in the IGF-I, EGF, and IGF-I+EGF treatment groups, respectively, compared to FCS cultured embryos. The level of SOD1 showed 0.8, 0.7, and 0.7-fold expression, respectively, relative to FCS control. Similarly, the level of SOD2 also fell to 0.8, 0.9, and 0.5-fold in the IGF-I, EGF, and IGF-I+EGF treatment groups, respectively, compared to FCS control.

2.4.4 Total Cells and Apoptotic Index

Representative digital images of TUNEL-positive cells in d 7 embryos are depicted in Figure 2-5 to 2-8.

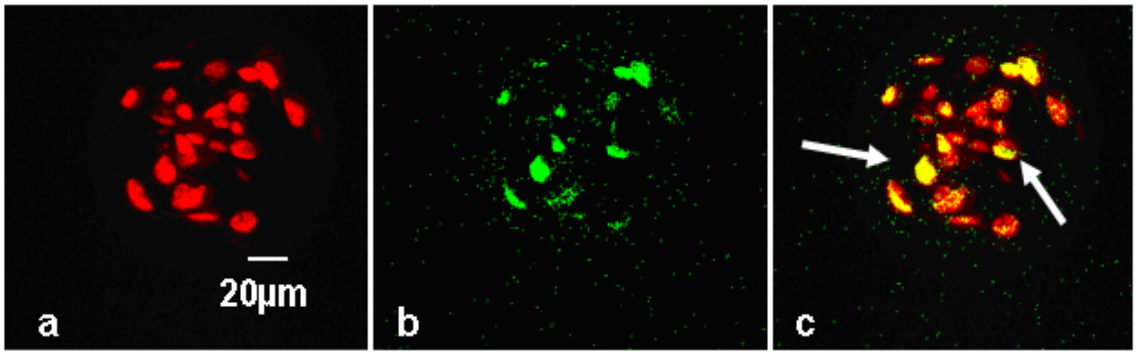

Figure 2- 6. Representative confocal image of a 7 d blastocyst in the FCS treatment illustrating the frequency of apoptotic nuclei subjected to TUNEL analysis. Reduced number of cells and many apoptotic nuclei. a) propidium iodide (red) b) fluorescein isothiocyanate (green) c) superimposed image. Arrows point to TUNEL positive nuclei (yellow).

Figure 2- 7. Representative confocal image of a 7 d blastocyst in the IGF-I treatment illustrating the frequency of apoptotic nuclei subjected to TUNEL analysis. Increased number of cells and few apoptotic nuclei. a) propidium iodide (red) b) no fluorescein isothiocyanate image c) superimposed image. No TUNEL positive nuclei.

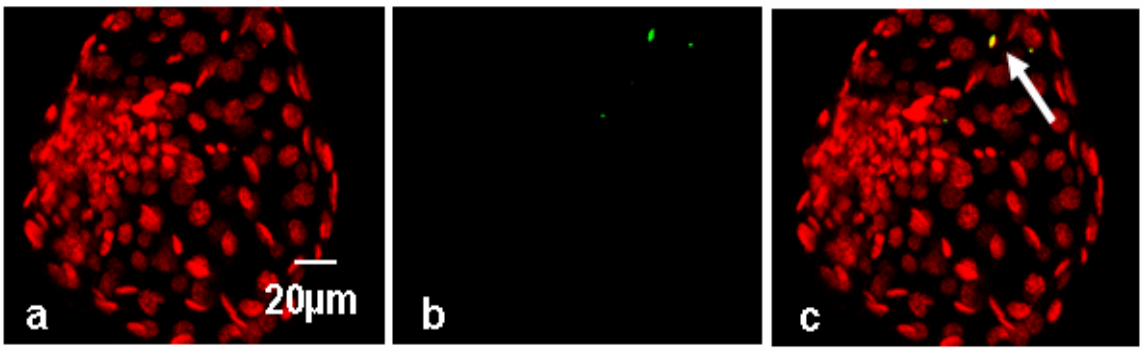

Figure 2- 8. Representative confocal image of a 7 d blastocyst in the IGF-I+EGF treatment illustrating the frequency of apoptotic nuclei subjected to TUNEL analysis. Increased number of cells and few apoptotic nuclei. a) propidium iodide (red) b) fluorescein isothiocyanate (green) c) superimposed image. Arrow points to TUNEL positive nucleus (yellow).

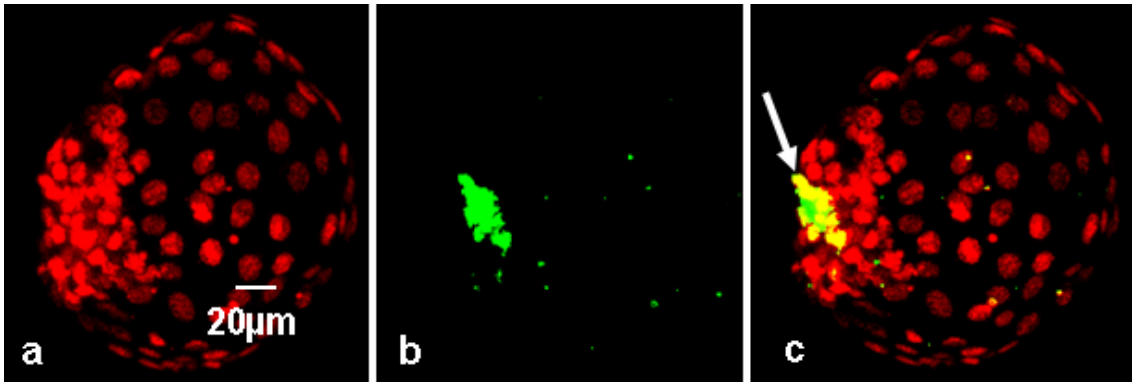

Figure 2- 9. Representative confocal image of a 7 d blastocyst in the EGF treatment illustrating the frequency of apoptotic nuclei subjected to TUNEL analysis. Increased number of cells and several apoptotic nuclei. a) propidium iodide (red) b) fluorescein isothiocyanate (green) c) superimposed image. Arrows point to TUNEL positive nuclei (yellow).

The least squares means and standard errors are shown in Table 2-8 for each culture treatment and the control. Data were analyzed excluding IVM treatments because there was no significant effect of cysteine supplemented during IVM at either 0 or 12 h of maturation on blastocyst development. There was no difference ($P < 0.05$) in the total cell number of blastocysts across treatments, but the combinatorial effect of IGF-I+EGF resulted in 152.2 total cells, while EGF and IGF-I alone resulted in 129.6 and 121.2 total cells, respectively, and FCS treatment yielded 98 total cells. There was no difference ($P < 0.05$) in the number of apoptotic cells in the blastocysts across treatments. The treatments of EGF and IGF-I+EGF resulted in 10 and 8 apoptotic nuclei, while IGF-I and FCS resulted in similar apoptotic nuclei of 6.4 and 6, respectively. The IGF-I treatment resulted in an apoptotic index of 5.0%, while IGF-I+EGF and EGF resulted in indices of 5.0% and 8.0, respectively. The FCS treatment resulted in an apoptotic index of 6.0%. Odds ratios for the likelihood that embryos in a particular treatment would develop apoptotic cells were not statistically different for treatments, as the odds ratios were within their lower and upper limits. The treatment IGF-I had an odds ratio of 0.86, and

was less likely to develop apoptotic cells when compared to FCS. The treatment of EGF had an odds ratio of 1.3, and was 0.3 times more likely to develop apoptotic cells when compared to FCS. The treatment of IGF-I+EGF had an odds ratio of 0.85, and was less likely to develop apoptotic cells when compared to FCS.

Table 2- 8. Least squares means (± SE) and odds ratios of total cells, apoptotic cells, and apoptotic index with treatment of FCS, IGF-I (IGF), epidermal growth factor (EGF).

Treatment	Total Cells	SE	Apoptotic Cells	SE	Apoptotic Index (%)	SE	Odds Ratio	Odds Ratio Lower Limit	Odds Ratio Upper Limit
FCS	98	23.5	6.0	3.1	6.0	0.40	-		
IGF	121.2	16.6	6.4	2.2	5.0	0.03	0.86	0.50	1.5
EGF	129.6	17.5	10.00	2.3	8.0	0.03	1.30	0.76	2.2
IGF+EGF	152.3	15.2	8.0	2.0	5.0	0.02	0.85	0.51	1.4
P-value	0.25		0.65		0.05				

($P < 0.05$)

2.5 DISCUSSION

Recent studies have focused on amino acid or growth factor supplementation to maturation and culture media of bovine oocytes and embryos. Preimplantation bovine embryos can develop in an in vitro system, but they are subjected to a number of stressors that retard or inhibit development. These embryos also require a precisely defined culture media, to which supplements are added for embryonic protection and development.

2.5.1 Influence of cysteine on embryonic development

The present study was carried out to determine the time dependent effect of cysteine (0.6 mM) in conjunction with growth factors in embryo culture on in vitro maturation, fertilization, and subsequent embryonic development of bovine oocytes. Our results showed that time-dependent cysteine supplementation during IVM in combination with growth factors in culture have no significant impact on cleavage or the development of embryos to blastocyst stages. Yet, there were differences in morula development from

cleaved embryos. The combinatorial effect of cysteine and growth factors added to IVM and IVC, respectively, improved early phases of in vitro development of bovine embryos. Cysteine is a required external substrate for GSH synthesis in maturing bovine oocytes (deMatos et al., 1996). Increases in GSH provide in vitro matured oocytes with large reserves of GSH available for protection of the embryo until it reaches blastocyst stage (deMatos et al., 1995; Gardiner et al., 1995). A higher developmental rate of bovine in vitro embryos was reported when maturation medium was supplemented with cysteine (de Matos et al., 1995, 1996; de Matos and Furnus 2000). Furthermore, a time dependant effect of cysteine was reported for porcine oocytes cultured in serum free maturation medium, but was not reported for bovine oocytes. The requirement was critical only between 42 and 48 h of maturation when the porcine oocytes are in the late M-I to M-II stage of development (Sawai et al., 1997). Our study did not support a time dependant influence of cysteine in IVM of bovine oocytes, possibly because the maturation period is about one-half the time needed for porcine oocytes. Yet, the beneficial effect of cysteine at 0 h in IVM in conjunction with IGF and IGF-I+EGF, and at 12 h with IGF-I+EGF in IVC on vitro development of embryos can not be disregarded at this point, because even though development to blastocyst results were not significantly different for these two treatments in comparison to FCS a greater percentage of embryos developed to the blastocyst stage.

2.5.2 Influence of growth factors on in vitro embryonic development

In the present study we investigated the effects of growth factors, IGF-I, EGF and IGF-I+EGF added to d 1 and d 4 media of embryo culture. Since we did not find significantly different effects of cysteine on in vitro embryonic development to the

blastocyst stage, we combined all culture treatments to exclude the maturation treatments (supplementation of cysteine to maturation media at either 0 h or 12 h of oocyte maturation) for analysis. There was not a significant difference in the cleavage rate among treatments, or for blastocyst development of the embryos that cleaved. The IGF-I+EGF combination resulted in highest cleavage rate (66.5%) in contrast to 61.2% for the FCS treatment, the least cleaved embryos among the treatments. However, IGF-I and IGF-I+EGF yielded similar blastocyst developmental rates (33.0 and 32.6% respectively), whereas the FCS treatment resulted in 30.5% blastocyst developmental rates. Growth factors, IGF-I and EGF were reported to have mitogenic effect, inducing a positive effect on pre-implantation development, and stimulating growth and metabolism of embryos (Martin et al., 1998). Present results contrast earlier reports of improvement in the blastocyst rate with the added IGF-I (Matusi et al., 1995; Prelle et al., 2001) without observing significant positive effects on the cleavage rates. It was reported that the EGF bound to embryonic cell surfaces at a later stage of growth (8-cell to blastocyst) and improved the blastocyst development (Paria and Dey, 1990; Schultz and Dardik, 1991). Furthermore, Herrler et al (1998) showed that in bovine embryos, IGF-I receptors were expressed from the morula stage onwards and the embryos were responding to IGF-I at this stage of embryonic growth. Our results indicate there is a synergistic action of IGF-I+EGF over either IGF-I or EGF alone for improved cleavage rates. This suggests that the defined growth factors either alone or in combination are at least as good or in some cases allow for faster development during early bovine embryo culture as the inconsistent FCS media supplements. As described by Burgaud and Baserga (1996) interactions or overlapping between IGF-I and EGF-signaling pathways might have exerted a positive

mitogenic effect on the post-fertilization embryos. The results indicate that the inclusion of growth factors either singly or in combination improved early embryonic development; IGF-I and EGF may act synergistically on bovine embryo development in vitro.

2.5.3 Expression of apoptotic and oxidative stress genes

Viuff et al. (2001) reported that suboptimal maturation, fertilization, and culture conditions can induce nuclear and chromosomal abnormalities, which can trigger apoptosis. Leunda Casi et al. (2002) found that embryonic stress during in vitro culture due to oxidative stress can lead to apoptosis. Taken together, these findings clearly show an interrelation between oxidative stress due to suboptimal conditions and apoptosis. So we tested the influence of IVC conditions on the abundance of 2 apoptotic genes, Bax and Bcl-2, and 2 oxidative stress genes, SOD1 and SOD2. A proper expression of developmentally important genes in a well-orchestrated manner is essential for appropriate development of an embryo. The up- or down-regulation of developmentally important genes can occur due to suboptimal culture conditions like nutritional imbalance and oxidative stress.

Due to the inconsistent expression of Bcl-2 we could not analyze fold expression of this gene. A similar failure of detection of Bcl-2 gene expression in viable human embryos was reported by Liu et al. (2000), suggesting the failure of detection of Bcl-2 may be due to the degradation of mRNA for Bcl-2, thereby becoming virtually undetectable. Bax is an apoptotic regulatory gene, which induces the release of cytochrome C from the mitochondria and further induces caspase-dependent DNA degradation. There were 0.6, 0.7, and 0.7-fold changes in the Bax transcript expression in

the IGF-I, EGF, and IGF-I+EGF treated blastocysts compared to control (FCS) blastocyst. This may be due to the role of IGF-I as an apoptotic survival factor in human embryos as explained by Spanos et al (2000). Similarly, there were 0.8, 0.7, and 0.7-fold changes in SOD1 and 0.8, 0.9, and 0.5-fold changes in the level of expression of SOD2 in the IGF-I, EGF, and IGF-I+EGF treated embryos relative to control (FCS). Super oxide dismutase enzymes are the first line of defense against oxygen-derived radicals (Michiels et al., 1994) essential for protecting embryos against oxidative insult (Johnson and Nasr-Esfahani, 1994). The expression of antioxidant enzyme genes is stimulated by oxidative stress (Allen and Tresini, 2000; Maitre et al., 1993). Kurzawa et al. (2004) reported that growth factors minimize the negative effects produced by the reactive oxygen species on cultured embryos. This is supported by transcript expression of SOD1 and SOD2, which could otherwise be stimulated by the accumulation of ROS. Similar effects of reducing the oxidative stress using EGF and IGF-I were reported (Kurzawa et al., 2004).

The arrested morulae at d 7 in IGF-I, EGF, and IGF-I+EGF inclusions showed a high level of expression of Bax, up to 4-fold, relative to FCS. Gutierrez-Adan et al. (2004) reported that genes that are stress-induced such as Bax were highly transcribed in slow developing in vitro produced embryos. In agreement with this view, it is noteworthy that in our study the IGF-I treatment had the lowest percentage of cleaved embryos that developed to morulae, but the highest percentage of blastocyst development. Hence, this transcription pattern may be connected to low quality of embryos with slow developmental speed, though we have not assessed the kinetics of embryo development. Kinetics of development can depend on culture conditions. In support, Hardy et al (1989) suggested that uncontrolled apoptosis or programmed cell death occurring earlier than a

blastocyst stage in pre-implantation embryos may lead to embryo demise. Yet, there was no concomitant increase in the transcript expression of SOD1 and SOD2 in the arrested morulae relative to FCS. A culture-dependant expression of SOD2 was reported (Lequarre et al., 2001) in bovine embryos and expression increased with serum for SOD2. The low level of SOD2 can be due to low mitochondrial activity as described by Farin et al (2001), who reported that the mitochondrial population in embryos produced using in vitro culture systems is compromised. In conclusion, the observed alterations in mRNA expression may be directly linked with the quality of embryos.

2.5.4 Total Cells and Apoptotic Index

DNA fragmentation as a hallmark of the final stage of apoptosis is one of the important quality markers for embryonic development. The occurrence of DNA fragmentation detectable with the TUNEL reaction was assessed in this study. Though there was no significant impact on the total cell number, apoptotic nuclei, and apoptotic index, our results showed that IGF-I+EGF treatment had 152.3 total number of cells compared to 98 for FCS, suggesting that the synergistic treatment effect had a mitogenic activity on the developing embryos. Our study also showed that the apoptotic index for IGF-I alone was 0.05 and was 0.06 for the IGF-I+EGF combination in contrast to 0.10 for FCS. The post implantation developmental potential or embryo quality is likely affected by apoptotic incidence in pre-implantation stages. This quality index could be further complemented by our other quality marker, apoptotic stress gene Bax expression, and developmental potential of embryos. Considering the quality marker results obtained for growth factors, our study shows that growth factor treatment provided a positive culture condition for production of quality embryos equal to FCS.

A negative correlation between embryonic cell number and incidence of the TUNEL reaction has been established in bovine embryos (Byrne et al., 1999). The incidence of apoptosis in bovine (Neuber et al., 2002) and human (Levy et al., 2001) blastocysts correlated with cell number and embryo quality. Blastocysts with fewer cells showed a range of TUNEL positive cells from 0 to 30%; whereas blastocysts with more cells had less than 10% TUNEL-positive cells (Levy et al., 2001). Our results support the findings of Wuu et al. (1999), whose culture conditions decreased embryonic cell number and increased the apoptotic incidence and also decreased implantation rates, increased fetal resorption, and resulted in lower fetal birth weight upon embryo transfer.

2.5.5 Summary

Although rates for blastocysts development were not different among treatments, an increased proportion of embryos attaining the morula stage of development was achieved when cysteine was added to the maturation media compared to the control (FCS). When cysteine treatments were combined because they did not affect blastocyst development, cleavage was greater for IGF-I+EGF compared to FCS. No significant treatment effect was observed on the expression of apoptotic and oxidative stress genes in morulae or blastocysts at d 7 of in vitro development. While Bax was expressed strongly (4-fold) in morulae with the addition of IGF-I, it was less prevalent in all other morula and blastocyst groups relative to FCS. There was slightly less expression of both SOD1 and SOD2 with treatments compared to FCS in morulae and blastocysts, which could indicate low mitochondrial activity and/or a low level of oxidative stress in treatments. There was no treatment effect on embryo quality based on total cell number, apoptotic nuclei, or apoptotic index. In conclusion, supplementation of cysteine during IVM of

oocytes, in conjunction with growth factors could effectively be used as a replacement for FCS.

2.5.6 Conclusions

Many attempts have been made to improve IVP of bovine embryos. Although FCS is the most common supplement used for IVM, IVF, and culture media, it is an undefined medium and highly variable. Therefore, creating defined media that result in embryonic development similar to that of the in vivo environment and the current in vitro situation, when supplemented with FCS, is important for developmental consistency and improvements for the IVP system. In our study, we identified supplements to IVM and IVC media that resulted in embryonic development similar to embryos only cultured with FCS. Additionally, our supplements yielded improvements on total cell numbers in comparison to FCS. Future studies of this nature should test a system where cysteine is included in oocyte maturation media over a greater time range and in embryo culture media along with growth factors at pre-designated time intervals. Also, the in vitro embryonic development using the defined supplements of cysteine, IGF-I and EGF should be compared to that of the in vivo situation. Additionally, oxidative stress (in normal IVC situations or induced) and GSH levels should be investigated.

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