# INSULIN-LIKE GROWTH FACTOR-I AND APOPTOSIS AS DETERMINANTS OF PREIMPLANTATION BOVINE EMBRYONIC DEVELOPMENT

By

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This dissertation is dedicated to my parents and family.

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| | |
|---|---|
| AI | Artificial insemination |
| Apaf-1 | Apoptotic protease activating factor-1 |
| ATP | adenosine triphosphate |
| bST | Bovine somatotropin |
| CCCP | Carbonyl cyanide 3-chlorophenylhydrazone |
| CL | Corpus luteum |
| COCs | Cumulus-oocyte complexes |
| DNA | deoxyribonucleic acid |
| ET | Embryo transfer |
| FSH | Follicle-stimulating hormone |
| GDP | Guanine diphosphate |
| GLUT | Glucose transporter |
| GnRH | Gonadotropin-releasing hormone |
| Grb2 | Growth factor receptor-bound protein 2 |
| GTP | Guanine triphosphate |
| hCG | Human chorionic gonadotropin |
| HIMO | 1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate |
| HSP | Heat shock protein |
| ICM | Inner cell mass |

| | |
|---|---|
| IGFBP | Insulin-like growth factor binding protein |
| IGF-I | Insulin-like growth factor-I |
| IRS-1 | Insulin receptor substrate-1 |
| KSOM | potassium simplex optimized medium |
| LH | Luteinizing hormone |
| MAPKK | Mitogen-activated protein kinase kinase |
| NF-κB | Nuclear factor kappa B |
| PBS | phosphate buffer saline |
| PDK-1 | Phosphoinositide-dependent kinase-1 |
| $PGF_{2\alpha}$ | Prostaglandin $F_{2\alpha}$ |
| PI3K | Phosphatidylinositol 3-kinase |
| PIP3 | Phosphatidylinositol 3,4,5-triphosphate |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| SH2 | src homology 2 |
| SOS | Son of Sevenless |
| ST | Somatotropin |
| TAI | Timed artificial insemination |
| TET | Timed embryo transfer |
| TNF-α | Tumor necrosis factor-α |
| TUNEL | Terminal deoxynucleotidyl transferase mediated dUTP nick end labeling |

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# INSULIN-LIKE GROWTH FACTOR-I AND APOPTOSIS AS DETERMINANTS OF PREIMPLANTATION BOVINE EMBRYONIC DEVELOPMENT

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The overall goal of this dissertation was to understand determinants of preimplantation bovine embryonic survival, particularly following heat shock. There were two main areas of focus – the role of insulin-like growth factor-I (IGF-I) as a cytoprotective molecule for the bovine embryo subjected to heat shock and the impact of apoptosis as a determinant of embryonic survival following heat shock as well as a predictor of embryonic potential for continued development. It was demonstrated in several experiments that IGF-I blocked the effects of heat shock on apoptosis, total cell number and blastocyst development. Actions of IGF-I to block heat shock-induced apoptosis required signaling through the phosphatidylinositol 3-kinase pathway while actions to prevent reduction in cell number required signaling through the mitogen activated protein kinase kinase pathway. Moreover, IGF-I was able to block effects of heat shock on blastocyst development independent of its ability to block apoptosis.

A field study was conducted to determine whether administration of bovine somatotropin (bST) to lactating cows during heat stress would enhance first- and second-service pregnancy rates. It was hypothesized that bST would have such effects because it increases IGF-I secretion. However, while bST increased circulating concentrations of IGF-I and milk yield, there was no effect on fertility, probably because fertility-enhancing actions of bST were counteracted by an increase in rectal temperature experienced in bST-treated cows.

While IGF-I protected embryos from heat shock independent of its actions to block apoptosis, results of the final experiment indicated that the degree of apoptosis can be a determinant of embryonic survival. In particular, it was shown that blastocysts at d 7 or 8 post-insemination that had low group II caspase activity had higher hatching rates at d 10 than blastocysts with medium or high caspase activity.

Taken together, results establish IGF-I as a cytoprotective factor for the bovine embryo subjected to heat shock and the importance of degree of apoptosis as a determinant of embryonic survival.

# CHAPTER 1
# INTRODUCTION

The fate of the mammalian embryo is first determined at fertilization by its cytoplasmic and nuclear inheritance from the oocyte and sperm. It is then further delineated as the embryo progresses through a series of developmental changes starting at the 1-cell stage. Errors in development, whether caused by genetic, epigenetic, or environmental factors, can result in failure to establish pregnancy or early termination of pregnancy. One of the most critical periods of development occurs during the first week of pregnancy when the embryo advances through a series of cleavage divisions from an undifferentiated 1-cell embryo to a blastocyst that has differentiated into two cell types, the inner cell mass (ICM) and the trophectoderm, and ultimately hatches from the confines of the zona pellucida. During this initial week of development, the embryo is dependent on the oviduct and uterus to establish an environment conducive for development, and early embryonic mortality may occur if such an environment is not established.

In a review of literature, Santos et al. (2004c) found that the fertilization rate of embryos flushed from the uterus of lactating dairy cattle between d 5 to 6 after insemination was 76%. That the period of early embryonic development is a critical one is shown by the fact that only 66% of the fertilized embryos recovered were considered viable, which equated to about 50% of the total structures recovered being classified as viable embryos (Santos et al., 2004c). This extensive early embryonic loss is a major

cause of the low conception rates at d 27 to 31 after artificial insemination (AI) in lactating dairy cattle (35 to 45%; Lucy, 2001; Stevenson, 2001; Santos et al., 2004c).

The already low fertility of lactating dairy cows can be compromised by various factors, including cyclic status (Cartmill et al., 2001), mastitis (Schrick et al., 2001), high crude protein content of the diet (Butler, 1998), and poor body condition (López-Gatius et al., 2002; Silke et al., 2002). Among the most deleterious environmental factors is thermal stress. Heat stress experienced by lactating dairy cows during the hotter months of the year compromises fertility (Rosenberg et al., 1977; Al-Katanani et al., 1999, López-Gatius, 2003) due to actions on the oocyte (Roth et al., 2001a; Sartori et al., 2002b) as well as the preimplantation embryo (Putney et al., 1988a). Attempts have been made to minimize the impact of heat stress on the fertility and performance of lactating cows through engineering evaporative cooling systems for cattle housing (Ray et al., 1992b; Ryan et al., 1992; West, 2003; Khongdee et al., 2006). Even when such cooling systems are employed, however, fertility is often compromised during the summer months (Hansen and Aréchiga, 1999; De Rensis and Scaramuzzi, 2003). It has been estimated that the total economic losses in Florida due to heat stress in dairy cows is $337/cow/yr (St-Pierre et al., 2003).

Our laboratory has utilized an in vitro heat shock model to study responses of the early bovine embryo to elevated temperatures. The advantage to using the model is that a large number of embryos can be produced, embryos can be selected at a particular developmental stage for study, and treatments can be easily and precisely applied. The model has been used to study the molecular and cellular basis for developmental changes in embryonic resistance to heat shock as well to understand how embryonic resistance to

heat shock can be enhanced. It appears that the model mimics, at least to some extent, the responses embryos undergo to hyperthermia in vivo. There are two lines of evidence supporting the model. First, the morphological responses embryos undergo in response to elevated temperature were similar for bovine 2-cell embryos produced in vitro and in vivo (Rivera et al., 2003). In addition, embryonic development was compromised by experimental application of diurnal changes in temperature to cultured bovine embryos (Rivera and Hansen, 2001; Sugiyama et al., 2003).

There are two major points of focus in this dissertation – on a cytoprotective molecule, insulin-like growth factor-I (IGF-I), and on a cellular process, apoptosis. Insulin-like growth factor-I is a polypeptide involved in endocrine and paracrine regulation of cellular function that has been implicated as a cytoprotective factor for many cells, including preimplantation embryos of the human (Lighten et al., 1998), rabbit (Herrler et al., 1998), mouse (Fabian et al., 2004), and bovine (Byrne et al., 2002b; Makarevich and Markkula, 2002). Insulin-like growth factor-I has been shown to block apoptosis in preimplantation embryos caused by ultraviolet irradiation (Herrler et al., 1998), campothecin and actinomycin D (Fabian et al., 2004), and tumor necrosis factor-α (TNF-α; Byrne et al., 2002a) and reduce the effects of hydrogen peroxide on the development of mouse preimplantation embryos (Kurzawa et al., 2002). If IGF-I exerts similar effects on preimplantation embryos, it is possible that approaches could be developed to expose the bovine embryo to IGF-I in vivo or in vitro to enhance its continued development during heat stress.

The cellular process of interest in this dissertation is apoptosis or programmed cell death. This process is a developmentally regulated one in bovine embryos, whether

determined by the appearance of spontaneous apoptosis (Gjørret et al., 2003) or in response to pro-apoptotic stimuli such as heat shock (Paula-Lopes and Hansen, 2002b), arsenic (Krininger et al., 2002), and TNF-α (Soto et al., 2003a). The consequences of apoptosis for the embryo are likely to depend upon the extent to which blastomeres undergo apoptosis. Extensive apoptosis encountered by the early embryo may result in decreased development. Slower-developing bovine embryos, based on the timing of first cleavage rate (36 or 48 h post-insemination), had reduced blastocyst rates at d 7 and a higher apoptotic cell ratio as compared to embryos that cleaved by 30 h post-insemination (Vandaele et al., 2005). Similarly, slower-cleaving bovine embryos, defined as being one cleavage division slower than faster-cleaving embryos (e.g., 8-cell versus 16-cell embryo on d 4 after insemination), had increased amounts of Bax transcripts as compared to faster-cleaving embryos that had higher amounts of IGF-I receptor and IGF-II mRNA (Gutiérrez-Adán et al., 2004). Limited induction of apoptosis in response to stress may be beneficial for continued development. Indeed, use of a group II caspase inhibitor to block heat-shock induced apoptosis had a detrimental effect on blastocyst development of heat-shocked embryos (Paula-Lopes and Hansen, 2002a). Perhaps embryonic development can be enhanced by regulating apoptosis of the preimplantation embryo through the addition of IGF-I to culture medium or by selecting embryos for continued development on the basis of caspase activity.

This dissertation will focus on the answer to this question, as well as determination of cytoprotective effects of IGF-I for the preimplantation embryo independent of apoptosis. In addition, practical consequences of these topics will be explored by determining whether administration of bovine somatotropin (bST) to enhance IGF-I

secretion can enhance fertility in lactating dairy cows and to determine whether analysis of blastocysts based on an index of apoptosis can be used to identify embryos with superior ability for continued development.

CHAPTER 2
LITERATURE REVIEW

## Heat Stress and Fertility in Cattle

### Effects of Maternal Heat Stress on Fertility in Lactating Dairy Cows

Fertility of lactating dairy cows is reduced during hot months of the year. Reduced pregnancy rates during hot weather have been observed in warm regions of the United States such as Florida (Gwazdauskas et al., 1975; Badinga et al., 1985; Cavestany et al., 1985; Al-Katanani et al., 1999; de Vries and Risco, 2005), Georgia (Al-Katanani et al., 1999; de Vries and Risco, 2005), Arizona (Ray et al., 1992a), and Texas (Thompson et al., 1996) as well as regions around the world such as Queensland, Australia (Orr et al., 1993), Spain (López-Gatius, 2003), and Israel (Zeron et al., 2001). Depression in fertility even occurs during hot weather in cool climates such as Minnesota (Udomprasert and Williamson, 1987).

There is evidence that the magnitude of heat stress effects on fertility is increasing. López-Gatius (2003) retrospectively analyzed pregnancy data over a 10-yr period from 1991 to 2000 for high-yielding dairy herds in northeastern Spain and found that fertility declined over time during the warm months of the year even though there was no change over time during the cool months of the year. It is likely that the increased susceptibility to heat stress has occurred because of increases in milk yield and the corresponding inability of the lactating female to regulate body temperature (Ray et al., 1992a). Indeed, the seasonal depression in 90-d non-return rate for lactating cows was more pronounced as milk yield increased (Al-Katanani et al., 1999).

## Mechanisms by Which Heat Stress Reduces Fertility

There are several mechanisms by which heat stress can decrease the fertility of lactating dairy cattle, including actions to disrupt estrus behavior, follicular development, function of reproductive tract tissues, oocyte competence and embryonic development. Based on in vitro studies, the embryo gains resistance to heat shock as development continues, and the effects of heat shock on the embryo may differ depending on the oxygen environment. Although estrus behavior does not impact fertility directly, failure to detect cows in estrus due to decreased expression of estrus caused by heat stress may reduce the cumulative increase in pregnancy rate over time.

### Estrus behavior

During summer, Holsteins in estrus had 4.5 mounts per estrus compared to 8.6 in winter (Nebel et al., 1997). The percentage of undetected estrus periods on a commercial Florida dairy was higher during June to September (76 to 82%) as compared to October to May (44 to 65%; Thatcher and Collier, 1986). Similarly, Rodtian et al. (1996) found that a greater frequency of silent estrus periods occurred for Holstein cows in the tropical environment of northern Thailand during the summer. One strategy to increase estrus behavior is to minimize the effects of heat stress on dairy cows, and short-term cooling of lactating Holstein cows was successful in increasing the number of cows showing standing estrus behavior while silent ovulations occurred more frequently for non-cooled cows (Her et al., 1988; Younas et al., 1993). While timed artificial insemination (TAI) programs eliminate the need for estrus detection and increase the proportion of heat-stressed cows eligible to be pregnant that become pregnant (Aréchiga et al., 1998; Cartmill et al., 2001), fertility itself is not enhanced.

Effects of heat stress on reduced expression of estrus could involve adrenocorticotropic hormones that interfere with estradiol-induced estrus behavior. Heat stress increased plasma cortisol secretion (Wise et al., 1988a; Elvinger et al., 1992) and injection of adrenocorticotropic hormone blocked estradiol-induced sexual behavior (Hein and Allrich, 1992). In other studies, though, heat stress did not increase serum cortisol concentration caused by heat stress (Wise et al., 1988b; West et al., 1991). Heat stress reduced peripheral estradiol concentrations at proestrus in some experiments (Gwazdauskas et al., 1981; Wilson et al., 1998a; Sartori et al., 2002a) but not in an experiment by Rosenberg et al. (1982). As there is a high correlation between plasma estradiol concentration and estrus behavior (Lyimo et al., 2000), the impact of heat stress to reduce estradiol concentrations could lead to an increased number of undetected estrus periods. It is also likely that reduced expression of estrus is due to heat stress causing more lethargy in an attempt to limit heat production.

**Follicular development**

The follicle destined to ovulate emerges as an antral follicle around 40-d before ovulation (Lussier et al., 1987) and exposure of lactating cows to heat stress prior to AI was associated with reduced conception rates (Chebel et al., 2004). Heat stress during this period of follicular growth can disrupt patterns of follicular development, follicular dominance, and oocyte competence. Initiation of heat stress on the day of ovulation reduced the diameter and volume of the first wave dominant follicle (Badinga et al., 1993), whereas heat stress starting at d 1 of the estrous cycle increased the number of follicles >10 mm in diameter, accelerated the decrease in size of the first wave dominant follicle, resulted in earlier emergence of the second dominant (preovulatory) follicle, and tended to reduce inhibin concentrations in plasma (Wolfenson et al., 1995). Trout et al.

(1998) found that lactating Holstein cows exposed to experimental heat stress had more 2 to 5 mm follicles with no effect of heat stress on populations of larger follicles.

A decreased period of follicular dominance and ovulation failure for heat-stressed cows was associated with a decrease in the proestrus rise in plasma estradiol concentrations and a smaller size of the second wave preovulatory follicle (Wolfenson et al., 1995; Wilson et al., 1998b). These effects caused a delay in luteolysis in heat-stressed cows (Wilson et al., 1998b; Sartori et al., 2002a), as estradiol produced by the preovulatory follicle is needed to up-regulate the synthesis of endometrial oxytocin receptor and activate enzymes associated with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) synthesis (Silvia et al., 1991). However, another group failed to observe an effect of heat stress on luteal function or estrous cycle length (Trout et al., 1998).

Alterations in follicular development and dominance caused by heat stress could be caused by disruption of endocrine and steroidogenic function of the granulosa and theca cells. Heat stress can alter luteinizing hormone (LH) secretion (Wise et al., 1988a; Gilad et al., 1993) and metabolic hormones that affect ovarian function such as somatotropin (ST). Indeed, plasma concentrations of ST tend to be reduced by heat stress (McGuire et al., 1991). Heat stress diminished gonadotropin receptor expression in rat granulosa cells and enhanced their susceptibility to apoptosis (Shimizu et al., 2005). Elevated temperature in vitro decreased estradiol and androstenedione production and increased progesterone secretion from heat-stressed dominant follicles, causing premature luteinization of follicular cells, which is a phenomenon that has been associated with reduced fertility in cattle with persistent follicles (Bridges et al., 2005). For cows that had low plasma estradiol concentrations, mean and basal concentrations and amplitude of

LH pulses were reduced by heat stress (Gilad et al., 1993) that could delay ovulation. There are even delayed effects of heat stress on follicular function during the autumn when heat stress has dissipated, as evidenced by impaired steroidogenesis in medium-sized follicles (lower estradiol production by granulosa cells and androstenedione production by thecal cells) and preovulatory follicles (lower androstenedione production by thecal cells; Roth et al., 2001b).

Alterations in the preovulatory follicle caused by heat stress could affect function of the corpus luteum (CL) and progesterone secretion, and heat stress could also affect progesterone secretion after formation of the CL. Reduced concentrations of plasma progesterone have been detected during the summer from lactating dairy cows (Wolfenson et al., 1993; Howell et al., 1994; Sartori et al., 2002a). Cultured luteinized granulosa cells collected from first-wave dominant follicles of Holstein cows during the summer or winter produced similar amounts of progesterone whereas cultured luteinized thecal cells collected during the winter produced three-fold more progesterone as compared to cultured luteinized thecal cells collected during the summer (Wolfenson et al., 2002).

**Reproductive tract**

Redistribution of blood flow from the internal organs to the periphery coincident with physiological efforts to increase heat loss to the environment could compromise reproductive tract function by reducing distribution of nutrients and hormones to the ovary, oviduct, and uterus. The effect of heat stress on the reduction in uterine blood flow has been observed for heat-stressed cows (Roman-Ponce et al., 1978), ewes (Bell et al., 1987; Dreiling et al., 1991), and rabbits (Lublin and Wolfenson, 1996). Additionally, reduced circulating concentrations of progesterone caused by heat stress could reduce its

support of the uterus (Rosenberg et al., 1982; Wise et al., 1988a; Howell et al., 1994). Alterations in uterine function caused by exposure of endometrial explants collected at estrus to elevated temperature (43°C) in culture (termed hereafter as heat shock) include reduced secretion of proteins (Malayer et al., 1988) and reduced DNA synthesis (Malayer and Hansen, 1990). Similarly, heat shock (42°C or 43°C) increased $PGF_{2\alpha}$ secretion of cultured endometrial explants collected at d 17 after estrus (Putney et al., 1988b; 1988c; Malayer et al., 1990). In contrast, Paula-Lopes et al. (2003b) found no change in quantity of protein secreted by oviductal or endometrial explants in response to heat shock (41°C), possibly due to the lower temperature used for heat shock.

**Oocyte competence and embryonic development**

The competence of bovine oocytes to develop to the morula and blastocyst stage of embryonic development following in vitro maturation, fertilization, and embryo culture was reduced during the summer in warm regions of the world (Rocha et al., 1998; Zeron et al., 2001; Al-Katanani et al., 2002b, 2002c) as well as in more temperate regions (Rutledge et al., 1999). Effects of heat stress on oocyte competence persist into the autumn (Roth et al., 2001a, 2001b), suggesting that the oocyte is damaged early in folliculogenesis. Oocyte competence in the autumn could be enhanced by use of transvaginal ultrasound-guided aspiration to remove 3 to 7 mm diameter follicles (Roth et al., 2001a). Combining oocyte aspiration with treatment with bST or follicle-stimulating hormone (FSH) to facilitate follicular turnover also improved oocyte morphology and, for the FSH-treated group, increased cleavage rates following in vitro fertilization (Roth et al., 2002). In addition to heat stress effects on oocyte morphology, fertilization rates can be compromised as lactating cows had reduced fertilization rates in summer as compared

to winter (Sartori et al., 2002b). Heat shock in vitro reduced cleavage rates (Roth and Hansen, 2004a, 2004b), and most embryos failed to continue with development past the 8- to 16-cell stage (Lawrence et al., 2004; Payton et al., 2004). Additionally, heat shock during bovine oocyte maturation reduced the proportion of oocytes that progressed to metaphase II and heat-shocked oocytes often possessed misshapen metaphase I spindles with disorganized microtubules and unaligned chromosomes, indicating that heat shock can disrupt the microtubular network (Roth and Hansen, 2005).

There is abundant evidence that the decline in fertility in lactating dairy cows during the summer is caused by heat stress and the resultant hyperthermia. When heat stress was applied to superovulated dairy cows between the onset of estrus and insemination, there was a reduction in embryonic development (Putney et al., 1989b). Similarly, experimental exposure of lactating cows to heat stress increased rectal temperature and decreased embryonic development (Putney et al., 1988a; Sartori et al., 2002b; Vasconcelos et al., 2006). Exposure of superovulated cows to direct solar radiation in the summer at d 1 following estrus compromised continued development of embryos when collected on d 8, but exposure to heat stress on d 3, 5, or 7 did not have a negative effect on embryonic development (Ealy et al., 1993). This result implies that the embryo becomes more resistant to heat shock as it progresses through development. Heat stress reduced secretion of interferon-τ needed to extend luteal lifespan and increased secretion of $PGE_2$ by the bovine conceptus (Putney et al., 1988c).

Heat shock causes a variety of damaging effects to the cellular function of preimplantation bovine embryos that presumably lead to disruption of development. One consequence of heat shock is alterations in the ultrastructure of the embryo. Exposure of

bovine 2-cell embryos produced in vivo or in vitro to 41°C for 6 h caused movement of organelles towards the center of each blastomere, leaving the periphery devoid of organelles (Rivera et al, 2003). This movement of organelles away from the periphery of the blastomere was due to disruptions in microtubules and microfilaments (Rivera et al., 2004).

Another reason why 2-cell bovine embryos are sensitive to heat shock could be due to a lack of induction of a heat shock protein (HSP) response. However, as early as the 2-cell stage, at a time when the embryo is largely transcriptionally inactive, the bovine embryo can respond to elevated temperatures by increasing synthesis of a heat-inducible form of HSP70 called HSP68 (Edwards and Hansen, 1996). Additionally, transcription of inducible HSP70 occurred at the 2-cell stage in bovine embryos, and addition of transcription inhibitors reduced amounts of HSP70 mRNA and cleavage rates, slowed development, and inhibited overall protein synthesis (Chandolia et al., 1999). There is evidence that amounts of HSP70 alter embryonic resistance to stress, as treatment of mouse embryos with antisense mRNA against the inducible form of HSP70 diminished the capacity for embryonic development to the blastocyst stage and heightened the sensitivity of embryos to arsenic exposure even in the presence of a sub-toxic dose (Dix et al., 1998). Furthermore, injection of HSP70 mRNA into mouse oocytes increased their resistance to heat shock (Hendrey and Kola, 1991). While the 2-cell bovine embryo can increase amounts of HSP70 in response to heat shock, it might not be enough to compensate for the deleterious effects of heat shock. Lack of a HSP70 response is probably not the reason as to why 2-cell bovine embryos are sensitive to heat shock. Other actions of heat shock that could lead to a reduced developmental potential include

mitochondrial depolarization (Rivera et al., 2003) and reduction in protein synthesis (Edwards and Hansen, 1996).

**Developmental changes in resistance to heat shock**

The maturing bovine oocyte is susceptible to in vitro thermal stress, resulting in compromised blastocyst development as the majority of embryos fail to develop past the 8- to 16-cell stage (Lawrence et al., 2004; Payton et al., 2004) and have reduced cleavage rates (Roth and Hansen, 2004a, 2004b). While the oocyte is susceptible to heat shock, these deleterious effects decrease as the embryo continues through development. This has been shown to occur for bovine embryos in vivo (Ealy et al., 1993) and in vitro (Ealy and Hansen, 1994; Ealy et al., 1995; Edwards and Hansen, 1997; Ju et al., 1999; Krininger et al., 2002; Sakatani et al., 2004). For example, exposure of bovine 2-cell embryos and 4- to 8-cell embryos to 41°C for 12 h reduced their ability to continue development to the blastocyst stage, and the effects of heat shock were more pronounced for 2-cell embryos than 4-to 8-cell embryos (Edwards and Hansen, 1997). However, the development of bovine morulae to the blastocyst stage was unaffected by heat shock (Edwards and Hansen, 1997). This study demonstrated that the early bovine embryo has increased sensitivity to heat shock that compromises continued development but the embryo becomes more resistant to heat shock later in embryonic development.

**Oxygen environment**

The oxygen content of the oviduct and uterus may mitigate the effects of heat shock. The oxygen concentration in the oviduct is about 40% or less than atmospheric concentration (Leese, 1995) whereas the oxygen concentration in the uterus is lower than that of the oviduct (Fischer and Bavister, 1993). Rivera and Hansen (2001) found that the deleterious effect of heat shock on in vitro development of bovine putative zygotes

and 2-cell embryos to the blastocyst stage was not dependent upon oxygen tension (5 vs 20%). In contrast, development to blastocysts was reduced when embryos were heat-shocked during culture using 20% (v/v) oxygen tension but not when cultured at 5% oxygen (Fujitani et al., 1997; Takahashi et al., 2000; Sakatani et al., 2004; de Castro e Paula and Hansen, 2005).

It is possible that heat shock is more deleterious to embryonic development at high oxygen concentrations because of involvement of free radicals. Heat shock of bovine embryos during culture increased the production of oxygen radicals (Fujitani et al., 1997; Sakatani et al., 2004) and increased DNA damage. In vivo studies indicate that heat stress does cause alterations in the reproductive tract through the generation of oxidative stress. Heat-stressed mice had increased amounts of hydrogen peroxide in the oviduct, and the percentage of collected zygotes that developed to morula and blastocysts was reduced while the majority of embryos arrested at the 2-cell stage (Ozawa et al., 2004; Matsuzuka et al., 2005a). Analysis of the arrested embryos revealed increased DNA damage and less Cdc2 activity in the maternally heat-stressed embryos (Ozawa et al., 2004; Matsuzuka et al., 2005a). Use of antioxidants alleviated the effects of oxidative stress caused by elevated temperature. Administration of melatonin to heat-stressed mice increased free radical scavenging activity in the oviduct, tended to increase glutathione content of zygotes and increased the percentage of zygotes that developed to the morula or blastocyst stage (Matsuzuka et al., 2005b). Administration of S-adenosyl-L-methionine, an inducer of glutathione synthesis, decreased the deleterious effects of heat shock of the percentage of murine embryos that became blastocysts (Aréchiga et al., 1995). Moreover, depletion of glutathione, by treatment with the glutathione synthesis

inhibitor, buthionine sulfoximine, increased embryonic susceptibility to heat shock (Aréchiga et al., 1995). Addition of various antioxidants to culture medium, including glutathione, taurine, and vitamin E, provided some thermoprotection to mouse morulae (Malayer et al., 1992; Aréchiga et al., 1994).

**Strategies to Increase Fertility during Heat Stress**

In addition to antioxidant therapy, many approaches have been explored to reduce the effects of heat stress experienced by the lactating dairy cow on fertility. Cooling of cows during heat stress increases pregnancy rates (Thatcher et al., 1974; Roman-Ponce et al., 1977; Wolfenson et al., 1988; Ryan et al., 1993; Ealy et al., 1994; Khongdee et al., 2006) but large seasonal variations in reproductive function can still persist on farms that use such cooling systems (Hansen and Aréchiga, 1999). Reasons for this variation in fertility could be attributed to the delayed effects on follicular development and oocyte competence caused by heat stress, differences in energy balance or milk yield between cows, or inadequate cooling systems.

Another attempt to increase fertility of lactating dairy cows during the summer is to increase post-ovulatory concentrations of progesterone. This strategy has had mixed results. Heat-stressed dairy cows treated with gonadotropin-releasing hormone (GnRH) or human chorionic gonadotropin (hCG) had increased pregnancy rates and serum progesterone concentrations (Ullah et al., 1996; Peters et al., 2000; Block et al., 2003; Willard et al., 2003; Franco et al., 2006). However, administration of GnRH or hCG to dairy heifers or lactating cows in the summer and winter subjected to TAI either failed to improve pregnancy rates or had a negative effect (Schmitt et al., 1996; Franco et al., 2006).

Given that the in vivo (Ealy et al., 1993) and in vitro (Ealy et al., 1995; Edwards and Hansen, 1997) bovine embryo becomes more resistant to heat shock as it progresses through development (by the morula stage), it follows that pregnancy rates following embryo transfer (ET) at the blastocyst stage should be higher during heat stress than following AI. Indeed, this has been demonstrated numerous times (Putney et al., 1989a; Ambrose et al., 1999; Drost et al., 1999; Al-Katanani et al., 2002a). Furthermore, development of a timed embryo transfer (TET) program using an in vitro-derived bovine blastocyst (d 7 after fertilization) transferred to a synchronized recipient female between d 6.5 to 7 after anticipated ovulation of the estrous cycle increased pregnancy rates of heat-stressed dairy cows (Ambrose et al., 1999; Al-Katanani et al., 2002a).

Induced thermotolerance is the process whereby cells are made more resistant to a severe heat shock by prior exposure to a mild heat shock. Induction of the induced thermotolerance response could conceivably improve survival of an embryo to an otherwise lethal heat shock. In cattle, it has been shown that induced thermotolerance can occur in blastocysts produced in vivo (Ealy and Hansen, 1994). Exposure of 8- to 16-cell embryos at d 4 post-insemination to heat shock increased the percentage of cells undergoing apoptosis while exposure to a mild heat shock blocked the apoptotic response to a subsequent, more severe heat shock (Paula-Lopes and Hansen, 2002b). However, practical methods to manipulate induced thermotolerance responses have not yet been devised.

Finally, genetic thermotolerance of bovine embryos could influence fertility during heat stress. For example, there was a less severe reduction in blastocyst development following heat shock during culture for Brahman and Romosinuano embryos than for

Angus and Holstein embryos (Paula-Lopes et al., 2003b; Hernández-Cerón et al., 2004). Additionally, Nelore embryos were better able to survive heat stress during culture than Angus heifers (Barros et al., 2006). Moreover, Nelore embryos that were heat-shocked in culture and became blastocysts were more likely to establish pregnancy than similarly treated Angus embryos (Barros et al., 2006). Genetic differences in cellular thermotolerance do not appear to be expressed until after activation of the embryonic genome, as no differences in resistance to heat shock were found between Brahman and Holstein embryos at the 2-cell stage (Krininger et al., 2003) when the genome of the embryo is largely inactive (Memili and First, 2000). While the identity of the specific gene(s) controlling cellular thermotolerance is not known, synthesis of HSP70 in response to heat shock did not differ between Brahman and Holstein lymphocytes (Kamwanja et al., 1994). It is unlikely that mitochondrial genes are involved in the genetic differences in thermotolerance because Brahman cattle contain *B. taurus* mitochondria (Meirelles et al., 1999).

## Apoptosis and Heat Shock in Embryos

### Occurrence of Apoptosis in Bovine Embryos

Apoptosis, or programmed cell death, is a conserved process that involves the controlled removal of cells in a distinct morphological manner. Those cells unable to go through apoptosis often undergo necrosis that could have more severe consequences for embryonic development. The apoptotic process occurs during normal mammalian development to remove damaged or unhealthy cells in an orderly fashion and plays a major role in preimplantation embryonic development (Paula-Lopes and Hansen, 2002b; Gjørret et al., 2003; Fabian et al., 2005). Apoptosis occurs for in vivo and in vitro produced embryos, and those embryos that reach the blastocyst stage often have cells that

have undergone apoptosis in both the ICM and trophectoderm (Jurisicova and Acton, 2004).

A common procedure utilized in determining if a blastomere of an embryo or cell has undergone apoptosis is the terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay, which involves the labeling of fragmented DNA at the free 3'OH end with nucleotides attached to a fluorescent probe. Apoptosis in bovine embryos begins at the 6-cell stage for in vitro embryos and the 21-cell stage for in vivo embryos (Gjørret et al., 2003). By the blastocyst stage, 97% of in vivo-derived embryos and 100% of in vitro-derived embryos had at least one nucleus displaying morphological evidence of apoptosis and TUNEL-positive labeling, and incidence of apoptosis was greater for in vitro embryos, primarily due to a higher degree of apoptosis confined to the ICM (Gjørret et al., 2003). Using the TUNEL assay, it has been demonstrated that heat shock can cause apoptosis in bovine oocytes and embryos (Paula-Lopes and Hansen, 2002a, 2002b; Roth and Hansen, 2004a; Brad and Hansen, 2006; Loureiro et al., 2006) and porcine embryos (Isom et al., 2003).

There is a relationship between embryonic development and proteins related to apoptosis. Good quality bovine oocytes and blastocysts had higher amounts of the anti-apoptotic Bcl-2 protein as compared to fragmented embryos, and denuded oocytes had the least amount of Bcl-2 (Yang and Rajamahendran, 2002). While the pro-apoptotic Bax protein was found in all oocytes and blastocysts analyzed, the greatest amount was found in denuded oocytes (Yang and Rajamahendran, 2002), indicating that the ratio of Bcl-2 to Bax could be used to gauge the tendency of oocytes or embryos towards continued development or apoptosis. Similarly, Bax transcripts were detected in higher

amounts in embryos with slower developmental speed (Gutiérrez-Adán et al., 2004). These experiments indicate that it might be possible to correlate a marker of apoptosis, ideally measured in a non-invasive, quick manner, with the ability of the embryo to continue development. Such a process could allow for refined selection of morphologically superior embryos in an attempt to increase pregnancy rates.

**Mechanism for Induction of Apoptosis by Heat Shock**

There are two major signaling pathways whereby apoptosis can occur – the mitochondrial- and death receptor-mediated pathways. Heat shock activates apoptosis through the mitochondrial (or intrinsic) pathway (Mirkes and Little, 2000; Yuen et al., 2000; Quan et al., 2004; Vera et al., 2004; Bettaieb and Averill-Bates, 2005). The pathway for induction of apoptosis by heat shock is described in Figure 2-1 based on several reviews (Basu and Kolesnick, 1998; Punyiczki and Fésüs, 1998; Fadeel et al., 1999; Hengartner, 2000; Gallaher et al., 2001; Wang, 2001; Mirkes, 2002; Bouchier-Hayes et al., 2005; Mohamad et al., 2005).

Induction of apoptosis caused by heat shock begins with alterations of plasma membrane phospholipids. Sphingomyelin, a phospholipid normally located in the outer leaflet of the membrane bilayer in a healthy cell, translocates to the inner leaflet where sphingomyelinase enzymes hydrolyze sphingomyelin to generate ceramide that causes mitochondrial dysfunction. In a healthy cell, anti-apoptotic proteins such as Bcl-2 heterodimerize with Bax or other pro-apoptotic proteins, thereby preventing their activity to cause mitochondrial depolarization and open channels for cytochrome c release to further drive the apoptotic cascade. However, heat shock-induced generation of ceramide reduces the amount of Bcl-2 in the cell and increases the translocation of Bax to mitochondria where Bax:Bax homodimers form that cause dysfunction of the

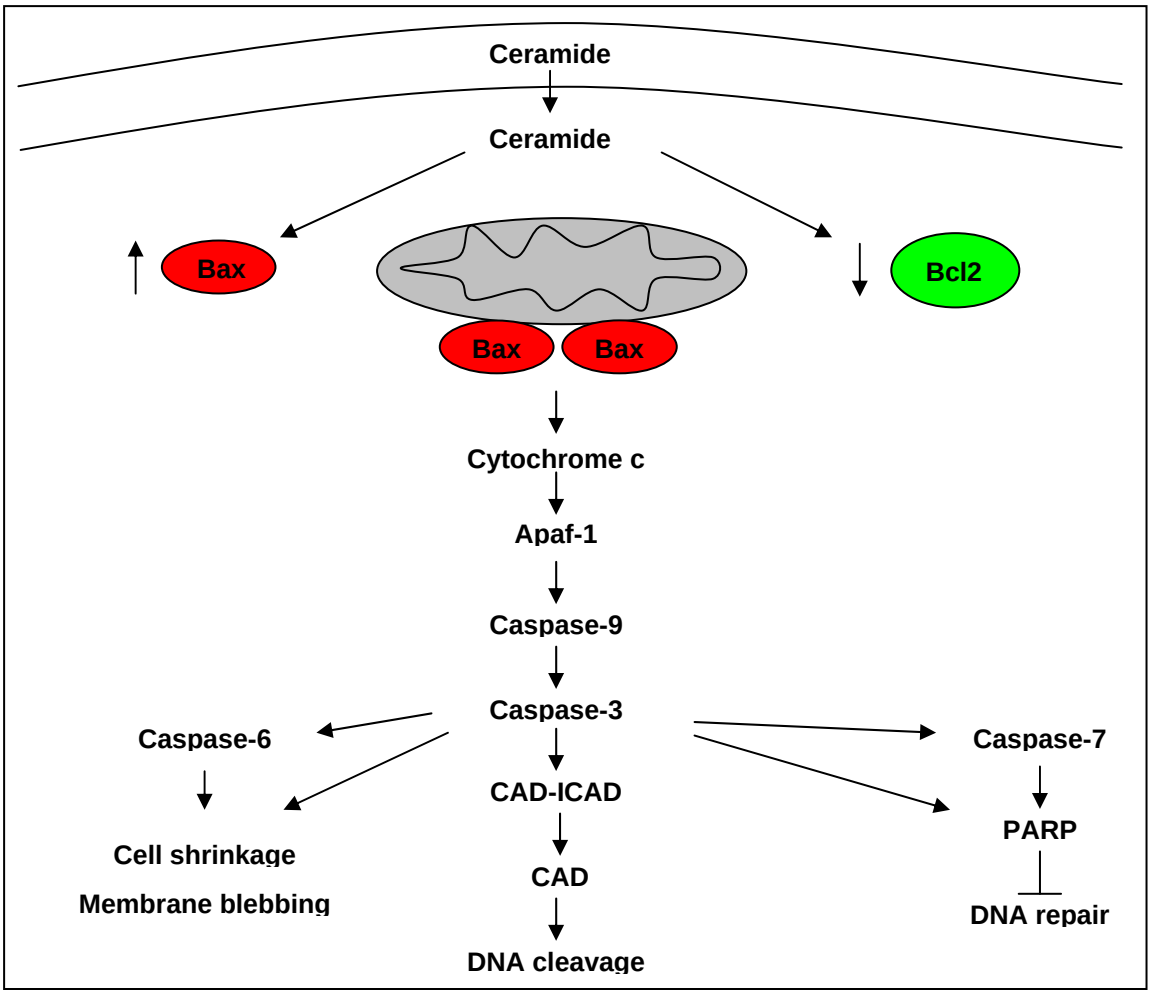


Figure 2-1. Heat shock-induced apoptosis involves the mitochondrial (intrinsic) pathway. The induction of apoptosis caused by heat shock results in alterations in plasma membrane phospholipids, as sphingomyelin is translocated to the inner leaflet of the plasma membrane where sphingomyelinase hydrolyzes sphingomyelin to generate ceramide. In healthy cells, anti-apoptotic proteins such as Bcl-2 heterodimerize with Bax or other pro-apoptotic proteins to prevent them from causing mitochondrial depolarization and opening mitochondrial permeability pores for cytochrome c release. However, ceramide causes mitochondrial depolarization and increases Bax translocation to mitochondria where Bax:Bax homodimers form to create permeability transition pores in the mitochondria to release cytochrome c. The apoptosome is formed through association of cytochrome c with Apaf-1 followed by conformational changes that allow Apaf-1 to bind ATP and procaspase-9 and eventually leads to cleavage of procaspse-9 to form caspase-9 that activates downstream caspases (including caspase-3, -6, and –7) that execute cell death. Active caspase-3 cleaves DNA into nucleosomal fragments by cleavage of DNA repair proteins, such as poly(ADP-ribose) polymerase, and destroys the inhibitory portion of caspase-activated DNase, thereby activating caspase-activated DNase.

mitochondria by opening permeability transition pores, thereby causing the release of cytochrome c into the cytoplasm. Permeability of mitochondrial transition pores can be caused by oxidative stress (Le Bras et al., 2005; Armstrong, 2006) to promote release of pro-apoptotic factors from the mitochondria (Blomgren and Hagberg, 2006). Treatment of heat-shocked bovine embryos during culture with the mitochondrial depolarizing agent, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), increased the proportion of TUNEL-positive blastomeres and induced activation of caspase-9 and group II caspases (Brad and Hansen, 2006).

Released cytochrome c associates with apoptotic protease activating factor-1 (Apaf-1) to induce a conformational change in Apaf-1 that allows it to bind adenosine triphosphate (ATP) and procaspase-9. The resultant complex is called the apoptosome and leads to cleavage of procaspse-9 to form active caspase-9. An inhibitor of caspase-9 (z-LEHD-fmk) blocked the induction of apoptosis in bovine embryos caused by heat shock (Loureiro et al., 2006). Caspase-9 in turn activates downstream caspases, including caspase-3, -6, and -7, which are the execution caspases that function in cell death. Active caspase-3 leads to cleavage of deoxyribonucleic acid (DNA) into nucleosomal fragments and involves at least two enzymatic actions of caspase-3. The first is cleavage of DNA repair proteins such as poly(ADP-ribose) polymerase and destruction of the inhibitory portion of caspase-activated DNase, thereby activating caspase-activated DNase. It is possible to block apoptosis in bovine embryos using the caspase-3 inhibitor z-DEVD-fmk, which blocked the detrimental effects of heat shock during bovine maturation on reduced cleavage and blastocyst rates (Roth and Hansen,

2004a) and the increase in TUNEL-positive blastomeres and caspase activity in d 4 bovine embryos (Paula-Lopes and Hansen, 2002a).

**Developmental Changes in Occurrence and Consequences of Apoptosis during Embryonic Development**

As discussed earlier, there is a developmental phenomenon whereby bovine oocytes and early embryos are more sensitive to heat shock than more developmentally advanced embryos. Apoptosis seems to be an important determinant of oocyte survival to heat shock. Heat shock can induce apoptosis in the oocyte during maturation (Roth and Hansen, 2004a), which would prevent embryonic development. Following fertilization, the embryo loses the capacity to undergo heat shock-induced apoptosis. For example, the bovine 2- and 4-cell embryo is incapable of undergoing apoptosis in response to a variety of pro-apoptotic stimuli, including heat shock (Paula-Lopes and Hansen, 2002b), arsenic (Krininger et al., 2002), and TNF-α (Soto et al., 2003). The inability of the 2-cell bovine embryo to undergo apoptosis is likely due to a block to caspase-9 activation and caspase-3 effector activity that can be overcome with treatment with the mitochondrial depolarizer CCCP (Brad and Hansen, 2006). By d 4 and 5 after fertilization, heat shock, arsenic, and TNF-α treatment can increase apoptosis (Krininger et al., 2002; Paula-Lopes and Hansen, 2002b; Soto et al., 2003a). Furthermore, comparison of group II caspase activity showed that heat shock increased caspase activity for bovine embryos ≥16-cells but not for 2-cell embryos (Paula-Lopes and Hansen, 2002a). It is possible that induction of apoptosis at early stages of bovine embryonic development is down regulated because continued development would be compromised by removal of even a few cells in the early embryo. As the embryo continues development and increases in cell number, however, removing those cells most damaged

by heat shock though apoptotic mechanisms could still leave enough healthy cells that can allow for continued development.

**Actions of IGF-I to Modify Function of the Preimplantation Bovine Embryo**

Exposure of the preimplantation bovine embryo to heat shock induces apoptosis (Paula-Lopes and Hansen, 2002a, 2002b; Roth and Hansen, 2004a; Brad and Hansen, 2006; Loureiro et al., 2006) and reduces the ability of the embryo to develop to the blastocyst stage (Lawrence et al., 2004; Payton et al., 2004). The fact that embryonic resistance to heat shock is such a plastic phenomenon implies that strategies can be found to manipulate embryonic responses to heat shock to enhance fertility during heat stress. Indeed, an embryo's propensity to resist actions of pro-apoptotic and anti-developmental agents in the environment can be modified by specific regulatory molecules, including epidermal growth factor (Cui and Kim, 2003; Sirisathien and Brackett, 2003; Sirisathien et al., 2003; Kurzawa et al., 2004; Makarevich et al., 2005), transforming growth factor-α (Brison and Schultz, 1997; Kawamura et al., 2005), granulocyte-macrophage colony-stimulating factor (de Moraes and Hansen, 1997; Sjöblom et al., 2002; Behr et al., 2005), interleukin-11 (Kochhar et al., 2003; Jousan et al., 2004), ST (Kölle et al., 2002; Moreira et al., 2002b; Markham and Kaye, 2003; Kidson et al., 2004), insulin (Pantaleon and Kaye, 1996; Herrler et al., 1998; Byrne et al., 2002b; Augustin et al., 2003), and IGF-I and -II (Herrler et al., 1998; Byrne et al., 1999, 2002a, 2002b; Kurzawa et al., 2002; Block et al., 2003; Fabian et al., 2004; Block and Hansen, 2006). Focus in this dissertation is on the last of these regulatory molecules – IGF-I because of the possibility of regulating IGF-I concentrations to enhance embryonic resistance to heat shock. This

section will discuss the actions of IGF-I on embryonic development and describe cytoprotective effects of IGF-I.

**Stimulation of Embryonic Development**

In cattle, IGF-I added at concentrations of 2 to 200 ng/ml increased the proportion of embryos becoming morulae at d 5 post-insemination (Matsui et al., 1995; Matsui et al., 1997) and blastocysts between d 6.5 and 8 post-insemination (Palma et al., 1997; Prelle et al., 2001; Sirisathien et al., 2001; Byrne et al., 2002b; Moreira et al., 2002b; Block et al., 2003; Sirisathien and Brackett, 2003; Sirisathien et al., 2003). Furthermore, IGF-I increased the proportion of blastocysts that were at advanced stages of development (i.e., expanded or hatched blastocysts) at d 8 post-insemination (Moreira et al., 2002b; Block et al., 2003). Additionally, IGF-I increased the total cell number of bovine blastocysts (Byrne et al., 2002b; Makarevich and Markkula, 2002; Moreira et al., 2002b) and differential staining revealed that IGF-I increased the number of ICM cells (Sirisathien et al., 2003).

Effects of IGF-I to promote increased cell number and blastocyst development in bovine embryos appears to be mediated through the IGF-I receptor because addition of a monoclonal antibody specific for the α-subunit of the IGF-I receptor prevented the ability of IGF-I to stimulate embryonic development to the morula stage (Matsui et al., 1997). In addition, IGF-I appears to act after the 2- to 4-cell stage because addition of IGF-I to culture medium of putative bovine zygotes did not increase the proportion of zygotes that cleaved or reached the 2- or 4-cell stage of development (Sirisathien et al., 2001; Makarevich and Markkula, 2002; Moreira et al., 2002b; Block et al., 2003; Sirisathien and Brackett, 2003; Sirisathien et al., 2003). Nonetheless, the IGF-I receptor is present at

all stages of bovine embryonic development (Lonergan et al., 2003). Transcript abundance remained relatively constant from the zygote to the 16-cell stage; at later stages, there was an increase in transcript abundance for the IGF-I receptor and amounts were higher for embryos placed within the sheep oviduct than for embryos cultured in vitro (Lonergan et al., 2003).

Like for the IGF-I receptor, IGF-I mRNA has been detected at all stages of bovine development from the zygote to the blastocyst stage (Schultz et al., 1992; Watson et al., 1992; Lonergan et al., 2000). Differences in the expression of IGF-I ligand and its receptor are correlated with the timing of first cleavage and speed of bovine embryonic development. For example, the IGF-I ligand was found in all 2-cell bovine embryos (6/6) that cleaved between 27 and 30 h post-insemination, only in some embryos (2/6) that cleaved between 33 and 36 h post-insemination, but not in embryos (0/6) that cleaved after 36 h (Lonergan et al., 2000). Among the population of fast-developing bovine embryos on d 4 post-insemination (16-cell stage), those embryos cultured in the ewe oviduct had increased IGF-I receptor mRNA as compared to embryos produced and cultured in vitro (Gutiérrez-Adán et al., 2004). Similarly, more murine embryos in which early IGF-I mRNA was detected formed blastocysts in culture and were of higher quality (based on morphological criteria from Dokras et al., 1993), suggesting that IGF-I mRNA in early cleavage stages may be associated with improved developmental potential (Kowalik et al., 1999).

As just alluded to, pro-developmental actions of IGF-I have also been observed in other species. In the mouse, IGF-I (ranging from 30 to 100 ng/ml) increased the percentage of in vivo fertilized zygotes and 2-cell embryos that developed to the

blastocyst (O'Neill, 1997; Lin et al., 2003; Głąbowski et al., 2005) and hatched blastocyst stages (Lin et al., 2003). Furthermore, IGF-I increased the number of ICM cells (Smith et al., 1993; Markham and Kaye, 2003). This effect required the IGF-I receptor because addition of an anti-IGF-I receptor monoclonal antibody blocked effects of IGF-I to increase the number of ICM cells (Markham and Kaye, 2003). Other species in which IGF-I has promoted embryonic development to the blastocyst stage and/or blastocyst cell number include the buffalo (Narula et al., 1996), pig (Kim et al., 2004a, 2005), rabbit (Makarevich et al., 2000), and human (Lighten et al., 1998; Spanos et al., 2000). However, not all studies have indicated a positive action of IGF-I on embryonic development, as IGF-I was without effect including experiments in cattle (Makarevich and Markkula, 2002) and mice (Fabian et al., 2004). It is possible that the discrepancies in the pro-developmental aspects of IGF-I in these studies are due to differences in culture medium, oxygen concentration, or amount of IGF-I supplemented during embryo culture.

While IGF-I can have pro-developmental effects to increase cell number and blastocyst development, elevated concentrations of IGF-I in vivo can have negative consequences on establishment of pregnancy in rats and mice. Superovulation of immature rats caused elevated uterine IGF-I concentrations (approximately 700 ng/g of tissue) from d 1 to 3 of pregnancy - treatment with the somatostatin analogue octreotide normalized uterine IGF-I concentrations (approximately 525 ng/g of tissue) and increased the number of normal embryos by 2.7-fold on d 3 of pregnancy (Katagiri et al., 1997). Also, intrauterine deposition of IGF-I-containing pellets in mice increased serum IGF-I concentrations (58 ± 11 ng/ml in control mice to 427 ± 70 ng/ml in IGF-I-treated mice)

and reduced implantation rates from 100% in control mice to 37% in implanted mice (Pinto et al., 2002b). Exposure of preimplantation mouse embryos to high concentrations of IGF-I (1 μg/ml) during culture resulted in extensive amounts of apoptosis in the ICM cells and decreased amounts of the IGF-I receptor (Chi et al., 2000b). Insulin-stimulated glucose uptake was also decreased upon exposure to high IGF-I and it was suggested that the mechanism for induction of apoptosis by high IGF-I concentrations involved decreased IGF-I receptor signaling (Chi et al., 2000b).

**Possible Mechanisms for Pro-Developmental Actions of IGF-I**

There are several possible mechanisms by which IGF-I could increase the proportion of embryos that develop to the blastocyst stage. Insulin-like growth factor-I is a well-characterized mitogen (Siddle et al., 2001; Bahr and Groner, 2005; Foulstone et al., 2005) and it could therefore increase blastomere proliferation rates. However, IGF-I did not increase the ratio of cells that were positive for proliferating cell nuclear antigen (Makarevich and Markkula, 2002). Another possibility is that IGF-I could increase energy uptake by bovine embryos. The bovine embryo is dependent on pyruvate and glutamine during early development but the major energy substrate utilized becomes glucose around the time of compaction (Rieger et al., 1992a, 1992b). Pantaleon and Kaye (1996) found that IGF-I and insulin increased glucose uptake in mouse blastocysts and IGF-I was 1,000-fold more potent than insulin. Actions of IGF-I to increase glucose uptake involves the up-regulation of glucose transporters (GLUT). Exposure of mouse blastocysts to GLUT1 antisense oligonucleotides decreased glucose uptake and increased apoptosis (Chi et al., 2000a).

One of the pathways activated by the IGF-I receptor is the phosphatidylinositol 3-kinase (PI3K)/Akt pathway that will be described in more detail in a later section. This pathway is present throughout embryonic development from the 1-cell to blastocyst stage in mouse embryos and is a regulator of growth, apoptosis, and glucose metabolism (Riley et al., 2005; Riley and Moley, 2006). Addition of the PI3K inhibitor LY 294002 to mouse blastocysts decreased glucose uptake and expression of GLUT1 and increased the percentage of TUNEL-positive nuclei (Riley et al., 2006). Furthermore, fetuses formed from blastocysts cultured with LY 294002 were more likely to undergo resorption (88.7%) compared to control embryos (12.5%; Riley et al., 2006). Antisense oligonucleotides towards the IGF-I receptor blocked actions of insulin to cause translocation of GLUT8 from intracellular compartments to the plasma membrane in murine blastocysts (Pinto et al., 2002a). In the same study, the IGF-I receptor antisense treatment increased blastocyst TUNEL-labeling (Pinto et al., 2002a). One possibility that is suggested by these experiments is that one of the critical actions for blastocyst survival is activation of the IGF-I receptor-signaling pathway to cause translocation of GLUT8.

Another possibility is that IGF-I has direct anti-apoptotic effects and that activation of these effects by IGF-I increases the proportion of embryos becoming blastocysts. Addition of IGF-I (ranging from 50 to 100 ng/ml) to cultured bovine zygotes reduced the spontaneous occurrence of apoptosis in blastocysts by 30 to 50% (Byrne et al., 2002b; Makarevich and Markkula, 2002; Sirisathien and Brackett, 2003; Kaya et al., 2006). Similar ability of IGF-I to decrease the occurrence of spontaneous apoptosis of blastocysts (approximately 30 to 50%) has been reported for human (Spanos et al., 2000) and mouse embryos (Fabian et al., 2004). Using another marker of apoptosis, annexin-V

staining, IGF-I (100 ng/ml) reduced the proportion of mouse blastocysts staining positive for annexin-V by approximately 50% (Lin et al., 2003).

**Cytoprotective Actions of IGF-I**

Given its anti-apoptotic and pro-developmental actions, it is not surprising that one of the properties of IGF-I is to reduce or block effects of adverse environmental factors that induce apoptosis and disrupt embryonic development. For example, IGF-I blocked the induction of apoptosis and decrease in cell number caused by culture of mouse zygotes for 5 d in the presence of TNF-α (Byrne et al., 2002a). Additionally, IGF-I blocked induction of apoptosis caused by exposure to camptothecin and actinomycin D in mice, although IGF-I did not reverse effects on blastocyst formation or cell number (Fabian et al., 2004). Effects of IGF-I to reduce anti-developmental effects of TNF-α in mouse embryos were seen by Głąbowski et al. (2005). Insulin-like growth factor-I also reduced effects of ultraviolet irradiation on development and cell proliferation in rabbit embryos (Herrler et al., 1998) as well as effects of hydrogen peroxide on mouse blastocyst development (Kurzawa et al., 2002). Additionally, IGF-I increased the percentage of thawed mouse morulae that survived freezing as indicated by the proportion of blastocysts that hatched following thawing and blastocyst cell number (Desai et al., 2000).

Cytoprotective effects of IGF-I can also result in increased survival of bovine blastocysts to establish and maintain pregnancy during heat stress in lactating dairy cows. During the summer, lactating dairy cows receiving bovine blastocysts previously cultured with 100 ng/ml of IGF-I had higher pregnancy rates at d 53 and 81 after expected ovulation and higher calving rates as compared to cows that received a control blastocyst

(Block et al., 2003). In a subsequent study, transfer of blastocysts previously cultured with 100 ng/ml of IGF-I to lactating dairy cows during the winter did not increase pregnancy rates, whereas pregnancy rates during the hot season were improved by addition of IGF-I to embryo culture (Block and Hansen, 2006).

## Biology of Insulin-like Growth Factor-I

### IGF-I Ligand

The IGF family arose from the duplication of an ancestral gene that caused the divergence of relaxin from the proto-IGF gene (McCusker, 1998). This was followed by a similar gene duplication event occurring before evolution of vertebrates that separated insulin from the IGF family (Zapf and Froesch, 1986). A third branching, occurring before the appearance of mammals, led to separate genes for IGF-I and -II (McCusker, 1998). There are thus three structurally-related peptides in the IGF family: IGF-I, -II, and insulin (Butler and LeRoith, 2001). Both IGF-I and -II are single chain polypeptides that consist of 70 and 67 amino acids with a molecular mass of 7.6 and 7.5 kDa, respectively, while insulin consists of 51 amino acids with a molecular mass of 6.0 kDa (McCusker, 1998; Dupont and Holzenberger, 2003).

Both IGF-I and -II contain four domains designated as A through D (Denley et al., 2005). The mature form of insulin resulted from cleavage of pro-insulin that results in a dimeric protein consisting of the A and B domains held together by two disulfide bonds and with the connecting peptide corresponding to the C domain removed (De Meyts, 2004; Denley et al., 2005). The small D domain is absent from pro-insulin (Dupont and Holzenberger, 2003). A comparison of the amino acid sequences of the A and B domains shared by all members of the IGF superfamily (human) revealed that IGF-I has a 67%

homology with IGF-II and IGF-I and -II have about a 50% homology to insulin (Rinderknecht and Humbel, 1978; Butler and LeRoith, 2001; Brzozowski et al., 2002).

**Regulation of IGF-I Synthesis by ST and Insulin**

Secretion of ST occurs by somatotroph cells in the anterior pituitary and is carried in the blood to the liver where it binds to its receptor and induces hepatic production of IGF-I (LeRoith et al., 2001; Butler et al., 2002; Etherton, 2004; Yaker et al., 2005). Even though ST concentrations in blood might be high during early lactation, the somatrophic axis is uncoupled because expression of the ST receptor is decreased in the liver and has become refractory to ST (Lucy, 2000; Butler et al., 2003). While ST is the main regulator of circulating amounts of IGF-I, insulin appears to be a key metabolic signal in the coupling of the ST/IGF-I axis. Infusion of insulin to lactating dairy cows during the early postpartum period elevated insulin to a physiological concentration even though the cows were in negative energy balance (Butler et al., 2003). Infusion of insulin also increased plasma IGF-I concentrations, amounts of mRNA for ST receptor 1A and IGF-I in the liver, and decreased the amounts of ST receptor and IGF-I mRNA in adipose tissue (Butler et al., 2003). The increase in hepatic IGF-I synthesis induced by insulin is mediated in part by increased ST receptor protein in liver (Rhoads et al., 2004).

The nutritional and physiological status of the dairy cow or heifer can influence circulating concentrations of IGF-I (McGuire et al., 1992; Thissen et al., 1994; Diskin et al., 2003; Hess et al., 2005). For example, concentrations of IGF-I in serum increased linearly for mid-lactation cows in positive energy balance, but decreased gradually for early lactation cows in negative energy balance during a 10-wk experimental period (Walters et al., 2002). The period of negative energy balance represents a state of under-

nutrition, as the energy requirements for body maintenance and milk production exceed the intake of utilizable energy (Zulu et al., 2002).

**Regulation of IGF-I Activity by Binding Proteins**

There are a total of six IGF binding proteins (IGFBP) that are designated by number from IGFBP-1 to -6 (Wetterau et al., 1999). Each of the binding proteins is organized into three domains, with the conserved N- and C-terminal domains being joined by a variable central linker domain that are all of approximately equal size (Bach et al., 2005). The mature IGFBPs have between 216 and 289 amino acids with a molecular mass of between 24 and 45 kDa (Firth and Baxter, 2002; Duan and Xu, 2005). The N-terminal domain of IGFBPs contains the major IGF-binding site that bind IGFs with a 10- to 1000-fold reduced affinity as compared to the full-length IGFBPs (Duan and Xu, 2005). However, the C-terminal domain also contributes to the ligand-binding capacity with a reduced affinity (3- to 1000-fold less) than their full-length counterparts (Bach et al., 2005). The C-terminal domain often mediates IGFBP interactions with other proteins, as IGFBP-3 and -5 bind to the acid-labile subunit through their C-domains (Firth and Baxter, 2002).

The main function of the IGFBPs is to bind IGFs and thereby regulate their transport, turnover, and tissue distribution (Wetterau et al., 1999). In circulation, most of the IGFs are complexed with IGFBP-3 (Hwa et al., 1999; Duan and Xu, 2005). Transcripts for IGFBP-2, -3, and -4 have been detected throughout bovine embryonic development while IGFBP-5 was detected only at the blastocyst stage (Winger et al., 1997).

Proteinases have the capability of cleaving IGFBPs into forms that have reduced or no affinity for the IGFs depending on the abundance of the proteinase, relative potency

with which the proteinase degrades a given IGFBP, and the presence or absence of activators or inhibitors of the proteinase (Mohan and Baylink, 2002). Some of the IGFBP proteinases degrade one IGFBP with greater potency than other IGFBPs (Lawrence et al., 1999; Busby et al., 2000) while other proteinases have the capability of degrading IGFBPs and other proteins (Rajaram et al., 1997).

Because the IGFs are capable of binding to the insulin receptor, one function of IGFBPs could be to prevent binding of IGF-I to the insulin receptor (Rajaram et al., 1997). However, the IGFBPs have little to no affinity for the IGF-I receptor (Kelley et al., 2002; Bunn and Fowlkes, 2003), suggesting that the formation of IGF-I to IGFBP complexes is favored and IGFBPs do not prevent the signaling of IGF-I through its receptor.

**IGF-I Receptor**

The IGF-I receptor is synthesized as a single polypeptide chain. It is modified post-translationally by removal of a 30 amino acid signal peptide and cleavage of the pro-receptor into a 706 amino acid extracellular α-subunit and a 626 amino acid transmembrane β-subunit linked together by disulfide bonds to form an αβ half-receptor (Jones and Clemmons, 1995). Two of these half-receptors are linked together by disulfide bonds between the two α-subunits to form the mature $\alpha_2\beta_2$ IGF-I receptor (Jones and Clemmons, 1995). The ligand binding specificity of the IGF-I receptor is found in the cysteine-rich regions of the α-subunit in the extracellular domain while the tyrosine kinase activity is found in the β-subunit in the cytoplasmic domain of the cell (Baserga, 2000). The tyrosine residues in the β-subunit are clustered into three domains, the juxtamembrane, catalytic, and C-terminal domain (Kim and Accili, 2002).

Ligand binding to the α-subunit of the IGF-I receptor causes a conformational change that results in phosphorylation of a cluster of three tyrosine residues (Y1131, Y1135, Y1136) in the activation loop within the catalytic domain of the β-subunit. As a result, ATP binding sites needed for biological activity are exposed (Kato et al., 1993; LeRoith et al., 1995). Initial phosphorylation at Y1135 is followed by stabilization of the receptor by phosphorylation at Y1131 and Y1136, resulting in increased catalytic activity of the IGF-I receptor (Foulstone et al., 2005). Besides initial phosphorylation and stabilization of the IGF-I receptor, autophosphorylation occurs on tyrosine residues in response to ligand binding, as the tyrosine kinase of one β-subunit phosphorylates tyrosine residues on the other β-subunit of the $\alpha_2\beta_2$ receptor (Frattali and Pessin, 1993). Additionally, phosphorylation of tyrosines outside of the catalytic domain serve as docking sites for proteins that contain phosphotyrosine binding motifs (Bell et al., 2005). Examples of proteins that dock to the IGF-I receptor include insulin receptor substrate-1 (IRS-1), growth factor receptor-bound protein 2 (Grb2), and Shc, an adaptor protein (Dews et al., 2000) that activates downstream signaling pathways (discussed in the next section).

Tyrosine phosphorylation is required for IGF-I receptor signaling as mutations in the catalytic domain or in the tyrosine clusters that become autophosphorylated eliminated the functional activity of the IGF-I receptor (Kato et al., 1994). Furthermore, mutation of the ATP-binding site or single tyrosine substitution in the triple tyrosine cluster in the catalytic domain blocked ligand-induced autophosphorylation of the IGF-I receptor (Stannard et al., 1995).

The insulin receptor has similar structure to the IGF-I receptor. Both have α- and β-subunits and are about 370 kDa in size (McCusker, 1998). Despite the low homology of the amino acid sequence between the α-subunit of the IGF-I and insulin receptor (48%), 24 out of 26 cysteine residues (which is the ligand-binding domain) present in the IGF-I receptor are conserved in the insulin receptor (Sepp-Lorenzino, 1998). In contrast to the lower homology in the α-subunits of the IGF-I and insulin receptor, there is a high degree of amino acid sequence homology (84%) in the tyrosine kinase domain (Foulstone et al., 2005). The less conserved C-terminal domain of the IGF-I receptor has four tyrosine residues, one of which is conserved in the insulin receptor sequence and has a potential binding site for src homology 2 (SH2) domains that can directly bind the regulatory (p85) subunit of PI3K. This C-terminal domain is needed for the anti-apoptotic effects requiring signaling through the IGF-I receptor (O'Connor et al., 1997).

The IGF-II receptor (300 kDa) is very different from the IGF-I receptor in terms of structure and function. The IGF-II receptor is a monomeric protein that binds to IGF-II and mannosylated proteins, but has no intrinsic tyrosine kinase activity (Dupont and Holzenberger, 2003; Denley et al., 2005). The only described function of IGF-II binding to its receptor is for clearance of IGF-II via uptake and degradation (Denley et al., 2005). However, there is some evidence that the IGF-II receptor may mediate the effects of IGF-II via activation of a G-protein coupled pathway to that can lead to protein kinase C (PKC)-induced phosphorylation of intracellular proteins or stimulate the mitogen activated protein kinase pathway (Zhang et al., 1997; McKinnon et al., 2001). However, Korner et al. (1995) found that the IGF-II receptor failed to interact with G-proteins. The

IGF-II receptor can also bind other growth factors including transforming growth factor-β (Scott and Firth, 2004).

The IGF-I receptor has highest affinity for IGF-I while the affinity of the IGF-I receptor for IGF-II is 2- to 15-fold lower than that for IGF-I and 500- to 1000-fold lower than that for insulin (Jones and Clemmons, 1995; Grothey et al., 1999). The insulin receptor has high-affinity binding to insulin, a 10-fold lower affinity binding to IGF-II, and a 50- to 100-fold lower affinity binding to IGF-I (Blakesley et al., 1996). The IGF-II receptor binds IGF-II with highest affinity while binding affinity for IGF-I is >500-fold lower and insulin is not bound (Jones and Clemmons, 1995).

**Signal Transduction Pathways Mediated by the IGF-I Receptor**

Much of the following information was gained from review articles (Blakesley et al., 1996; Adams et al., 2000; Schlessinger, 2000; Kim and Accili, 2002; Vincent and Feldman, 2002; Foulstone et al., 2005).

Once IGF-I has bound to and activated the IGF-I receptor, phosphorylated domains on the receptor serve as a recruitment site for proteins to initiate the signaling cascade. In this way, the cytoplasmic signal is propagated through signal transduction pathways that lead to a series of multiple intracellular phosphorylation cascades that ultimately serve various physiological functions within the cell. Effector actions mediated through activation of the IGF-I receptor include progression through the cell cycle, proliferation, differentiation, and inhibition of apoptosis.

There are two well-characterized pathways for signal transduction events mediated through the IGF-I receptor. Each utilizes a separate adaptor protein, Shc and IRS-1. There are various isoforms of Shc derived by splicing and translational modifications and

at least four genes (IRS-1 to -4) that encode IRS proteins. Both Shc and IRS-1 bind to the same phosphorylated tyrosine (Tyr-950) located in the juxtamembrane region on the cytoplasmic side of the IGF-I receptor and become phosphorylated in turn after binding. Although these two proteins bind to the same site on the receptor, maximal phosphorylation of IRS-1 is reached within 1 to 2 min following IGF-I binding to its receptor as compared to 5 to 10 min for Shc. Phosphorylation of the two adaptor proteins leads to two separate series of phosphorylation cascades. Activation and phosphorylation of Shc causes the formation of a complex between Shc and a second adaptor protein, Grb2. The adaptor protein IRS-1 can also facilitate the activation of two SH2 proteins capable of continuing different signaling cascades, Grb2 and the p85 regulatory subunit of PI3K.

From this point onward, the signaling events mediated through Grb2 and PI3K will be discussed separately. A summary of the IGF-I signaling pathway can be found in Figure 2-2. Activation of Grb2 occurs by forming of a complex with either Shc or IRS-1. Both complexes require the guanine nucleotide exchange factor Son of Sevenless (Sos) for activation of the membrane-bound protein Ras activating factor that exchanges guanine diphosphate (GDP) for guanine triphosphate (GTP). Activation of Ras initiates a cascade of phosphorylation events involving the intermediary kinases Raf and MEK that ultimately leads to the phosphorylation and activation of mitogen-activated protein kinase kinase (MAPKK) pathway. Signaling through this pathway is associated with cell differentiation, migration, proliferation, and in some instances, regulation of apoptosis. Indeed, IGF-I blocked the induction of apoptosis caused by serum withdrawal in rat pheochromocytoma PC12 cells that involved the MAPKK pathway, as an inhibitor of

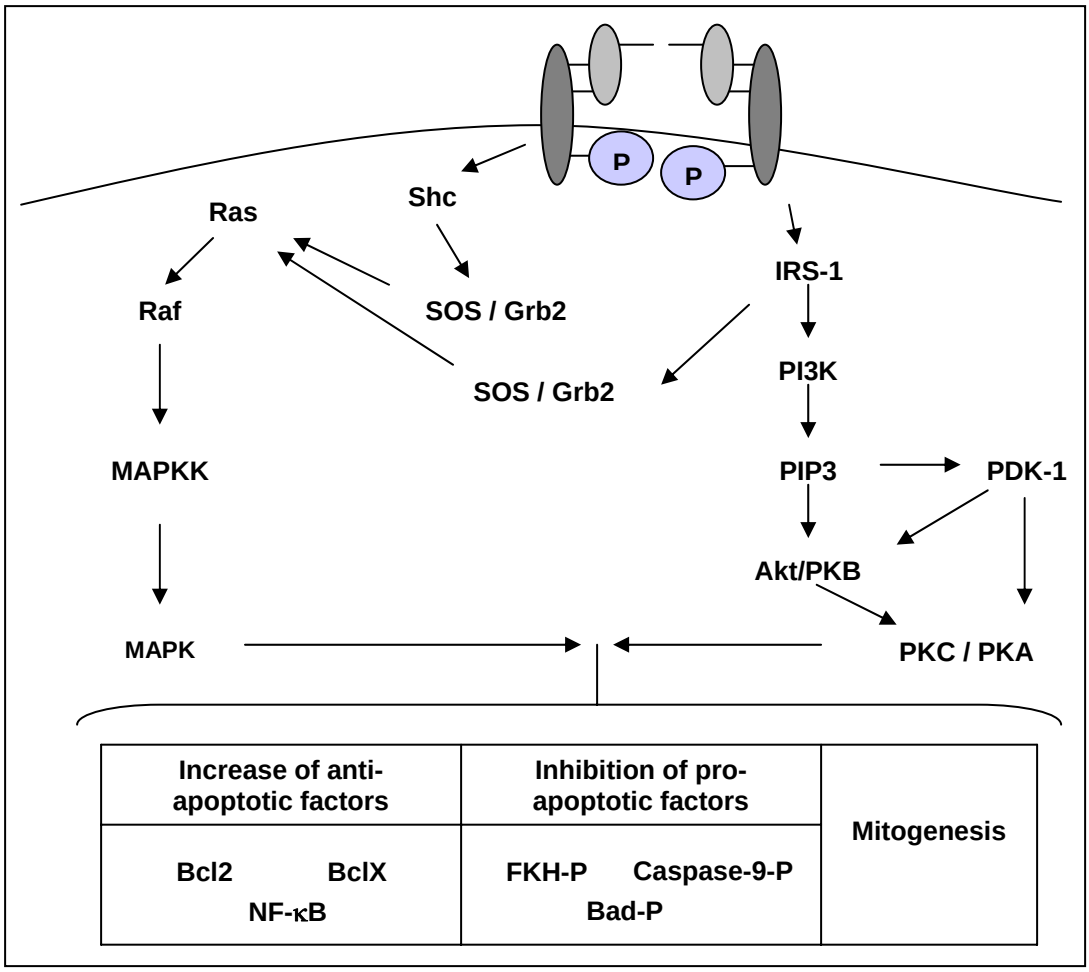


Figure 2-2. Signal transduction pathway mediated through the IGF-I receptor. Following the binding of IGF-I to its receptor, a series of signaling cascades can be activated that leads to the increased proliferation and anti-apoptotic functions of IGF-I. The best characterized pathways activated by the IGF-I receptor are the PI3K/Akt pathway and the MAPKK pathway that can lead to increased activity of anti-apoptotic factors (e.g., Bcl2, BclX, and NF-κB) and inhibition of pro-apoptotic factors (e.g., forkhead transcription factor, caspase-9, and Bad), thereby decreasing the incidence of apoptosis and increasing cell proliferation. Signaling through IRS-1 can also activate the MAPKK pathway using SOS/Grb2 independent of Shc signaling. A third pathway not shown in this figure involves the 14.3.3 protein that is involved in the anti-apoptotic actions mediated by the IGF-I receptor as it binds to pro-apoptotic proteins and renders them inactive.

MAPKK signaling (PD 98059) prevented the ability of IGF-I to block apoptosis (Párrizas et al., 1997).

The other pathway activated as a result of IGF-I binding to its receptor is activation of PI3K caused either by IRS-1 or direct binding to the C-terminal domain of the IGF-I receptor. Activation of PI3K leads to a series of enzymatic conversions of the membrane phosphoinositols that increases phosphatidylinositol 3,4,5-triphosphate (PIP3) and other phosphorylated phospholipids that can bind to the pleckstrin homology domain of at least two proteins, protein kinase B (also referred to as Akt) and phosphoinositide-dependent kinase-1 (PDK-1). This binding in turn leads to the phosphorylation of Akt, PKC, and protein kinase A (PKA). Once Akt becomes phosphorylated, it prevents apoptosis by altering pro- and anti-apoptotic proteins (Gallaher et al., 2001; Nitta et al., 2004; Zheng and Quirion, 2004; Bridgewater et al., 2005; Scott et al., 2005; Zaka et al., 2005). The ability of IGF-I to prevent apoptosis through the PI3K/Akt pathway caused by serum withdrawal in rat PC12 cells was blocked by the PI3K inhibitors wortmannin and LY 294002 (Párrizas et al., 1997). Additionally, IGF-I blocked the induction of apoptosis in human fetal glomeral epithelial cells caused by treatment with etoposide, an inducer of apoptosis, which involved activation of the PI3K/Akt pathway, as LY 294002 blocked the protective effect of IGF-I (Bridgewater et al., 2005). Moreover, the ability of IGF-I to prevent apoptosis in Baf3 cells involved regulation of caspase activity that was abolished when cultured with the PI3K inhibitor LY 294002 (Leverrier et al., 1999). Finally, IGF-I reduced hypoxia-induced DNA fragmentation and caspase-3 activity in mouse neurons involving the PI3K pathway, as these protective effects of IGF-I were prevented by treatment with wortmannin (Yamaguchi et al., 2001).

One of the primary targets of Akt is Bad, a Bcl-2 family member, which in its non-phosphorylated state resides in the outer mitochondrial membrane and interacts with anti-

apoptotic proteins, such as Bcl-2. Activated Akt phosphorylates serine 126 of Bad and causes it to become inactivated by favoring association with the cytosolic protein 14.3.3. Additionally, Akt inhibits other pro-apoptotic factors through phosphorylation, including caspase-9 and members of the forkhead transcription factor family (del Peso et al., 1997; Cardone et al., 1998; Brazil and Hemmings, 2001; Kim and Chung, 2002; Vara et al., 2004; Song et al., 2005). Furthermore, Akt can phosphorylate and activate the transcription factor cyclic AMP response element-binding protein and the IκB kinase α, which phosphorylates IκB and targets it for degradation, consequently activating nuclear factor-kappa B (NF-κB) and the transcription of anti-apoptotic genes, such as Bcl-2 and Bcl-xL. For instance, IGF-I increased amounts of Bcl-x and Bcl-xL mRNA in PC12 cells (Párrizas and LeRoith, 1997) and in Baf-3 cells derived with bone marrow, but this action of IGF-I was inhibited in the presence of LY 294002 (Leverrier et al., 1999). Additionally, IGF-I decreased apoptosis in human fetal glomeral epithelial cells caused by etoposide by increasing Bad phosphorylation (Bridgewater et al., 2005). Signaling through Akt also can phosphorylate and stabilize X-linked inhibitor of apoptosis proteins involved in cell survival (Cheng et al., 2002; Dan et al., 2004).

Signaling through Akt can also regulate repair of broken DNA. The ability of IGF-I to increase Akt activity of mesangial cells cultured to induce hyperglycemia was blocked with LY 294002 (Kang et al., 2003). Moreover, IGF-I-treated mesangial cells increased the repair of DNA double-strand breaks by nearly 2-fold (Yang et al., 2005). Additionally, IGF-I enhanced DNA repair by facilitating the colocalization of Rad51, a key enzyme in the repair of DNA (Trojanek et al., 2003), with foci of nuclear damage,

confirming that IGF-I-signaling can rescue mesangial cells from hyperglycemia-induced danger signals by enhancing DNA repair (Yang et al., 2005).

A final pathway for IGF-I signaling involves the 14.3.3 proteins. There are three isoforms of this protein (β, ε, and ζ) that can bind to the cytoplasmic portion of the IGF-I receptor and have a functional tyrosine kinase catalytic domain. The 14.3.3 protein is thought to interact with the IGF-I receptor since the serine quartet at positions 1280-1283 (1283 in particular) bind to the 14.3.3 family of proteins (Craparo et al., 1997). The 14.3.3 protein is involved in the sequestration of pro-apoptotic proteins, such as Bad (Peruzzi et al., 1999), and inhibits their pro-apoptotic actions.

**Presence of IGF-I and Its Receptor in Reproductive Tissues**

**Follicle**

Messenger RNA for the IGF-I receptor has been detected in granulosa cells and oocytes of preantral and antral follicles, but mRNA for IGF-I or -II was not detected in preantral or antral follicles (Armstrong et al., 2000, 2002). In bovine cumulus-oocyte complexes (COCs), transcripts were detected for IGF-I and IGF-I receptor isolated from 3 to 6 mm antral follicles and from larger 8 to 16 mm antral follicles while only the mRNA encoded for the IGF-I receptor was detected in denuded oocytes (Nuttinck et al., 2004). However, mRNA for IGF-I and its receptor were not detected in unfertilized oocytes (Watson et al., 1992). In the bovine, addition of IGF-I to culture medium improved oocyte quality in the largest follicles (281 to 380 μm; Walters et al., 2006).

One function of IGF-I is to increase follicular growth. Culture of small bovine follicles (165 to 215 μm) with IGF-I increased follicular diameter and estradiol production (Walters et al., 2006). Additionally, IGF-I alone or in combination with FSH

treatment stimulated cumulus cell proliferation in antral follicles that were 1 to 5 mm in diameter (Armstrong et al., 1996). Besides increasing proliferation of cumulus cells, IGF-I suppressed apoptosis in cumulus cells in small (≤4 mm) follicles and had an additive effect with FSH on suppression of apoptosis (Yang and Rajamahendran, 2000).

The importance of the ST-IGF-I axis in follicular growth is indicated by a study of Brahman cows with a genetic deficiency in the ST receptor. These animals had a >7 fold decrease in IGF-I concentration as compared to control Brahman cattle, had a decreased number of 2 to 5 mm follicles, and a reduced number of follicles >5 mm diameter (Chase et al., 1998). These changes were seen without any change in peripheral concentrations of FSH, LH, and estradiol.

Insulin-like growth factor-I may play a role in selection of the dominant follicle in cattle. Follicular fluid concentrations of IGF-I decreased in the second-largest follicle between the 7.0 to 7.9 and 8.0 to 8.9 mm diameter range (Beg et al., 2002), which is when deviation of the dominant follicle occurs in cattle (Ginther, 2000; Ginther et al., 2003). In the presence of LH, IGF-I increased the production of progesterone and androstenedione from thecal cells of large bovine follicles (>7.9 mm; Spicer et al., 2004). The amount of IGF-I and -II mRNA was increased in dominant follicles as compared to subordinate follicles collected at the same time in the first follicular wave in cattle (Yuan et al., 1998).

**Corpus luteum**

In the bovine CL, the greatest amount of mRNA for IGF-I and its receptor occurred during the early luteal phase of the estrous cycle (d 1 to 4; Schams et al., 1999, 2002). Amounts of IGF-I and its receptor decreased from d 5 to 7, and then increased during the

mid-to-late luteal phase from d 8 to 18 (Schams et al., 2002). Immunolocalization for IGF-I was mainly in large luteal cells with lower amounts in small luteal cells (Amselgruber et al., 1994; Schams et al., 1999).

One role of IGF-I in the CL is to stimulate progesterone secretion. Both IGF-I and -II are potent stimulators of progesterone production by cultured luteal cells (Einspanier et al., 1990; Liebermann et al., 1996; Brown and Baden, 2001). Similarly, IGF-I stimulated progesterone production in a dose-dependent manner from mid-cycle bovine CL, and this stimulatory effect of progesterone production by IGF-I was blocked when human recombinant IGFBP-2 and -3 were added to culture (Brown and Braden, 2001).

In the bovine CL, as in other tissues, IGFBPs can interfere with actions of IGF-I to promote progesterone production. Both IGFBP-2 and -3 blocked the binding of radiolabeled IGF-I to its receptor (Brown and Braden, 2001). Luteolysis may involve IGFBP-mediated inhibition of the luteotropic actions of IGF-I. Amounts of IGFBP-1 mRNA and protein isolated from the bovine CL were increased by treatment of cyclic cows with $PGF_{2\alpha}$ and serum progesterone concentrations were reduced (Sayre et al., 2000). It is possible that increased production of IGFBPs could antagonize actions of IGF-I that could alter progesterone production by the CL.

As mentioned earlier, the energy balance of the dairy cow can influence IGF-I synthesis. Indeed, lactating Holstein cows in a positive energy balance during the first 12 wk postpartum had greater serum concentrations of IGF-I and increased progesterone secretion at diestrus in the first two postpartum estrous cycles than cows in negative energy balance (Spicer et al., 1990). This indicates that increased luteal activity during

positive energy balance may be associated with increased IGF-I concentrations as compared to cows in a negative energy balance.

**Oviduct**

Transcripts for IGF-I were found in the oviduct during the bovine estrous cycle with increased abundance detected after ovulation (Schmidt et al., 1994). Additionally, IGF-I was localized in the secretory cells of the oviductal epithelium (Schmidt et al., 1994). Similarly, transcripts for IGF-I, IGF-I receptor, and IGFBP-3 and -5 were found to be present in the mucosa and musclar regions of the bovine oviduct (Pushpakumara et al., 2002); however, amounts of mRNA for IGF-I and its receptor were not different among pregnant heifers on d 3, 6, or 16 after insemination (Pushpakumara et al., 2002). Somatotropin addition to cultured bovine oviductal cells increased the release of IGF-I into medium (Makarevich and Sirotkin, 1997). Additionally, IGF-I stimulated [$^{3}$H] thymidine incorporation of cultured bovine oviductal cells (Tiemann and Hansen, 1995). The fact that the oviduct produces IGF-I means that this tissue is a source of IGF-I for possible regulation of early embryonic development. Little is known about whether IGF-I also exerts actions on oviductal cells.

**Uterus**

The uterus is also a source of IGF-I that could regulate the embryo. Amounts of IGF-I mRNA in the bovine uterus were greatest at the time of estrus and lowest during early and late luteal phases of the estrous cycle (Robinson et al., 2000). There are conflicting reports as to whether pregnancy status affects IGF-I synthesis in the uterus. There was no influence of pregnancy status at d 16 after breeding on the steady-state amount of IGF-I mRNA in one study (Robinson et al., 2000). In contrast, pregnant cows had higher amounts of IGF-I mRNA in endometrium at d 16 of pregnancy than non-

pregnant cows (Kirby et al., 1996). Concentrations of IGF-I from bovine uterine luminal flushings were greater on d 0 and 5 as compared to the other days of the cycle and early pregnancy; however, amounts of endometrial IGF-I mRNA did not differ between cyclic or pregnant animals or during the days of the estrous cycle (Geisert et al., 1991). This is in contrast to Meikle et al. (2001) where amounts of endometrial IGF-I mRNA were greater on d 0 and 5 of the estrous cycle as compared to d 12, suggesting a role for IGF-I in the early stages of embryonic development. Expression of IGFBP-1, -2, and -3 mRNA was also found in the uterus of pregnant and non-pregnant females (Keller et al., 1998).

## Effects of bST in the Dairy Cow

Although the focus of this dissertation is on IGF-I, the potential to use bST to regulate IGF-I secretion in the lactating dairy cow makes a discussion of the role of bST in regulating IGF-I secretion, milk yield, and reproduction warranted.

### IGF-I Concentrations

Plasma concentrations of IGF-I in dairy cattle gradually decrease 2 wk prior to parturition in a manner that closely follows the decrease in amounts of IGF-I mRNA in liver (Radcliff et al., 2003). Synthesis and secretion of IGF-I require ST receptor 1A in the liver (Etherton and Bauman, 1998). The periparturient decline in IGF-I secretion is probably caused by the loss of this receptor because there was a precipitous decline in amounts of ST receptor 1A mRNA 3-d prior to parturition that preceded the decline in amounts of IGF-I mRNA and protein (Kobayashi et al., 1999; Radcliff et al., 2003; Kim et al., 2004b).

Plasma IGF-I concentrations remain low during the initial week of lactation (Vega et al., 1991; Kobayashi et al., 1999; Pushpakumara et al., 2003; Radcliff et al., 2003) but gradually increase during the second week of lactation (Pushpakumara et al., 2003;

Radcliff et al., 2003). Nonetheless, ST receptor 1A expression in liver remains low during early lactation (Kobayashi et al., 1999; Radcliff et al., 2003; Kim et al., 2004b) and the IGF-I response to bST administration is also low during this period (Vicini et al., 1991). Plasma concentrations of IGF-I are higher in non-lactating cows compared to lactating cows, presumably due to the increased energy demands associated with lactation and negative energy balance (De La Sota et al., 1993). However, daily administration of bST (20 to 40 mg/d) to lactating dairy cows increased plasma IGF-I concentrations (Cohick et al., 1989; De La Sota et al., 1993; Sharma et al., 1994; Molento et al., 2002; Rose et al., 2005). Similar increases in plasma IGF-I concentrations occur in lactating dairy cows following administration of the slow-release form of bST (500 mg slow-release preparation given at 14-d intervals; Newbold et al., 1997; Bilby et al., 1999; Cushman et al., 2001).

**Milk Yield**

Increased milk yield of lactating dairy cows has been reported following daily treatment with bST (Cohick et al., 1989; Burton et al., 1990a; Johnson et al., 1991; Elvinger et al., 1992; Sharma et al., 1994; Rose et al., 2005) or administration of the slow-release form of bST at 14-d intervals (Lotan et al., 1993; Newbold et al., 1997; Tarazón-Herrera et al., 1999, 2000; Keister et al., 2002; Santos et al., 2004b). Increased glucose needed for lactose synthesis in lactating dairy cows treated with bST occurs by reducing the uptake of glucose by muscle and adipose tissue if in positive energy balance, increasing gluconeogenesis in the liver, and releasing glucose from glycerol during increased lipolysis if in negative energy balance (Peel and Bauman, 1987; Miller et al., 1991; Bauman, 1992, 1999; Bauman and Vernon, 1993). Reduction in lipogenesis in body tissues allows more free fatty acids to be used directly in milk fat production in the

mammary gland (Etherton et al., 1993; Etherton and Bauman, 1998). Treatment with bST also reduces protein turnover and the oxidation of amino acids for energy production to make available more amino acids for milk protein synthesis (Peel and Bauman, 1987; Bauman, 1992, 1999; Bauman and Vernon, 1993). These actions of bST are matched by an increase in feed intake to match the nutrient needs for increased milk synthesis (Peel and Bauman, 1987; Hartnell et al., 1991; Bauman, 1992, 1999; Gibson et al., 1992; Bauman and Vernon, 1993; Lotan et al., 1993; Renaville et al., 2002). Although bST-treated cows did not have increased dry matter intake, they had increased feed efficiency for milk yield (Burton et al., 1990a; Tarazón-Herrera et al., 1999, 2000). Increased milk yield following treatment of lactating dairy cows with bST might be due to increased cell proliferation of mammary epithelial cells. Indeed, lactating, bST-treated Holstein cows in mid-lactation had an increased proportion of mammary epithelial cells labeled with the nuclear proliferation antigen Ki-67, but did not alter the frequency of apoptosis, indicating that bST can induce the proliferation of mammary epithelial cells during lactation (Capuco et al., 2001).

The metabolic heat associated with increased lactation could result in an increased rectal temperature of the lactating dairy cow, especially during the hot months of the year when the ambient temperature and humidity are increased and lactating cows are more susceptible to heat stress. Exposure of lactating, bST-treated Holstein cows to heat stress during the summer caused an increase in rectal temperature (0.6ºC) over control cows (Elvinger et al., 1992). Similarly, lactating, bST-treated Holstein and Jersey cows had increased body temperature (measured as milk temperature) at the morning and afternoon milkings during heat stress (West et al., 1990a). Moreover, rectal temperature was 0.5ºC

higher for bST-treated cows compared to control cows, but bST-treated cows produced 23.0 kg/d of 3.5% fat corrected milk compared to 15.9 kg/d for non-treated cows (Vicini et al., 1990). In some environments, for example in an environmental chamber, lactating, bST-treated cows were able to offset their greater heat production by increased heat loss primarily through skin and respiratory vaporization so that there was no difference in rectal temperature between bST-treated and non-treated lactating cows (Manalu et al., 1991).

Whether or not the increased milk yield and body temperature associated with bST treatment to lactating dairy cows has negative impacts on herd health is unclear. Numerous studies have reported no adverse effects of bST treatment to lactating dairy cows on herd health (Burton et al., 1990b; Stanisiewski et al., 1992; Downer et al., 1993; Collier et al., 2001). However, a meta-analysis by Dohoo et al. (2003) concluded that bST-treated cows had an increased risk for developing clinical mastitis by approximately 25% during the treatment period. Similarly, while lactating, multiparous Holstein cows treated with bST did not have increased incidence of mastitis or somatic cell counts in milk, bST treatment increased somatic cell counts after 81-d in milk for primiparous cows (Santos et al., 2004b). Additionally, bST-treated cows diagnosed with mastitis had increased treatment duration (Burton et al., 1990a).

**Reproduction**

There have been conflicting reports on the impact of bST treatment on reproductive performance of lactating dairy cows. Differences in the findings of these studies could be due to the dose of bST administered, the timing of initiation of bST treatment, energy balance of the cow, and milk yield. For example, lactating Holstein cows administered a high daily dose of bST (14 mg/d) had decreased pregnancy rates as compared to lactating

Holstein cows that received a low dose of bST (5 mg/d) from 14 to 130 d postpartum (Stanisiewski et al., 1992). Similarly, Esteban et al. (1994) reported that the odds of a cow becoming pregnant decreased linearly as the dose of bST treatment increased. At 180-d after calving, lactating, bST-treated Holstein cows had reduced pregnancy rates as compared to non-treated cows (Luna-Dominguez et al., 2000). In addition to compromised pregnancy rates, bST-treated cows had decreased expression of estrus and delayed estrus behavior, resulting in a decreased detection of cows in estrus, fewer mounting events, and reduced inseminations (Morbeck et al., 1991; Cole et al., 1992; Lefbreve and Block, 1992; Kirby et al., 1997b; Dohoo et al., 2003). In other studies, however, there were no negative effects of bST treatment on first-service conception rates, pregnancy rates, number of days open, twinning rates, incidence of cystic ovaries, or abortions (Bilby et al., 1999; Luna-Dominquez et al., 2000; Collier et al., 2001).

Beneficial effects of bST treatment have been reported on fertility of lactating dairy cows. In large-scale field trials, lactating, bST-treated dairy cows had increased conception and pregnancy rates following TAI than control cows (Moreira et al., 2000b, 2001; Santos et al., 2004b). Additionally, bST treatment to repeat-breeding Holstein cows at estrus improved conception rates (Morales-Roura et al., 2001). However, there was a non-significant reduction in conception rate for bST-treated cows following the second AI after detected estrus (37.5% for control cows to 31.3% for bST cows; Santos et al., 2004b). Thus, either the beneficial effect of bST is more likely for the first insemination after calving or prolonged treatment with bST causes other changes that limit its fertility-enhancing effect. One effect of bST is to reduce the period of dominance for the dominant follicle in the first follicular wave, resulting in emergence of

the second follicular wave 24 to 48 h before that of the untreated cows (Kirby et al., 1997a; Lucy, 2000). The resultant prolonged dominance of the second wave dominant follicle could reduce oocyte viability and subsequent fertility (Austin et al., 1999).

The positive effect of bST on fertility likely occurs in the post-ovulatory period since cows treated with bST 10-d before TAI or coincident with TAI had similar pregnancy rates (Moreira et al., 2001). Moreover, increased conception rates in lactating, bST-treated Holstein cows was due to reduced pregnancy loss that occurred between d 31 and 45 following the first postpartum AI (6.6% loss for bST-treated cows compared to 14.1% loss for non-treated cows; Santos et al., 2004b). Further evidence for post-ovulatory effects on the embryo and uterus comes from a superovulation study (Moreira et al., 2002a), as bST-treated lactating and non-lactating Holstein superovulated cows treated with a single injection of bST at insemination had a reduced number of unfertilized ova per flush and an increased percentage of transferable embryos and number of blastocysts per flush. In the same study, recipients that received a transferable embryo from a bST-treated donor cow had increased pregnancy rates compared to recipients that received a control embryo (Moreira et al., 2002a). Moreover, bST-treated recipient cows (a single injection at 12 h after detected estrus) had increased pregnancy rates over control recipients that received a control embryo (Moreira et al., 2002a). There was, however, no additive effect on increased pregnancy rates for bST-treated recipients that received an embryo from a bST-treated donor. Cushman et al. (2001) also observed a beneficial effect of bST on superovulation response. In particular, bST-treated Angus heifers or non-lactating Angus cows for 5 or 6 injections had increased number of total

ova and transferable embryos following superovulation when collected between d 6 and 8 post-insemination (Cushman et al., 2001).

While bST treatment seems to have effects during the post-ovulatory period, it is also possible that bST could have effects on follicular dynamics and reproductive tissues. Treatment of cows with bST can influence the ovary, as lactating, bST-treated cows had increased CL weight (progesterone concentrations in plasma were similar to controls, however), number of largest follicles 10 to 15 mm in diameter, and concentrations of IGF-I in follicular fluid (Lucy et al., 1995). Additionally, lactating and non-lactating bST-treated dairy cows had a greater number of follicles 6 to 15 mm in diameter for lactating cows and increased size of the second largest follicle for lactating and non-lactating cows (De La Sota et al., 1993). Treatment of lactating cows with bST restored plasma IGF-I concentrations equal to that of non-lactating cows, and this corresponded to the development of follicle populations in lactating cows that were more characteristic of non-lactating cows (De La Sota et al., 1993). While administration of bST to previously heat-stressed lactating dairy cows improved the percentage of quality grade 1 oocytes harvested by ovum pick-up, cleavage rates following in vitro maturation and parthenogenetic activation were similar to cows not treated with bST (Roth et al., 2002).

A few studies have evaluated effects of bST on gene expression in the endometrium and oviduct. Non-lactating, bST-treated Holstein cows had increased amounts of oxytocin receptor mRNA, estradiol receptor $\alpha$ (ER$\alpha$) protein, and prostaglandin H synthase-2 (PSTS-2) protein in endometrial tissues of cows at d 17 of pregnancy (Guzeloglu et al., 2004). These effects might be expected to enhance luteolysis and thereby decrease pregnancy rates. Consistent with these results, non-

lactating bST-treated cows had a decreased pregnancy rate as compared to cows not treated with bST (Bilby et al., 2004a). In contrast to these results, lactating, bST-treated Holstein cows had minimal effects on endometrial amounts of ERα and PSTS-2 mRNA (Balaguer et al., 2005), indicating that the physiological state of the female might influence its responsiveness to bST treatment. Similarly, there was no change in the amount of IGF-I mRNA at d 3 following a synchronized estrous cycle for lactating, bST-treated cows (Pershing et al., 2002).

These studies indicate that bST treatment can influence reproductive function, in part due to changes in IGF-I. Thatcher et al. (2006) proposed that there might be an optimal range of circulating IGF-I concentrations for establishment of pregnancy in lactating dairy cows treated with bST and that IGF-I concentrations above or below this range could result in decreased pregnancy rates. Experimental support for this idea comes from a comparison of two independent studies involving the administration of bST to non-lactating Holstein cows (Bilby et al., 2004a) or lactating Holstein cows (Bilby et al., 2004b). Non-lactating, bST-treated pregnant cows had an increased plasma concentration of IGF-I (over 600 ng/ml) and a lower pregnancy rate whereas non-lactating pregnant cows not treated with bST had a decreased plasma concentration of IGF-I (~350 ng/ml), but a higher pregnancy rate (Bilby et al., 2004a). Conversely, lactating, bST-treated pregnant cows had an increased concentration of plasma IGF-I (approximately 300 ng/ml) that was similar to the non-lactating pregnant cows not treated with bST (Bilby et al., 2004b). These lactating, bST-treated cows had a higher pregnancy rate while lactating pregnant cows not treated with bST had a decreased concentration of plasma IGF-I (<150 ng/ml) and a lower pregnancy rate (Bilby et al., 2004b). Thus,

manipulation of the circulating IGF-I concentrations of lactating cows with bST treatment to increase their IGF-I concentrations similar to that of non-lactating cows could be beneficial in increasing their pregnancy rates (Thatcher et al., 2006). Similar negative effects of high concentrations of IGF-I have been associated with increased embryonic loss in the rat (Katagiri et al., 1997), increased apoptosis in mouse blastocysts (Chi et al., 2000b), and reduced implantation rates in mice (Pinto et al., 2002b) as described earlier.

## Hypotheses for Dissertation

There are two major points of focus in this dissertation – the role of IGF-I as a cytoprotective molecule for the bovine embryo subjected to heat shock and the role of apoptosis as a determinant of embryo survival. The hypothesis for the experiments in Chapter 3 was that IGF-I would act as a cytoprotective factor for the early bovine embryo by reducing the effects of heat shock on embryo total cell number, the presence of apoptotic cells, and the percentage of selected embryos that would develop to the blastocyst stage. As a result of the findings in Chapter 3, the first objective of Chapter 4 was to determine the signaling pathway whereby IGF-I blocked the effects of heat shock on total cell number and apoptosis in bovine embryos. Availability of inhibitors to the signaling pathways mediated through the IGF-I receptor (LY 294002 and PD 98059 that inhibit PI3K and MAPKK signaling, respectively) allowed for such determinations. The second objective of Chapter 4 was to determine if the ability of IGF-I to block heat shock-induced apoptosis was the reason that IGF-I prevented the reduction in bovine blastocyst development caused by heat shock.

Because embryos were continuously cultured with IGF-I in Chapters 3 and 4, it was possible that IGF-I could have made embryos more resistant to heat shock because of

long-term effects on embryo differentiation rather than receptor-mediated activation of a cytoprotective mechanism. Thus, it was hypothesized in Chapter 5 that short-term treatment with IGF-I would still be effective in blocking the effects of heat shock on total cell number and the induction of apoptosis in bovine embryos. Since IGF-I had cytoprotective effects on heat-shocked bovine embryos the hypothesis of Chapter 6 was that administration of bST to lactating Holstein cows during the summer would increase pregnancy rates during heat stress. Given the potential role of apoptosis as a determinant of embryonic survival, the final objective of this research, achieved in Chapter 7, was to test whether measurement of group II caspase activity could predict embryo potential for continued development.

# CHAPTER 3
# INSULIN-LIKE GROWTH FACTOR-I AS A CYTOPROTECTIVE FACTOR FOR THE BOVINE PREIMPLANTATION EMBRYO EXPOSED TO HEAT SHOCK

## Introduction

The fate of the developing preimplantation embryo depends on its ability to adapt to the microenvironment established by the mother. In some instances, such as when the female has experienced heat stress, the embryo might not be able to successfully adjust to the increased reproductive tract temperature and embryonic development can become terminated (Putney et al., 1988a; Rivera and Hansen, 2001). In cattle, studies using in vitro-produced embryos demonstrated that the oocyte and early preimplantation embryo are very sensitive to detrimental effects of heat shock and that the embryo acquires some ability to protect itself from heat shock as it progresses in development (Edwards and Hansen, 1997; Krininger et al., 2002; Paula-Lopes et al., 2003a; Sugiyama et al., 2003).

A cell's resistance to stress also depends upon the array of extracellular signals that can modify cytoprotective responses. Several molecules have been demonstrated to be beneficial for protecting preimplantation embryos from deleterious effects of stress including transforming growth factor-α (Brison and Schultz, 1997) and epidermal growth factor (Levy et al., 2000; Kurzawa et al., 2002). Among these cytoprotective factors, IGF-I has been widely studied for its effects on embryonic development in the presence and absence of stress. In numerous species (human (Lighten et al., 1998; Spanos et al., 2000), rabbit (Herrler et al., 1998), mouse (Lin et al., 2003; Markham and Kaye, 2003), and bovine (Byrne et al., 2002b; Moreira et al., 2002b; Block et al., 2003; Sirisathien et

al., 2003)), development to the blastocyst stage was increased when embryos were stimulated with IGF-I. In addition, IGF-I increased embryonic ICM number (Lighten et al., 1998; Markham and Kaye, 2003) and total cell number (Herrler et al., 1998; Byrne et al., 2002b; Lin et al., 2003; Sirisathien et al., 2003).

Addition of IGF-I to culture medium reduced the incidence of spontaneous apoptosis in embryos of various species, including the human (Lighten et al., 1998), rabbit (Herrler et al., 1998), mouse (Fabian et al., 2004), and bovine (Byrne et al., 2002b; Makarevich and Markkula, 2002). In addition, IGF-I blocked apoptosis in preimplantation embryos induced by ultraviolet irradiation (Herrler et al., 1998), camptothecin and actinomycin D (Fabian et al., 2004), and TNF-α (Byrne et al., 2002a). Addition of IGF-I to culture medium also reduced the detrimental effect of hydrogen peroxide on development of mouse preimplantation embryos (Kurzawa et al., 2002).

For the current experiments, it was hypothesized that IGF-I serves as a cytoprotective factor for bovine preimplantation embryos subjected to heat shock by reducing the effect of heat shock on embryo cell number, the proportion of blastomeres that undergoes apoptosis, and the percentage of embryos developing to the blastocyst stage.

## Materials and Methods

### Materials

The media HEPES-Tyrodes Lactate (TL), IVF-TL, and Sperm-TL were purchased from Cell and Molecular Technologies Inc. (Lavallete, NJ) or Caisson (Sugar City, ID) and used to prepare HEPES-Tyrodes albumin lactate pyruvate (TALP), IVF-TALP, and Sperm-TALP as previously described (Parrish et al., 1986). Oocyte collection medium (OCM) was Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol

red (Atlanta Biologicals, Norcross, GA) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR) containing 2 U/ml heparin, 100 U/ml penicillin-G, 0.1 mg/ml streptomycin, and 1mM glutamine. Oocyte maturation medium (OMM) was TCM-199 (BioWhittaker, Walkersville, MD) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 μg/ml estradiol 17-β, 20 μg/ml bovine FSH (Folltropin-V; Vetrepharm Canada, London, ON), 22 μg/ml sodium pyruvate, 50 μg/ml gentamicin sulfate, and 1 mM L-glutamine. Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Frozen semen from various bulls was donated by Southeastern Semen Services (Wellborn, FL).

Potassium simplex optimized medium (KSOM) that contained 1 mg/ml bovine serum albumin (BSA) was obtained from Cell and Molecular Technologies or Caisson. Essentially fatty-acid free (EFAF) BSA was from Sigma (St. Louis, MO). Recombinant human IGF-I was purchased from Upstate Biotechnology (Lake Placid, NY). On the day of use, KSOM was modified for bovine embryos to produce KSOM-BE2 as described elsewhere (Soto et al., 2003b). For the addition of IGF-I to KSOM-BE2, a vial containing 25 μg of lyophilized IGF-I was rehydrated with 100 μl of 0.1 M acetic acid. This solution was then stored at -20°C in 5 μl aliquots until use when a single aliquot of IGF-I was diluted with KSOM-BE2 to a concentration of 100 ng/ml. Control media contained an equivalent volume of acetic acid.

In Situ Cell Death Detection Kit (fluorescein) was obtained from Roche Diagnostics Corporation (Indianapolis, IN). Hoechst 33342 and glycerol were purchased from Sigma. Polyvinylpyrrolidone (PVP) was purchased from Eastman Kodak (Rochester, NY) and RQ1 RNase-free DNase was from Promega (Madison, WI).

Paraformaldehyde (8%) was purchased from Electron Microscopy Sciences (Fort Washington, PA). A 10x solution of phosphate buffer saline (PBS; 0.1 M $KPO_4$, 9% NaCl, and 0.2% $NaN_3$ at pH 7.4) was diluted 1:10 with double distilled water to make a 1x solution of PBS. All other reagents were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

**In Vitro Production of Embryos**

Embryo production was performed as previously described (Paula-Lopes and Hansen, 1998; Rivera and Hansen, 2001) except for the culture medium used in the current study. Briefly, COCs were obtained by slicing 2 to 10 mm follicles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from Central Beef Packing Co. (Center Hill, FL). Those COCs with at least one complete layer of compact cumulus cells were washed two times in OCM and used for subsequent steps. Groups of 10 COCs were placed in 50 μl drops of OMM overlaid with mineral oil and matured for 20 to 22 h at 38.5°C in an atmosphere of 5% (v/v) $CO_2$ in humidified air. Matured COCs were then washed once in HEPES-TALP and transferred in groups of 30 to 4-well plates containing 600 μl of IVF-TALP and 25 μl of PHE [0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% (w/v) NaCl] per well and fertilized with approximately 1 x $10^6$ Percoll-purified spermatozoa from a pool of frozen-thawed semen from 3 bulls. After 8 to 10 h at 38.5°C in an atmosphere of 5% (v/v) $CO_2$ in humidified air, putative zygotes were removed from fertilization wells, denuded of cumulus cells by vortex mixing in 1 ml of 1000 U/ml hyaluronidase in HEPES-TALP, and placed in groups of 30 in 50 μl drops of KSOM-BE2.

Approximately half of the putative zygotes were cultured in KSOM-BE2 containing 100 ng/ml of IGF-I for the entire culture period whereas the remaining zygotes were cultured in KSOM-BE2. All drops of embryos were overlaid with mineral oil and cultured at 38.5°C in an atmosphere of 5% (v/v) $CO_2$ in humidified air until selected for treatment at d 5 after insemination. At this time, embryos ≥16 cells were harvested from culture drops according to the specific experimental design. In addition, each replicate contained an undisturbed drop of 15 embryos cultured ± IGF-I that was used to assess cleavage rates at d 3 after insemination and development to the blastocyst stage at d 8 after insemination.

**TUNEL and Hoechst 33342 Labeling**

The TUNEL assay was used to detect DNA fragmentation associated with late stages of the apoptotic cascade. The enzyme terminal deoxynucleotidyl transferase is a DNA polymerase that catalyzes the transfer of a fluorescein isothiocyanate-conjugated dUTP nucleotide to a free 3' hydroxyl group characteristic of DNA strand breaks. Embryos were removed from KSOM-BE2 ± IGF-I and washed two times in 50 μl drops of 10 mM $KPO_4$, pH 7.4 containing 0.9% (w/v) NaCl (PBS) and 1 mg/ml PVP (PBS-PVP) by transferring the embryos from drop to drop. Zona pellucida-intact embryos were fixed in a 50 μl drop of 4% (w/v) paraformaldehyde in PBS for 15 min at room temperature, washed twice in PBS-PVP, and stored in 600 μl of PBS-PVP at 4°C until the time of assay. All steps of the TUNEL assay were conducted using microdrops in a humidified box. On the day of the TUNEL assay, embryos were transferred to a 50 μl drop of PBS-PVP and then permeabilized in 0.1% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 10 min at room temperature. Controls for the TUNEL assay

were incubated in 50 μl of RQ1 RNase-free DNase (50 U/ml) at 37°C in the dark for 1 h. Positive controls and treated embryos were washed in PBS-PVP and incubated with 25 μl of the TUNEL reaction mixture (containing fluorescein isothiocyanate-conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase as prepared by and following the guidelines of the manufacturer) for 1 h at 37°C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Embryos were then washed three times in PBS-PVP and incubated in a 25 μl drop of Hoechst 33342 (1 μg/ml) for 12 min in the dark. Embryos were washed three times in PBS-PVP to remove excess Hoechst 33342, mounted on 10% (w/v) poly-L-lysine coated slides using 3-4 μl drops of glycerol, and coverslips were placed on the slides. Labeling of TUNEL and Hoechst 33342 nuclei was observed using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany). Each embryo was analyzed for total cell number (blue nuclei) and TUNEL-positive blastomeres (green nuclei) with a DAPI and FITC filter, respectively, using a 20X objective. Digital images were acquired using AxioVision software and a high-resolution black and white Zeiss AxioCam MRm digital camera.

**Experiments**

**Protective effect of IGF-I on heat-shock induced apoptosis**

Embryos ≥16-cells were collected on d 5 after insemination and transferred to a fresh drop of KSOM-BE2 ± IGF-I. Embryos were maintained at 38.5°C for 24 h or were heat-shocked at 41°C for 9 h followed by culture at 38.5°C for 15 h. All harvested embryos were fixed on d 6 and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated 7 times using 86 to 100 embryos per treatment.

**Developmental competence of IGF-I-treated embryos subjected to heat shock**

Bovine embryos ≥16-cells were collected on d 5 after insemination and transferred to a fresh drop of KSOM-BE2 ± IGF-I. Embryos were maintained at 38.5°C or were heat-shocked at 41°C for 9 h followed by culture at 38.5°C until d 8 when development to the blastocyst stage was assessed. All blastocysts were fixed and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated 11 times using 191 to 236 embryos per treatment.

**Developmental capacity of undisturbed embryos cultured continuously with or without IGF-I**

For each replicate of the above-mentioned experiments to test IGF-I effects on resistance to heat shock, an additional, undisturbed drop of 15 embryos were cultured in KSOM-BE2 ± IGF-I (100 ng/ml) for assessment of cleavage and blastocyst development rates on d 3 and 8 after insemination, respectively. A total of 18 replicates were completed using 270 embryos per treatment.

**Statistical Analysis**

Data were analyzed by least-squares analysis of variance using the General Linear Models (GLM) procedure of SAS (SAS for Windows, Version 8, 1999-2001, Cary, NC). Percentage data were transformed by arcsin transformation before analysis. Independent variables included IGF-I, heat shock treatment, and replicate. The mathematical model included main effects and all interactions. Tests of significance for IGF-I and heat shock were performed using IGF-I x replicate and heat shock x replicate as the error term, respectively. All values reported are least-squares means ± SEM. Probability values for percentage data are based on analysis of arcsin-transformed data while least-squares

means are from analysis of untransformed data. Level of significance was set at $P \leq 0.05$.

## Results

### Cleavage and Development as Modified by IGF-I

Culture of embryos continuously in the presence of IGF-I beginning after fertilization increased the percentage of oocytes that cleaved on d 3 after fertilization ($P < 0.01$), as well as the percentage of oocytes that developed to the blastocyst stage on d 8 after fertilization ($P < 0.06$; see Figure 3-1). There was no significant difference in the percentage of cleaved embryos that became blastocysts.

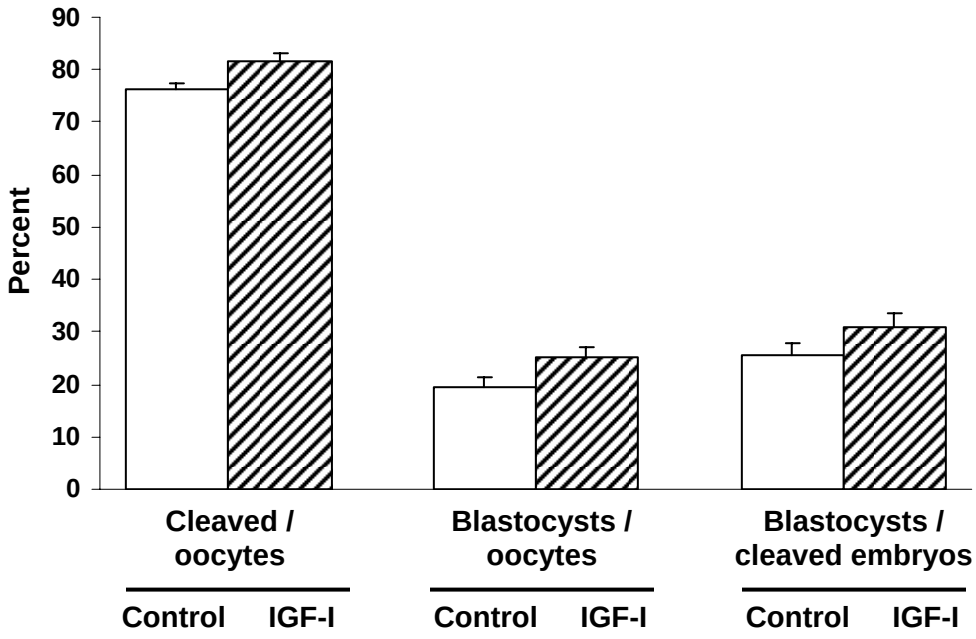


Figure 3-1. Cleavage rates and blastocyst development on day 3 and 8 after insemination, respectively, for embryos cultured with or without 100 ng/ml of IGF-I. Blastocyst development was expressed as the proportion of oocytes and cleaved embryos that became blastocysts. Results are least-squares means ± SEM. The percentage of oocytes that cleaved ($P < 0.01$), as well as the percentage of oocytes that developed to the blastocyst stage ($P < 0.06$), was affected by IGF-I. There was no significant treatment effect on the percentage of cleaved embryos that developed to the blastocyst stage.

**Protective Effect of IGF-I on Induction of Apoptosis by Heat Shock**

Representative fluorescent images illustrating the effect of IGF-I on the frequency of apoptotic nuclei for d 5 embryos ≥16-cells exposed to 38.5°C for 24 h or to 41°C for 9 h and 38.5°C for 15 h are shown in Figure 3-2. In the absence of IGF-I, heat shock caused a reduction in the total number of nuclei and an increase in the proportion of nuclei that were apoptotic (compare Figure 3-2A for an embryo cultured continuously at 38.5°C with Figure 3-2B for an embryo exposed to 41.0°C). These deleterious effects of heat shock were not apparent in embryos cultured with IGF-I (compare Figure 3-2C of an embryo cultured continuously at 38.5°C and Figure 3-2D of an embryo exposed to 41.0°C). Quantitative analysis of total cell number and percentage of cells that were TUNEL-positive are illustrated in Figure 3-3. Exposure of embryos to 41°C for 9 h on d 5 after insemination reduced total cell number ($P < 0.05$; Figure 3-3A) and increased the percentage of nuclei that were TUNEL-positive ($P < 0.001$; Figure 3-3B) on d 6 after insemination. Effects of heat shock were less for IGF-I-treated embryos for both total cell number (heat shock x IGF-I, $P = 0.07$) and for the percentage of nuclei that were TUNEL-positive (heat shock x IGF-I, $P < 0.01$).

**Reduction by IGF-I of the Heat Shock-Induced Inhibition in Blastocyst Development**

Insulin-like growth factor-I increased the percentage of embryos ≥16-cells at d 5 after insemination that developed to the blastocyst stage ($P < 0.001$; Figure 3-4A). Heat shock caused a reduction in the percentage of embryos that developed to the blastocyst stage for both control and IGF-I treated embryos ($P < 0.001$; Figure 3-4A). As shown in Figure 3-4B, however, the reduction in development caused by heat shock, expressed as a percentage of development for embryos not subjected to heat shock, was less ($P < 0.05$)

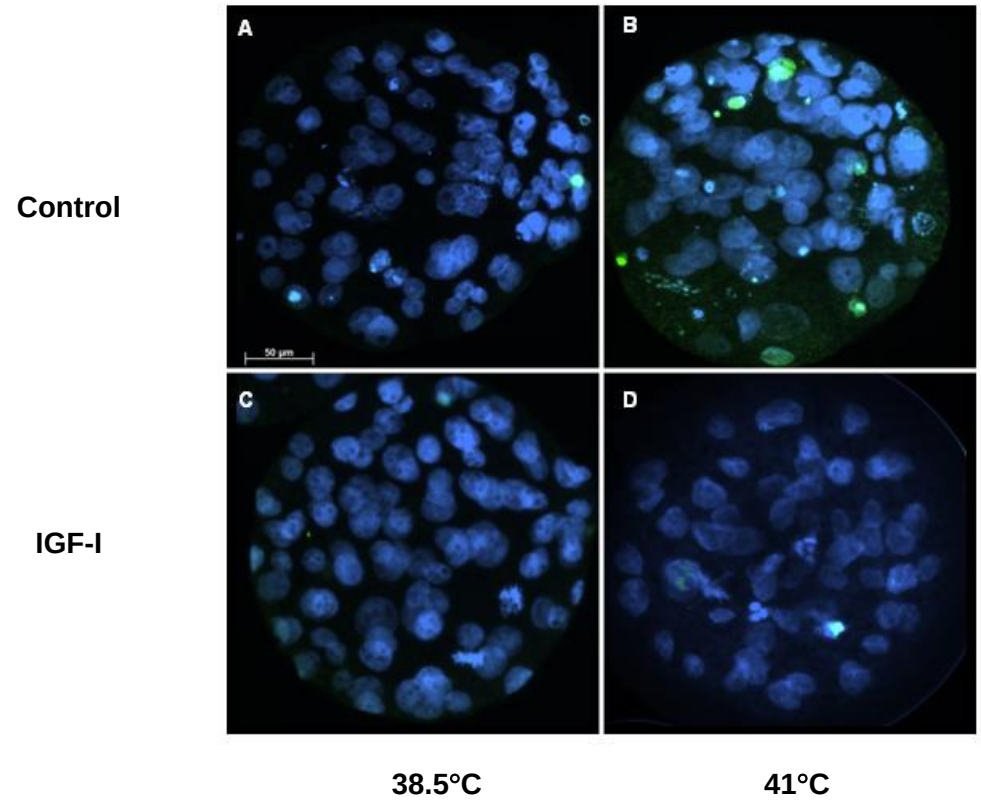


Figure 3-2. Representative images illustrating the effect of IGF-I on frequency of apoptotic nuclei in bovine embryos. Embryos ≥16-cells were cultured continuously at 38.5°C (Panels A and C) or were subjected to heat shock for 9 hours at 41°C (Panels B and D) on day 5 after insemination. Labeling at 15 h after the end of heat shock was performed for nuclei using Hoechst 33342 (blue) and labeling for apoptotic nuclei was performed using the TUNEL reaction and fluorescein isothiocyanate-conjugated dUTP (green). Bar = 50 µM.

for embryos cultured in the presence of IGF-I (19.4%) than for embryos cultured without IGF-I (46.4%).

Properties of blastocysts as affected by IGF-I and heat shock treatments are summarized in Figure 3-5. Heat shock reduced total blastocyst cell number ($P < 0.01$; Figure 3-5A) and increased the percentage of blastomeres that were TUNEL-positive ($P < 0.05$; Figure 3-5B). The lack of a significant heat shock x IGF-I interaction indicated that effects of heat shock were apparent for embryos with or without IGF-I.

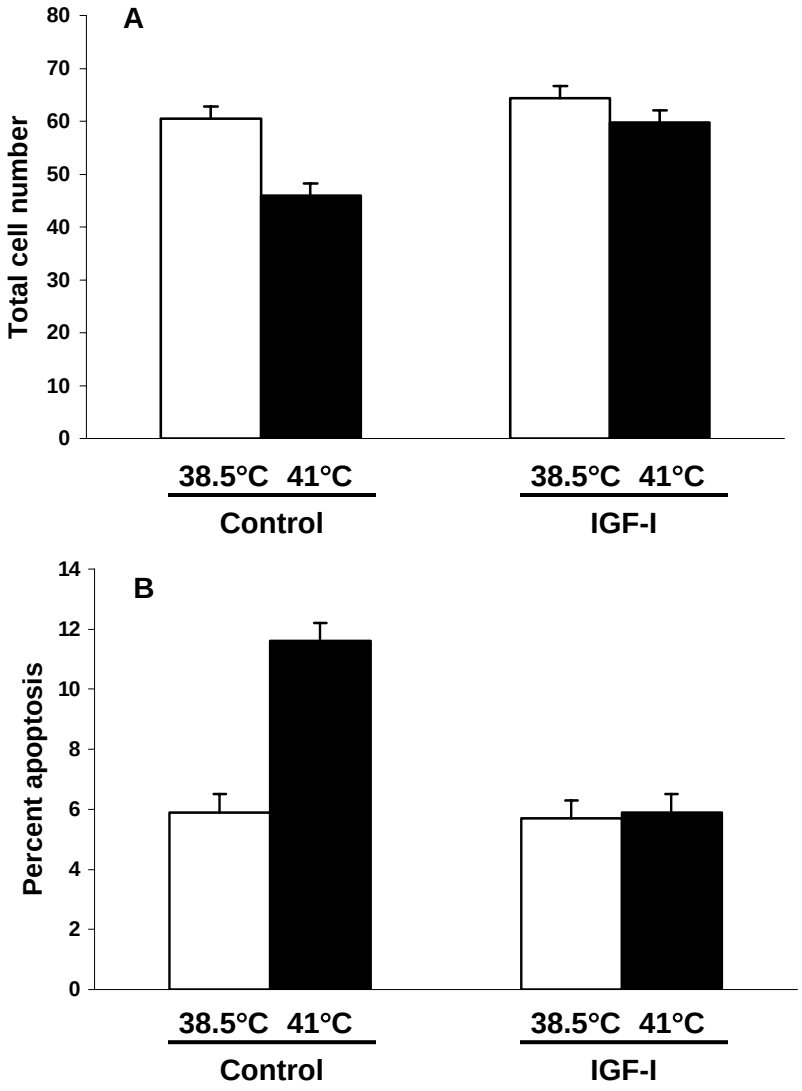


Figure 3-3. Inhibition of heat-shock induced apoptosis and growth retardation in embryos ≥16-cells by IGF-I. Open bars represent embryos maintained continuously at 38.5°C while filled bars represent embryos subjected to heat shock at 41°C for 9 h followed by culture at 38.5°C for 15 h. Results are least-squares means ± SEM. Heat shock reduced total cell number ($P < 0.05$; Panel A) and increased the percentage of nuclei that were TUNEL-positive ($P < 0.001$; Panel B). Effects of heat shock were less for IGF-I-treated embryos as determined by heat shock x IGF-I effects for total cell number ($P = 0.07$; Panel A) and the percentage of nuclei that were TUNEL-positive ($P < 0.01$; Panel B).

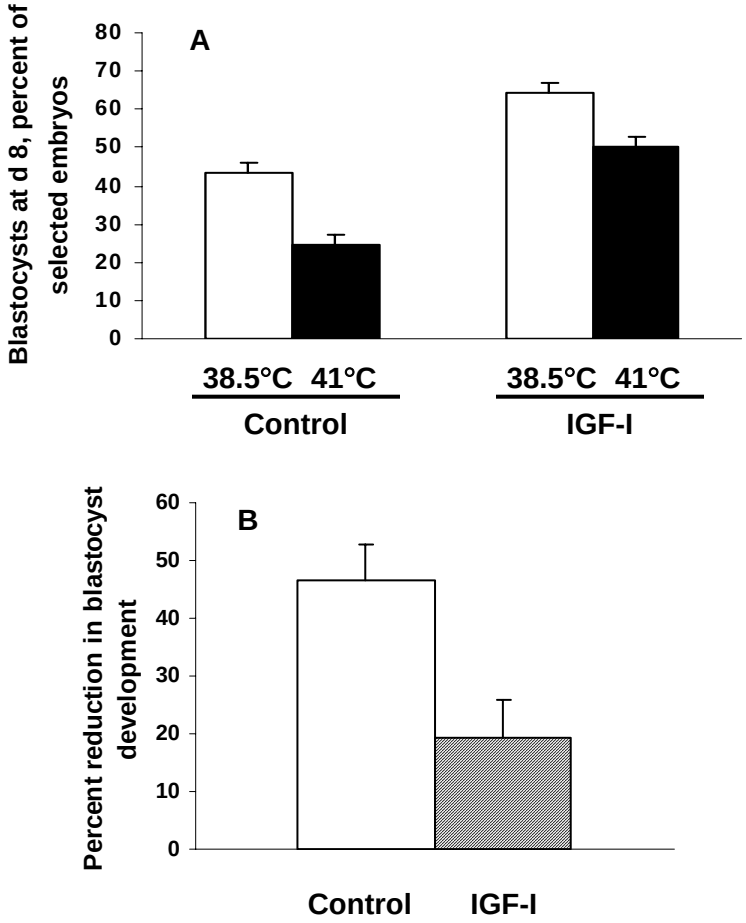


Figure 3-4. Inhibition of the deleterious effects of heat shock on development of embryos ≥16-cells to the blastocyst stage by IGF-I. Results are least-squares means ± SEM. For Panel A, open bars represent embryos maintained continuously at 38.5°C while filled bars represent embryos subjected to heat shock at 41°C for 9 h followed by culture at 38.5°C until day 8 after insemination. Development was affected by the presence of IGF-I ($P < 0.001$) and heat shock ($P < 0.001$). Panel B depicts the percent reduction in blastocyst development for embryos caused by heat shock expressed as a percentage of development for non-shocked embryos [100-((percent development at 41°C ÷ percent development at 38.5°C) x 100)]. The percent reduction in blastocyst development caused by heat shock was less ($P < 0.05$) for embryos cultured in the presence of IGF-I than for embryos cultured without IGF-I.

However, IGF-I-treated blastocysts had increased total cell number ($P < 0.001$; Figure 3-5A) and reduced percentage of blastomeres that were TUNEL-positive ($P <$

0.01; Figure 3-5B) as compared to control embryos, and these effects of IGF-I occurred regardless of heat shock (i.e., the heat shock x IGF-I interaction was not significant).

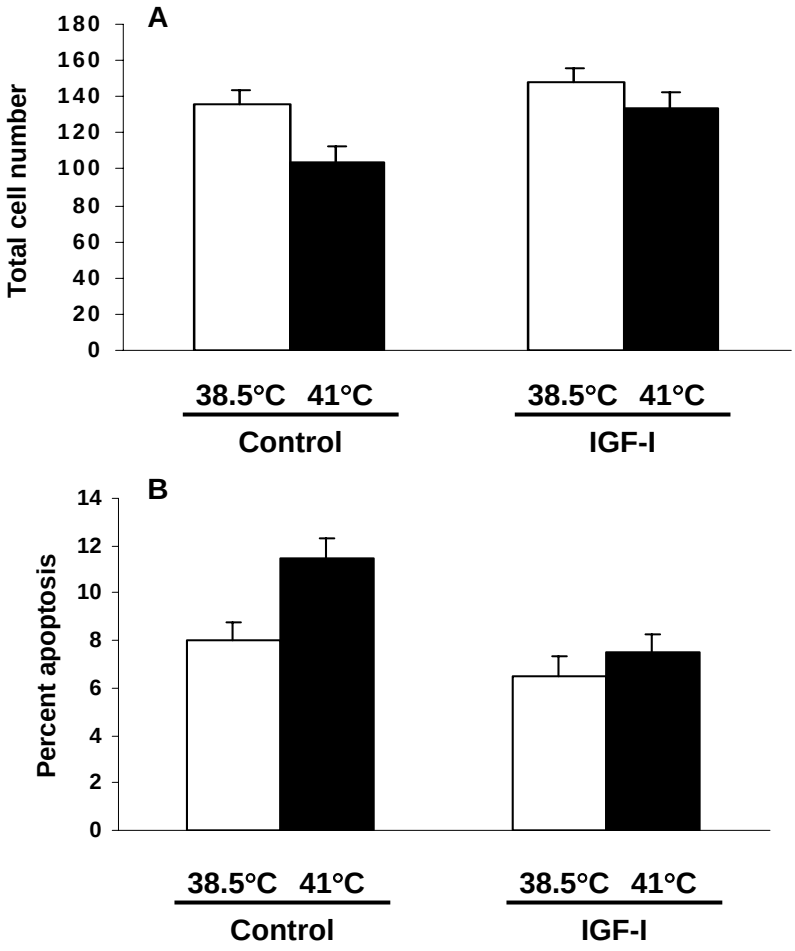


Figure 3-5. Cell number and apoptosis in day 8 blastocysts as affected by heat shock on day 5 after insemination and IGF-I. Open bars represent embryos maintained continuously at 38.5°C while filled bars represent embryos subjected to heat shock at 41°C for 9 h followed by culture at 38.5°C until day 8 after insemination. Results are least-squares means ± SEM. Heat shock reduced total cell number ($P < 0.01$; Panel A) and increased the percentage of nuclei that were TUNEL-positive ($P < 0.05$; Panel B). However, IGF-I-treated blastocysts had increased total cell number ($P < 0.001$; Panel A) and a lower percentage of nuclei that were TUNEL-positive ($P < 0.01$; Panel B).

## Discussion

Results from the current study implicate IGF-I as a cytoprotective factor for preimplantation embryos exposed to heat shock. In particular, IGF-I blocked the effects of heat shock on cell number and development to the blastocyst stage and prevented induction of apoptosis in response to elevated temperature. These results have implications for understanding the range of biological systems that regulate embryonic responses to stress and point the way to possible therapeutic interventions for enhancing embryonic development during stress.

There could be several mechanisms whereby IGF-I blocked effects of heat shock on cell number 24-h after heat shock and on the proportion of embryos that became blastocysts on d 8 after insemination. A direct mitogenic action of IGF-I is possible but not likely since IGF-I did not increase the proportion of blastomeres positive for proliferating cell nuclear antigen in bovine embryos (Makarevich and Markkula, 2002). Also, there was no major change in percent development to the blastocyst stage for non-heat-shocked embryos cultured continuously with IGF-I (see Fig. 3-1). The slight increase in development to the blastocyst stage caused by IGF-I, although non-significant, is similar in magnitude to that seen in an earlier study in our laboratory (Block et al., 2003). It is more likely that the inhibition of heat-shock induced apoptosis caused by IGF-I prevented the reduction in cell number caused by heat shock and allowed for a greater proportion of embryos to successfully develop to the blastocyst stage. Indeed, IGF-I has been demonstrated to block apoptosis in many cell types (Peruzzi et al., 1999; Su et al., 2003), including preimplantation embryos (Lighten et al., 1998; Spanos et al., 2000; Byrne et al., 2002a; Makarevich and Markkula, 2002; Fabian et al., 2004). Inhibition of apoptosis by IGF-I is mediated through activation of PI3K/Akt

and subsequent phosphorylation and inactivation of pro-apoptotic proteins such as Bad that cause mitochondrial membrane depolarization and release of cytochrome c (Gallaher et al., 2001).

It is conceivable, however, that the cytoprotective function of IGF-I occurred independently of its actions on apoptosis. Inhibition of apoptosis using the caspase inhibitor z-DEVD-fmk actually increased susceptibility of d 4 bovine preimplantation embryos to heat shock as determined by capacity to develop into blastocysts (Paula-Lopes and Hansen, 2002a). These data were interpreted to mean that a limited degree of apoptosis was beneficial for an embryo at this stage exposed to stress because apoptosis removed those cells most damaged by heat shock. One possibility is that actions of IGF-I that are proliferation-independent enhanced developmental competence and made these embryos more likely to survive stress. For example, IGF-I up-regulated glucose uptake in preimplantation mouse embryos (Pantaleon and Kaye, 1996) and such an effect might make embryos more able to survive cellular damage caused by heat shock. Insulin-like growth factor-I has been reported to reduce effects of hydrogen peroxide on development of mouse preimplantation embryos (Kurzawa et al., 2002), and given induction of peroxide production by heat shock in bovine embryos (Sakatani et al., 2004), it is possible that IGF-I protected embryos from heat shock in the current study by blocking the deleterious actions of free radicals.

Bovine embryos collected on d 5 post-insemination have been demonstrated to be more resistant to heat shock than embryos collected at earlier stages (Edwards and Hansen, 1997; Krininger et al., 2002; Paula-Lopes and Hansen, 2002a; Sakatani et al., 2004). Heat shock at this stage sometimes (Paula-Lopes and Hansen, 2003b), but not

always (Edwards and Hansen, 1997; Krininger et al., 2002; Paula-Lopes and Hansen, 2002a), reduced the potential for subsequent development to the blastocyst stage. In the present study, heat shock at this stage did reduce the proportion of embryos that developed to the blastocyst stage, but IGF-I reduced this effect. In the absence of IGF-I, moreover, blastocyst cell number was lower and the proportion of blastomeres classified as apoptotic was higher for blastocysts formed from heat-shocked embryos. Thus, it is likely that the potential for these blastocysts to continue development and establish pregnancy was compromised by the earlier heat shock. However, blastocyst cell number and apoptosis for IGF-I treated embryos exposed to heat shock was similar to values for control embryos not exposed to heat shock. These results imply that IGF-I might increase blastocyst development and pregnancy rates of cows exposed to periods of heat stress during early embryonic development. Pregnancy rates of ET recipients in the summer were higher for cows that received embryos cultured with IGF-I than for cows that received control embryos (Block et al., 2003). Additional studies to evaluate effects of IGF-I on fertility of heat-stressed cows are warranted.

One implication of the present results is that the IGF-I status within the reproductive tract is likely to be an important criterion for embryonic development. Both the oviduct and uterus produce IGF-I (Wathes et al., 1998) and content of IGF-I in the uterine lumen (but not the oviduct) was increased through injection of ST (Pershing et al., 2003). Consistent with the idea of IGF-I as a determinant of embryonic development are findings that fertility of lactating dairy cows, which have reduced concentrations of IGF-I in circulation, can be increased by treatment with bST (Thatcher et al., 2001; Moreira et al., 2002a). Perhaps manipulation of the IGF-I system with ST could enhance embryonic

development in females exposed to heat stress or other stresses. In such a scheme, effects of ST on hyperthermia (Elvinger et al., 1992) would have to be overcome.

In conclusion, IGF-I protected the preimplantation embryo from the detrimental effects of heat shock. This protective action of IGF-I, which was associated with an increase in total cell number and a reduction in the number of blastomeres that become apoptotic, may involve inhibition of apoptosis. These results imply that manipulation of the IGF-I system may enhance embryonic development in females exposed to heat stress or other stresses. Additional studies are needed to characterize the mechanistic function whereby IGF-I enhances the development of embryos exposed to heat shock.

# CHAPTER 4
# INSULIN-LIKE GROWTH FACTOR-I PROMOTES RESISTANCE OF BOVINE PREIMPLANTATION EMBRYOS TO HEAT SHOCK THROUGH ACTIONS INDEPENDENT OF ITS ANTI-APOPTOTIC ACTIONS REQUIRING PI3K SIGNALING

## Introduction

Cows that are pregnant during the hotter months of the year can experience maternal hyperthermia as a consequence of elevated temperatures and this insult can impede continued embryonic development and possibly terminate the pregnancy (Putney et al., 1988a; Ealy et al., 1993; García-Ispierto et al., 2005). Successful embryonic development requires that the embryo adjust its internal biochemistry to continue development following stresses such as heat stress that can perturb cellular function. Experiments using heat shock of the bovine preimplantation embryo have indicated that acquisition of resistance to cellular stress is in part a process inherent to the embryo's developmental program. In particular, embryos become more resistant to heat shock as they advance in development (Edwards and Hansen, 1997; Krininger et al., 2002; Sakatani et al., 2004).

Signals from the maternal environment can also regulate embryonic resistance to stress. The best-characterized example is IGF-I, which is secreted by the oviduct (Makarevich and Sirotkin, 1997) and uterus (Robinson et al., 2000) and produced by the embryo (Lonergan et al., 2000). In addition to stimulating embryonic growth, as measured by effects on embryo cell number and development to the blastocyst stage (Byrne et al., 2002b; Block et al., 2003; Sirisathien et al., 2003), IGF-I can block effects

of heat shock on bovine embryos including induction of apoptosis and reduction in competence to develop to the blastocyst stage (Chapter 3). In addition, IGF-I has been shown to reduce the effects of hydrogen peroxide on the continued development of mouse preimplantation embryos (Kurzawa et al., 2002). Anti-apoptotic actions of IGF-I are observed in a wide variety of cell types and IGF-I can block apoptosis caused by exposure to ultraviolet irradiation (Herrler et al., 1998), camptothecin and actinomycin D (Fabian et al., 2004), and TNF-α (Byrne et al., 2002a; Kurzawa et al., 2002).

The anti-apoptotic effects of IGF-I are generally considered to be mediated through a distinct signaling pathway from the proliferative actions of IGF-I (Vincent and Feldman, 2002; Mauro and Surmacz, 2004; Foulstone et al., 2005). Once the IGF-I receptor has become activated, it serves as a docking site for the phosphorylation of IRS-1, which then binds to the regulatory subunit of PI3K. Activated PI3K leads to the phosphorylation of Akt/PKB that plays a major role in the prevention of apoptosis through phosphorylation and subsequent inactivation of pro-apoptotic proteins involved in apoptosis. Examples of inactivated pro-apoptotic proteins include Bad, caspase-9, and forkhead transcription factors, which increase the activity of anti-apoptotic proteins, such as Bcl-2 and Bcl-x and the transcription factor NF-κB. An additional signaling pathway through the activated IGF-I receptor involves activation of another adaptor protein Shc that activates a secondary adaptor protein Grb2 that can also be activated by IRS-1. A guanine nucleotide exchange factor SOS-1 and Grb2 subsequently activate GTP-bound Ras, which is necessary for the continued activation of MAPKK. Signaling through this pathway can increase cell proliferation and also prevent induction of apoptosis (Jones and Clemmons, 1995; Rubinfeld and Seger, 2005).

It is not known whether IGF-I reduces the detrimental actions of heat shock on development to the blastocyst stage through its anti-apoptotic effects or through actions independent of its ability to block apoptosis, for example by promoting proliferation. However, there is a report that limited activation of apoptosis is beneficial for the embryo in order to continue development to the blastocyst stage following heat shock. Specifically, addition of z-DEVD-fmk, which inhibits apoptosis, exacerbated the reduction in development to the blastocyst stage caused by heat shock (Paula-Lopes and Hansen, 2002b). Given this observation, which indicates that apoptosis represents a protective response to heat shock to facilitate continued development, it was hypothesized that the actions of IGF-I to block effects of heat shock on development are independent of its anti-apoptotic effects.

The first objective of the current experiments was to determine the signaling pathway whereby IGF-I blocks heat shock-induced apoptosis and promotes an increase in embryo cell number. The approach was to test the effects of inhibitors of the PI3K and MAPKK pathways, LY 294002 and PD 98059, respectively, on actions of IGF-I signaling. The second objective was to determine if the anti-apoptotic actions of IGF-I block the effects of heat shock on blastocyst development. This objective was accomplished by testing whether an inhibitor to the anti-apoptotic signaling pathway of IGF-I (LY 294002) blocks actions of IGF-I on blastocyst development and by determining whether another molecule that inhibits apoptosis (the caspase-3 inhibitor z-DEVD-fmk) mimics the pro-developmental actions of IGF-I in heat-shocked embryos.

## Materials and Methods

### Materials

The materials used for these experiments were as described in Chapter 3 with the following additions. Dimethyl sulfoxide (DMSO), PD 98059, and LY 294002 were obtained from Sigma. For the addition of the PI3K inhibitor, a vial containing 1 mg of LY 294002 was rehydrated with 32.5 μl of 100% DMSO and stored at -20°C in 3 μl aliquots until use when a single aliquot was diluted in KSOM-BE2 ± IGF-I to a concentration of 100 μM. For the addition of the MAPKK inhibitor, a vial containing 1 mg of PD 98059 was rehydrated with 37.4 μl of 100% DMSO and stored at -20°C in 3 μl aliquots until use when a single aliquot was diluted in KSOM-BE2 ± IGF-I to a concentration of 100 μM. For the addition of the caspase-3 inhibitor, a vial containing 1 mg of z-DEVD-fmk was rehydrated with 15 μl of 100% DMSO and stored at -20°C in 1.25 μl aliquots until use when a single aliquot was diluted in KSOM-BE2 ± IGF-I to a concentration of 100 μM. The final concentration of DMSO in all treatments was 0.1% (v/v). The In Situ Cell Death Detection Kit (rhodamine) was obtained from Roche Diagnostics Corporation. The SYTOX green nucleic acid stain was purchased from Molecular Probes.

### In Vitro Production of Embryos

Embryo production was performed using in vitro maturation of oocytes and in vitro fertilization as described in Chapter 3. Approximately half of the putative zygotes were cultured in KSOM-BE2 containing 100 ng/ml of IGF-I for the entire culture period, whereas the remaining zygotes were cultured in KSOM-BE2 containing an equivalent amount of acetic acid as for IGF-I treated zygotes. All drops of embryos were overlaid

with mineral oil and cultured at 38.5°C in an atmosphere of 5% (v/v) $CO_2$ in humidified air until selected for treatment at d 5 after insemination. At this time, embryos ≥16-cells were harvested from culture drops according to the specific experimental design.

**TUNEL and Sytox Green Nucleic Acid Labeling**

The TUNEL assay used to detect DNA fragmentation associated with late stages of the apoptotic cascade was as described in Chapter 3 except that TUNEL-positive blastomeres were labeled with TMR red-conjugated dUTP nucleotides (red nuclei) and SYTOX green nucleic acid stain (1 mg/ml) was used for determination of total cell number (green nuclei).

**Experiments**

**Role of MAPKK and PI3K signaling pathways in IGF-I mediated actions to block heat shock-induced apoptosis and promote development**

Embryos ≥16-cells were collected on d 5 after insemination and transferred to a fresh drop of KSOM-BE2 ± IGF-I (containing the same treatment as received from d 0 to 5). Embryos were then assigned randomly to treatments. The experiment was designed as a 2 x 2 x 3 factorial arrangement of treatments to determine the role of PD 98059 and LY 294002 in blocking the anti-apoptotic effects of IGF-I on the embryo subjected to heat shock. Embryos were maintained at 38.5°C for 24 h or were heat-shocked at 41°C for 9 h followed by culture at 38.5°C for 15 h. Additionally, embryos were cultured in the presence of 0.1% (v/v) DMSO (vehicle), 100 μM of PD 98059 reconstituted in 0.1% (v/v) DMSO, or 100 μM of LY 294002 reconstituted in 0.1% (v/v) DMSO. Both heat shock and inhibitor treatments were initiated simultaneously. All harvested embryos were fixed on d 6 and stored in PBS-PVP at 4°C until analysis by TUNEL and SYTOX green labeling. The experiment was replicated 12 times using DMSO (112 to 123

embryos per treatment), 5 times using PD 98059 (42 to 52 embryos per treatment), and 7 times using LY 294002 (69 to 79 embryos per treatment).

A second experiment similar to the one previously described was performed using a 2 x 2 x 2 factorial arrangement of treatments with main effects of IGF-I (0 vs 100 ng/ml), temperature (38.5°C for 24 h or 41°C for 15 h followed by 38.5°C for 9 h), and inhibitor (vehicle vs LY 294002). The experiment was replicated 5 times using 42 to 52 embryos per treatment.

**Role of PI3K signaling in the cytoprotective actions of IGF-I to promote developmental competence of heat-shocked embryos**

Bovine embryos ≥16-cells were collected on d 5 after insemination and transferred to a fresh drop of KSOM-BE2 ± IGF-I (containing the same treatment as received from d 0 to 5) and then assigned randomly to treatment. The experiment was designed as a 2 x 2 x 2 factorial arrangement of treatments to determine the role of the anti-apoptotic actions of IGF-I in preventing effects of heat shock on development to the blastocyst stage. Embryos were maintained at 38.5°C or were heat-shocked at 41°C for 15 h followed by culture at 38.5°C while being cultured with 0.1% (v/v) DMSO (vehicle) or 100 μM of LY 294002 reconstituted in 0.1% (v/v) DMSO. Both heat shock and inhibitor treatments were initiated simultaneously. Culture continued until d 8 when development to the blastocyst stage was assessed. Each embryo that developed to the blastocyst stage was classified as a blastocyst, expanded blastocyst, or hatched blastocyst. The experiment was replicated 10 times using 112 to 142 embryos per treatment.

**Developmental capacity of IGF-I-treated embryos subjected to heat shock in the presence of z-DEVD-fmk, a caspase-3 inhibitor**

Bovine embryos ≥16-cells were collected on d 5 after insemination and transferred to a fresh drop of KSOM-BE2 ± IGF-I (containing the same treatment as received from d 0 to 5) and then assigned randomly to treatment. The experiment was designed as a 2 x 2 x 2 factorial arrangement of treatments. Embryos were maintained at 38.5°C or were heat-shocked at 41°C for 15 h followed by culture at 38.5°C while being cultured with 0.1% (v/v) DMSO (vehicle) or 100 μM of z-DEVD-fmk reconstituted in 0.1% (v/v) DMSO until d 8 when development to the blastocyst stage was assessed. Both heat shock and inhibitor treatments were initiated simultaneously. Each embryo that developed to the blastocyst stage was classified as a blastocyst, expanded blastocyst, or hatched blastocyst. The experiment was replicated 12 times using 114 to 137 embryos per treatment.

**Statistical Analysis**

Data on embryo cell number and percent apoptosis and blastocyst development were calculated for each replicate and analyzed by least-squares analysis of variance using the GLM procedure of SAS. Percentage data were transformed by arcsin transformation before analysis. Independent variables included IGF-I, heat shock treatment, inhibitor treatment, and replicate. The mathematical model included main effects and all interactions. Tests of significance for the main effects of IGF-I, heat shock, and inhibitor treatment were performed using IGF-I x replicate, heat shock x replicate, and inhibitor treatment x replicate as the error terms, respectively. Tests of significance for the interactions of IGF-I x heat shock, IGF-I x inhibitor treatment, and heat shock x inhibitor treatment were performed using IGF-I x heat shock x replicate,

IGF-I x inhibitor treatment x replicate, and heat shock x inhibitor treatment x replicate as the error terms, respectively. All values reported are least-squares means ± SEM. Level of significance was set at $P \leq 0.05$. Probability values for percentage data are based on analysis of arcsin-transformed data while least-squares means are from analysis of untransformed data. Furthermore, for each experiment, data were analyzed for the effects of IGF-I and heat shock treatments in subsets based on inhibitor treatment used (i.e. LY 294002, PD 98059, or DMSO in Exp. 1, LY 294002 or DMSO in Exp. 2 and 3, and z-DEVD-fmk or DMSO in Exp. 4). The results will largely reflect such analyses and instances where combined data analysis was used will be noted.

## Results

### Role of MAPKK and PI3K Signaling Pathways in IGF-I Mediated Actions to Block Heat Shock-Induced Apoptosis and Promote Development

Representative fluorescent images depicting patterns of TUNEL labeling as affected by interactions of temperature, IGF-I, and the inhibitors PD 98059 and LY 294002 are shown in Figure 4-1 while quantitative analysis of the percentage of blastomeres that were TUNEL-positive is shown in Figure 4-2A. For embryos cultured in the DMSO vehicle, heat shock increased the percentage of blastomeres that were TUNEL-positive ($P < 0.01$) and IGF-I was effective in blocking the induction of apoptosis caused by heat shock (Figure 4-2A; IGF-I x heat shock, $P < 0.05$; compare Figure 4-1B for a control embryo subjected to 41°C and Figure 4-1D for an IGF-I embryo subjected to 41°C). For embryos cultured in LY 294002, heat shock increased the number of TUNEL-positive embryos ($P < 0.05$) and IGF-I was no longer effective in blocking apoptosis (Figure 4-2A; non-significant effects of IGF-I or the interaction of IGF-I x heat shock; see Figure 4-1E-H). However, for embryos cultured in PD 98059,

IGF-I still blocked the induction of apoptosis caused by heat shock (Figure 4-2A; IGF-I x heat shock, $P = 0.09$; compare Figure 4-1J for a control embryo exposed to 41°C and Figure 4-1L for an IGF-I embryo exposed to 41°C).

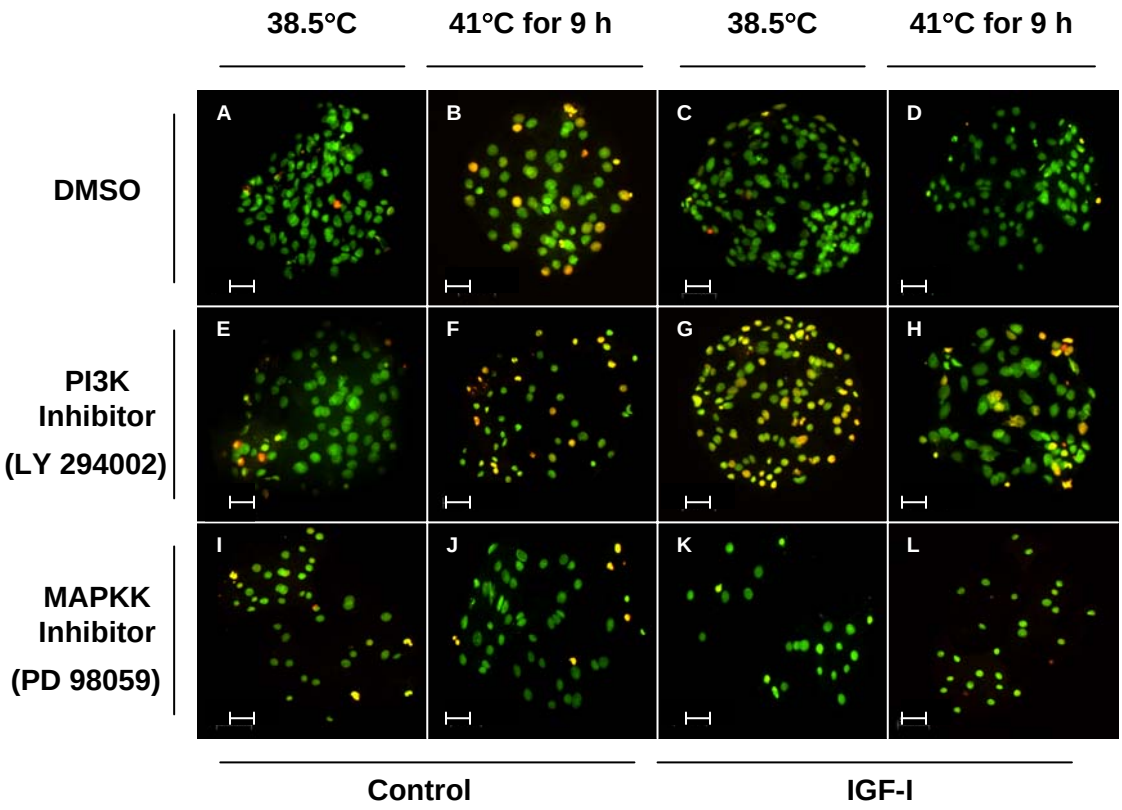


Figure 4-1. Representative images illustrating the effect of IGF-I on the frequency of apoptotic nuclei. Embryos ≥16-cells on day 5 after insemination were cultured at 38.5°C for 24 hours or were subjected to heat shock for 9 hours at 41°C followed by culture at 38.5°C for 15 hours while being cultured with DMSO vehicle (**A**-**D**), the phosphatidylinositol 3-kinase (PI3K) inhibitor LY 294002 (**E**-**H**), or the mitogen activated protein kinase kinase (MAPKK) inhibitor PD 98059 (**I**-**L**). Labeling was performed for nuclei using the SYTOX green nucleic acid stain (green) and for apoptotic nuclei using the TUNEL reaction with TMR red-conjugated dUTP (red). Bar = 50 μm.

The other endpoint determined in this experiment was total cell number at d 6. Analysis of combined data revealed that embryo total cell number was increased by IGF-I ($P < 0.01$) whereas heat shock reduced embryo cell number ($P < 0.05$; Figure 4-2B).

The ability of IGF-I to increase cell number was eliminated when embryos were cultured with PD 98059 (Figure 4-2B; non-significant effect of IGF-I in the PD 98059 subset). In the presence of LY 294002, in contrast, IGF-I increased embryo total cell number ($P < 0.05$; Figure 4-2B).

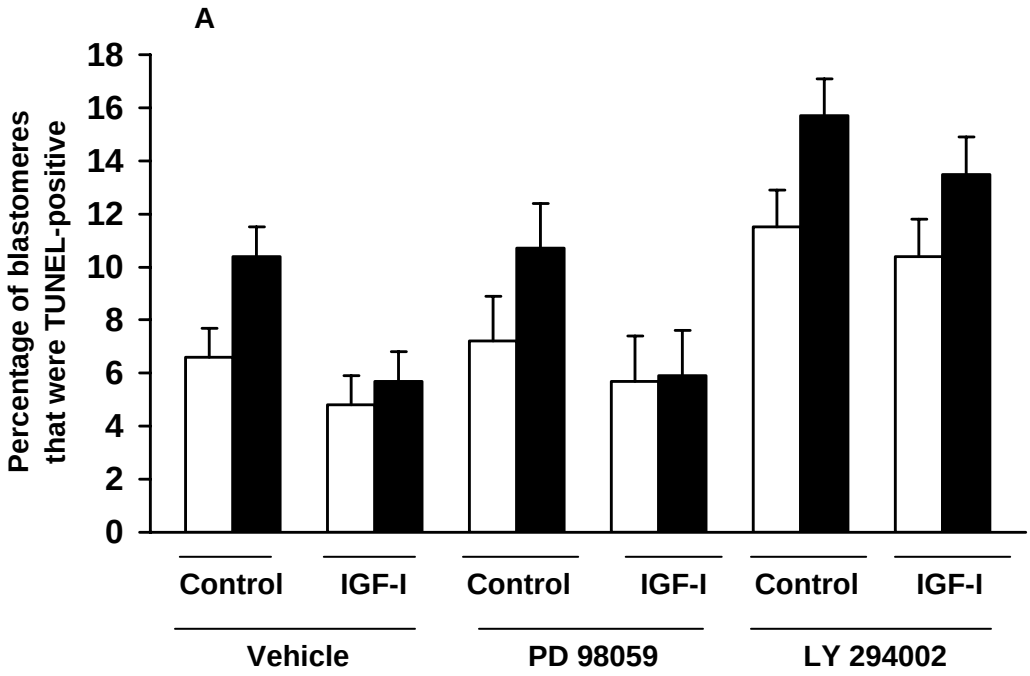


Figure 4-2. Effects of phosphatidylinositol 3-kinase (PI3K) inhibitor (LY 294002) and mitogen activated protein kinase kinase (MAPKK) inhibitor (PD 98059) on ability of IGF-I to modify resistance of day 5 embryos to heat shock. Open bars represent embryos maintained continuously at 38.5°C for 24 h while filled bars represent embryos subjected to 41°C for 9 h followed by culture at 38.5°C for 15 h. In Panel A, heat shock increased the percentage of TUNEL-positive blastomeres for DMSO-treated embryos ($P < 0.01$) and IGF-I was effective in blocking the induction of apoptosis caused by heat shock for DMSO- and PD 98059-treated embryos (IGF-I x heat shock, $P < 0.05$; $P = 0.09$). However, while heat shock increased the percentage of TUNEL-positive blastomeres for embryos cultured with LY 294002 ($P < 0.05$), IGF-I was no longer effective in blocking apoptosis (non-significant interaction of IGF-I x heat shock). In Panel B, embryo total cell number was increased by IGF-I ($P < 0.01$) whereas heat shock reduced embryo cell number ($P < 0.05$) determined by analysis of combined data. However, PD 98059 eliminated the ability of IGF-I to increase cell number (non-significant effect of IGF-I in the PD 98059 subset). In contrast, IGF-I increased total cell number in the presence of LY 294002 ($P < 0.05$).

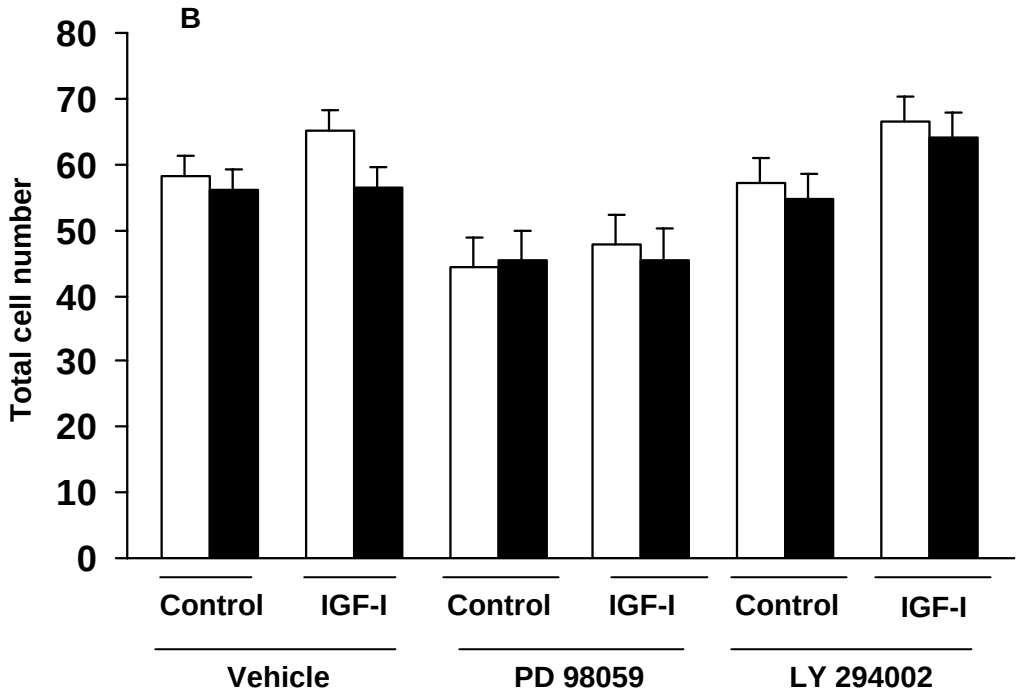


Figure 4-2 Continued

A second experiment was conducted to verify the actions of the PI3K inhibitor LY 294002 in preventing the anti-apoptotic effects of IGF-I. Analysis of the entire dataset revealed that heat shock of control embryos caused a two-fold increase in the percentage of nuclei that were TUNEL-positive ($P < 0.05$), but IGF-I was still effective in blocking the induction of apoptosis caused by heat shock (IGF-I x heat shock, $P = 0.09$; Figure 4-3A). As in the previous experiment, culture with LY 294002 increased the percentage of TUNEL-positive nuclei in all groups and the effect of IGF-I to block induction of apoptosis caused by heat shock was abolished (IGF-I x heat shock x LY 294002 treatment, $P < 0.01$).

Analysis of the entire dataset revealed that heat shock of control embryos reduced embryo cell number ($P = 0.07$), but IGF-I was effective in blocking the reduction in embryo total cell number caused by heat shock (IGF-I x heat shock, $P < 0.05$; Figure 4-3B). In contrast to the previous experiment, LY 294002 blocked the stimulatory effect of

IGF-I treatment to increase embryo total cell number (IGF-I x LY 294002 treatment, $P$ = 0.08).

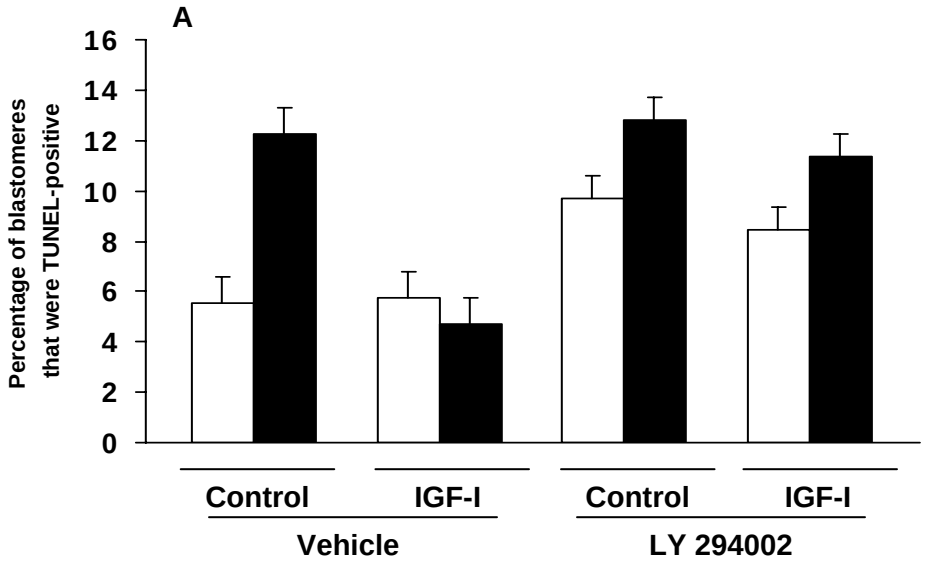


Figure 4-3. Effects of the phosphatidylinositol 3-kinase (PI3K) inhibitor LY 294002 on ability of IGF-I to modify resistance of embryos collected at day 5 of culture. Open bars represent embryos maintained continuously at 38.5°C for 24 h while filled bars represent embryos subjected to heat shock at 41°C for 15 h followed by culture at 38.5°C for 9 h. Results are least-squares means ± SEM. For Panel A, combined analysis revealed that heat shock increased the percentage of TUNEL-positive blastomeres for control embryos ($P < 0.05$) but IGF-I blocked this increase (IGF-I x heat shock, $P = 0.09$). However, LY 294002 treatment abolished the ability of IGF-I to block induction of apoptosis caused by heat shock (IGF-I x heat shock x LY 294002 treatment, $P < 0.01$). In Panel B, combined analysis revealed that heat shock reduced embryo cell number ($P = 0.07$) but IGF-I blocked the reduction in total cell number caused by heat shock (IGF-I x heat shock, $P < 0.05$). However, LY 294002 blocked the stimulatory effect of IGF-I treatment to increase total cell number (IGF-I x LY 294002 treatment, $P = 0.08$).

**Role of PI3K Signaling in the Cytoprotective Actions of IGF-I to Promote Developmental Competence of Heat-shocked Embryos**

Developmental competence was assessed as the proportion of embryos ≥16 cells selected on d 5 of culture that became blastocysts on d 8. Analysis of the entire dataset revealed that heat shock reduced blastocyst development ($P < 0.05$) and IGF-I was able to block this reduction in development (IGF-I x heat shock, $P < 0.05$; Figure 4-4). There

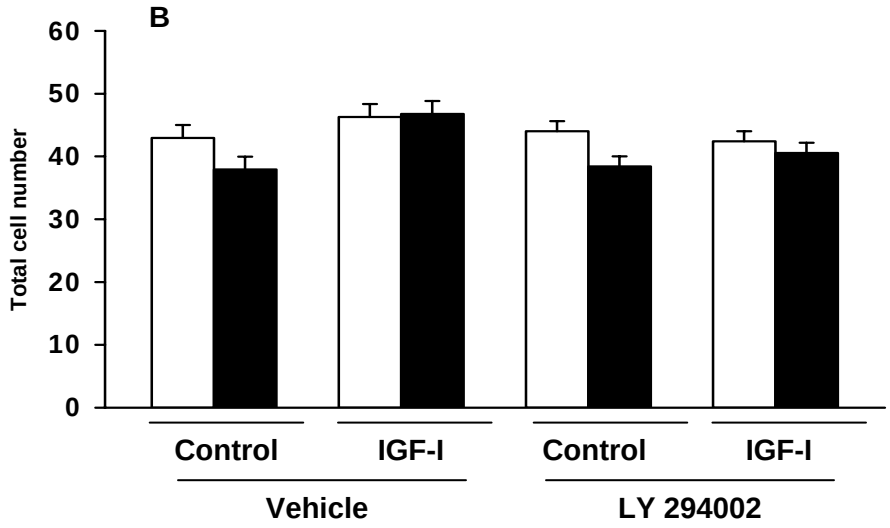


Figure 4-3 Continued

was no IGF-I x heat shock x LY 294002 interaction because the protective effects of IGF-I occurred in both the presence and absence of the inhibitor.

**Developmental Capacity of IGF-I-treated Embryos Subjected to Heat Shock in the Presence of z-DEVD-fmk, a Caspase-3 Inhibitor**

Here it was reasoned that, if IGF-I allows development of heat-shocked embryos by blocking apoptosis, then addition of the group II caspase inhibitor, z-DEVD-fmk, should still allow for increased development of heat-shocked embryos. For embryos cultured in the presence of DMSO vehicle, exposure to heat shock tended to reduce blastocyst development ($P < 0.06$) while IGF-I treatment tended to increase blastocyst development (IGF-I, $P < 0.06$; non-significant IGF-I x heat shock interaction; Figure 4-5). While heat shock reduced ($P < 0.01$) blastocyst development for embryos treated with z-DEVD-fmk, analysis of the entire dataset revealed that IGF-I was no longer effective in promoting blastocyst development or blocking the reduction in development due to heat shock for embryos cultured in the presence of z-DEVD-fmk (IGF-I x z-DEVD-fmk inhibitor: $P < 0.01$).

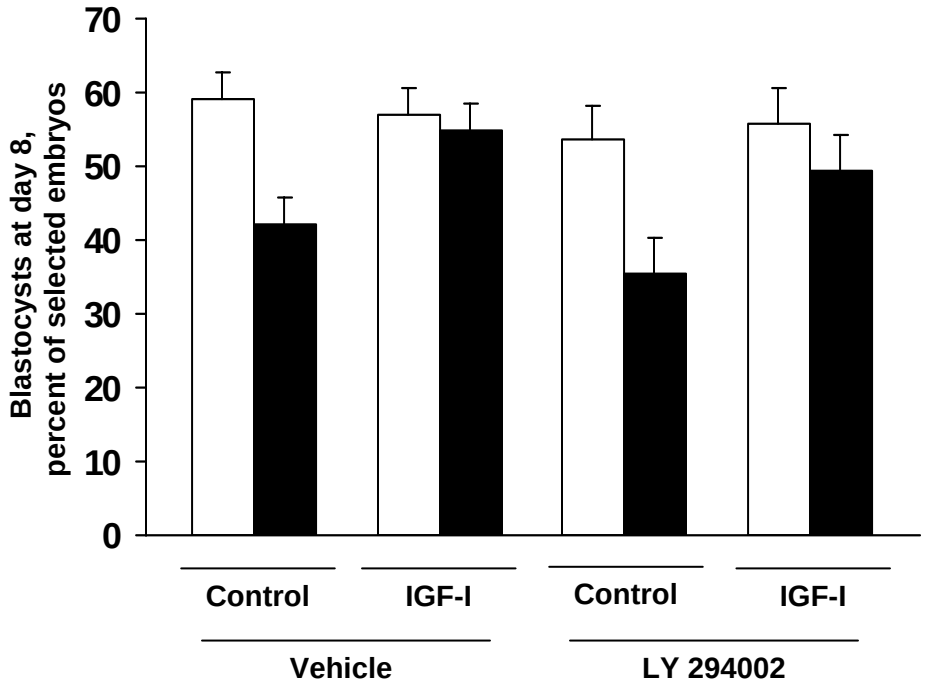


Figure 4-4. Effects of the phosphatidylinositol 3-kinase (PI3K) inhibitor LY 294002 on ability of IGF-I to block effects of heat shock on development of day 5 embryos to the blastocyst stage at day 8. Open bars represent embryos maintained continuously at 38.5°C until day 8 while filled bars represent embryos subjected to heat shock at 41°C for 15 h followed by culture at 38.5°C until day 8. Results are least-squares means ± SEM. Combined analysis revealed that heat shock reduced blastocyst development ($P < 0.05$) and IGF-I was able to block this reduction in development (IGF-I x heat shock, $P < 0.05$). This protective effect of IGF-I occurred in the presence and absence of LY 294002 (no IGF-I x heat shock x LY 294002 interaction).

## Discussion

Results of the current study confirm the cytoprotective actions of IGF-I in the preimplantation bovine embryo seen in Chapter 3, demonstrate that the anti-apoptotic actions of IGF-I are mediated through the PI3K pathway while the pro-proliferative actions involve the MAPKK pathway (and perhaps the PI3K pathway), and show that the action of IGF-I allowing heat-shocked embryos to develop to the blastocyst stage is independent of its actions to block apoptosis induced by heat shock. Thus, IGF-I acts as a cytoprotective factor for the bovine preimplantation embryo by inducing a

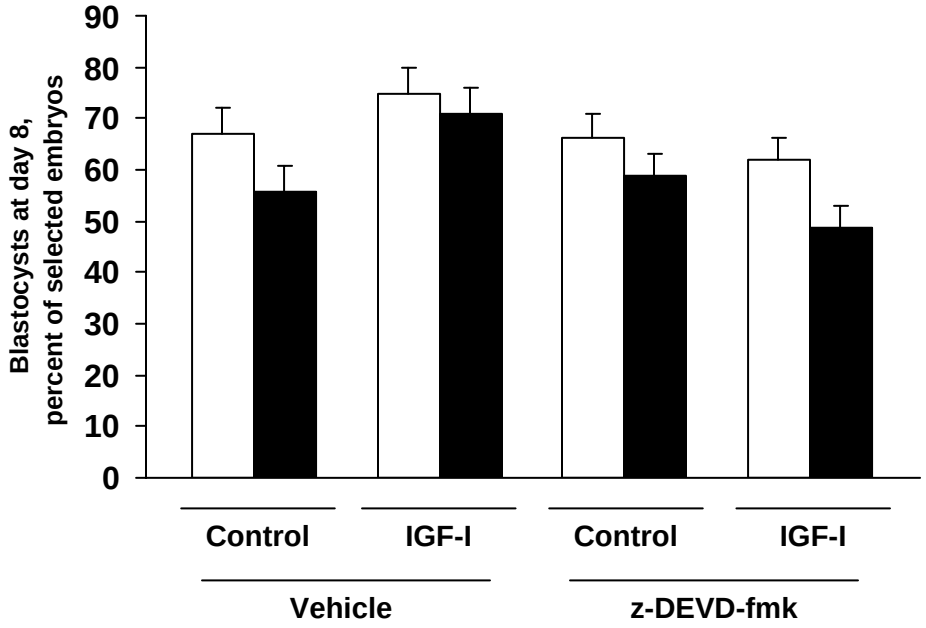


Figure 4-5. Effects of the caspase inhibitor z-DEVD-fmk and IGF-I on development of day 5 embryos to the blastocyst stage at day 8 of culture. Open bars represent embryos maintained continuously at 38.5°C until day 8 while filled bars represent embryos subjected to heat shock at 41°C for 15 h followed by culture at 38.5°C until day 8. Results are least-squares means ± SEM. Heat shock tended to reduce blastocyst development ($P < 0.06$) of DMSO-treated embryos whereas IGF-I tended to increase blastocyst development of DMSO-treated embryos ($P < 0.06$). Heat shock reduced blastocyst development of z-DEVD-fmk-treated embryos ($P < 0.01$). However, z-DEVD-fmk treatment prevented the ability of IGF-I to promote blastocyst development or block the reduction in development due to heat shock (IGF-I x z-DEVD-fmk inhibitor: $P < 0.01$).

multiplicity of cellular changes including those that maintain embryonic competence for development following heat shock.

Like a variety of other cell types (Nitta et al., 2004; Zheng and Quirion, 2004; Bridgewater et al., 2005; Scott et al., 2005; Zaka et al., 2005), the bovine preimplantation embryo utilizes the PI3K pathway for mediating actions of IGF-I to block apoptosis. This is so because the inhibition to heat shock-induced apoptosis was blocked by the PI3K inhibitor, LY 294002, but not by the MAPKK inhibitor, PD 98059. In contrast, the MAPKK pathway was a prerequisite for actions of IGF-I to increase embryo cell number

at d 6 because PD 98059 blocked these effects of IGF-I. The role of the PI3K pathway in pro-proliferative effects of IGF-I were less clear because effects of IGF-I on cell number were blocked by LY 294002 in one experiment but not in another.

In the same manner as observed in Chapter 3, IGF-I reduced the impact of heat shock on the proportion of embryos that developed to the blastocyst stage. This action of IGF-I was not the result of the decreased apoptosis caused by IGF-I. There are two lines of evidence to support this contention. First, the cytoprotective actions of IGF-I to maintain embryonic competence for blastocyst development after heat shock was not blocked by LY 294002 even though this inhibitor did block effects of IGF-I on apoptosis. Secondly, addition of z-DEVD-fmk, which inhibits activity of caspases-2, -3, and -7, and which blocks induction of apoptosis caused by heat shock in bovine embryos (Paula-Lopes and Hansen, 2002b), did not mimic the effects of IGF-I on embryonic development. In control embryos, z-DEVD-fmk had no effect on development while it magnified the effects of heat shock on development of IGF-I treated embryos to the blastocyst stage at d 8 of culture.

In fact, occurrence of apoptosis in preimplantation embryos is not necessarily an indicator of compromised development. There is some evidence that limited apoptosis, such as occurs in embryos exposed to heat shock, can allow embryos to recover from stress and continue development. In particular, the deleterious effects of heat shock at d 4 of culture on development to the blastocyst stage were exacerbated when embryonic capacity for apoptosis was blocked by z-DEVD-fmk (Paula-Lopes and Hansen, 2002b). In the present study, using embryos more advanced in development (d 5 of culture), z-DEVD-fmk only exacerbated effects of heat shock on development to the blastocyst stage

if embryos were cultured with IGF-I. Perhaps the degree of apoptosis was so low in IGF-I-treated embryos cultured with z-DEVD-fmk that the resultant inability of the embryo to remove damaged cells compromised blastocyst development. Failure of z-DEVD-fmk to alter embryonic potential for development in control embryos at d 5 of culture, even though it reduces development of heat-shocked embryos at d 4, could reflect developmental changes in responses of embryos to heat shock. Further work is needed to verify whether the consequences of inhibition of apoptosis are actually different between d 4 and 5 embryos.

It was not possible to determine whether the cytoprotective actions of IGF-I on blastocyst development are mediated through actions on proliferation mediated by MAPKK signaling pathways. While this seems plausible, it is not feasible to test the hypothesis using PD 98059 because the inhibitor blocks blastocyst development at 38.5$^{o}$C (Jousan and Hansen, unpublished). There may also be other actions of IGF-I that preserve ability of embryos exposed to heat shock to continue development to the blastocyst stage. For example, IGF-I causes up-regulation of glucose uptake in preimplantation mouse blastocysts (Pantaleon and Kaye, 1996) and such an effect could conceivably aid the damaged embryo in continuing development.

One implication of the present studies is the potential for manipulation of the IGF-I system to enhance embryonic development and pregnancy rates of cows that are exposed to heat stress. This idea is also supported by the literature as pregnancy rates of embryo transfer recipients in the summer were higher for those cows that received an embryo that had been cultured with IGF-I than for cows that received a control embryo (Block et al., 2003). In addition, fertility of lactating dairy cows can be increased by treatment with

bST (Thatcher et al., 2001; Moreira et al., 2002a). One action of ST is to increase IGF-I secretion (Lucy, 2000). Perhaps, increasing systemic concentrations of IGF-I by treatment with bST could enhance embryonic development of females that are exposed to heat stress. There is precedent for such a cytoprotective effect of ST as peripheral blood lymphocytes harvested from heifers treated with ST were more resistant to heat shock in vitro than lymphocytes from control heifers (Elvinger et al., 1991a).

In conclusion, IGF-I blocked the induction of apoptosis and reduction in blastocyst development for the bovine preimplantation embryo subjected to heat shock. The actions of IGF-I to block the induction of apoptosis caused by heat shock require signaling via the PI3K pathway while actions to promote an increase in embryo cell number require signaling through the MAPKK pathway and perhaps the PI3K pathway. It is evident that IGF-I does not promote blastocyst development due to its anti-apoptotic effects, as inhibition of PI3K signaling did not eliminate the ability of IGF-I to promote blastocyst development of heat-shocked embryos and cytoprotective actions of IGF-I on development could not be mimicked by treatment with z-DEVD-fmk. Studies are needed to demonstrate that IGF-I activates the signaling pathways studied in this current research, to determine which genes are regulated by IGF-I to exert its anti-apoptotic and growth-promoting effects to increase blastocyst development of embryos subjected to stress, and to experimentally determine whether manipulation of the IGF-I system can enhance fertility of heat-stressed cows.

# CHAPTER 5
# SHORT-TERM CULTURE OF BOVINE PREIMPLANTATION EMBRYOS WITH INSULIN-LIKE GROWTH FACTOR-I PROMOTES RESISTANCE TO HEAT SHOCK-INDUCED APOPTOSIS REQUIRING PI3K/AKT SIGNALING

## Introduction

One of the best-characterized regulatory molecules for the preimplantation bovine embryo is IGF-I, which in the cow is secreted by the oviduct (Pushpakumara et al., 2002), endometrium (Geisert et al., 1991), and embryo (Lonergan et al., 2000). Bovine embryos cultured with IGF-I leads to an increased proportion that reach the blastocyst stage and an increased blastocyst cell number (Byrne et al., 2002b; Block et al., 2003; Sirisathien and Brackett, 2003; Sirisathien et al., 2003). Additionally, IGF-I is a cytoprotective molecule for the embryo. In mice, IGF-I reduced effects of hydrogen peroxide on development (Kurzawa et al., 2002). Furthermore, IGF-I can block apoptosis in rabbit embryos caused by exposure to ultraviolet irradiation (Herrler et al., 1998) and in mouse embryos caused by exposure to camptothecin and actinomycin D (Fabian et al., 2004) and TNF-$\alpha$ (Kurzawa et al., 2001; Byrne et al., 2002a).

The pathway by which IGF-I regulates apoptosis in preimplantation embryos is not known. In other cells studied, anti-apoptotic actions of IGF-I are mediated through a signaling pathway distinct from that used to promote proliferation (Peruzzi et al., 1999; Vincent and Feldman, 2002; Mauro and Surmacz, 2004; Foulstone et al., 2005). Inhibition of apoptosis involves activation of the regulatory subunit of PI3K, which in turn leads to the phosphorylation and activation of Akt/PKB. This kinase plays a major role in the prevention of apoptosis through phosphorylation and subsequent inactivation

of pro-apoptotic proteins involved in apoptosis, including Bad, caspase-9, and forkhead transcription factors (Brazil and Hemmings, 2001; Kim and Chung, 2002; Vara et al., 2004; Song et al., 2005). Additionally, Akt activates by phosphorylation the transcription factor cyclic AMP response element-binding protein and the IκB kinase α, which phosphorylates IκB and targets it for degradation, thereby leading to activation of NF-κB and the transcription of anti-apoptotic genes such as Bcl-2 and Bcl-XL (Pugazhenthi et al., 2000; Mitsiades et al., 2002; Osaki et al., 2004; Thompson and Thompson, 2004). Insulin-like growth factor-I also activates the MAPKK pathway to increase cell proliferation and also contributes to inhibition of apoptosis (Jones and Clemmons, 1995; Rubinfeld and Seger, 2005).

In the preimplantation bovine embryo, continuous culture with IGF-I beginning after fertilization blocked the detrimental effects of heat shock at d 5 after fertilization on induction of apoptosis and inhibition of development as described in Chapter 3. Studies with PI3K (LY 294002) and MAPKK inhibitors (PD 98059) indicated that the anti-apoptotic actions of IGF-I required the PI3K pathway whereas the actions of IGF-I to promote proliferation and blastocyst development required the MAPKK pathway (Chapter 4).

In experiments in which embryos were chronically exposed to IGF-I (Chapters 3 and 4), it is possible that some of the anti-apoptotic actions of IGF-I represent differentiation events mediated by IGF-I rather than acute activation of signaling pathways. The objective of the present series of studies was to test whether short-term addition of IGF-I to culture medium of bovine preimplantation embryos would block

induction of apoptosis caused by heat shock and, if so, whether these anti-apoptotic actions of IGF-I involve the PI3K/Akt pathway.

## Materials and Methods

### Materials

The materials used for these experiments were as described in Chapter 3 with the following additions. The PI3K inhibitor wortmannin was obtained from Sigma and the Akt inhibitor (1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate; HIMO) was from Calbiochem (San Diego, CA). For the addition of the PI3K inhibitor, a vial containing 1 mg of wortmannin was rehydrated with 23.34 μl of 100% DMSO and stored at -20°C in 1.25 μl aliquots until use when a single aliquot was diluted in KSOM-BE2 ± IGF-I to a concentration of 100 nM. For the addition of the Akt inhibitor, a vial containing 1 mg of HIMO was rehydrated with 172 μl of 100% DMSO and stored at -20°C in 1.25 μl aliquots until use when a single aliquot was diluted in KSOM-BE2 ± IGF-I to a concentration of 10 μM. The final concentration of DMSO in all treatments was 0.1% (v/v). The In Situ Cell Death Detection Kit (rhodamine) was obtained from Roche Diagnostics Corporation (Indianapolis, IN). The Zenon Alexa Fluor 488 rabbit IgG labeling kit was purchased from Molecular Probes (Eugene, OR) while Hoechst 33342 was purchased from Sigma. The rabbit polyclonal antibody to phosphorylated Akt1 (phospho S473) was obtained from Abcam Inc. (Cambridge, MA). Rabbit IgG was purchased from Sigma.

### In Vitro Production of Embryos

Embryo production was performed using in vitro maturation of oocytes and in vitro fertilization as described in Chapter 3. Putative zygotes were cultured in KSOM-BE2

overlaid with mineral oil and cultured at 38.5°C in an atmosphere of 5% (v/v) $CO_2$ in humidified air until selected for treatment at d 5 after insemination. At this time, embryos ≥16-cells were harvested from culture drops according to the specific experimental design.

**TUNEL and Hoechst 33342 Labeling**

The TUNEL assay used to detect DNA fragmentation associated with late stages of the apoptotic cascade was as described in Chapter 3 except that TUNEL-positive blastomeres were labeled with TMR red-conjugated dUTP nucleotides (red nuclei) and Hoechst 33342 for determination of total cell number (blue nuclei).

**Experiments**

**Effectiveness of acute exposure of preimplantation bovine embryos to IGF-I in blocking apoptosis caused by heat shock**

Embryos ≥16-cells were collected on d 5 after insemination. Approximately half of the harvested embryos were randomly transferred to a fresh drop of KSOM-BE2 containing 100 ng/ml of IGF-I while the other half were cultured in a fresh drop of KSOM-BE2 containing an equivalent amount of acetic acid as for IGF-I-treated embryos. The experiment was designed as a 2 x 2 factorial arrangement of treatments to determine if addition of IGF-I starting coincidently with heat shock was effective in blocking the induction of apoptosis. Embryos were maintained at 38.5°C for 24 h or were heat-shocked at 41°C for 15 h followed by culture at 38.5°C for 9 h. All harvested embryos were fixed in 4% (v/v) paraformaldehyde (by diluting 8% paraformaldehyde 1:1 with PBS) on d 6. Embryos were then stored in PBS containing PVP (1 mg/ml; PBS-PVP) at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated 5 times using 68 to 74 embryos per treatment.

**Inhibition of PI3K**

Embryos ≥16-cells were collected on d 5 after insemination. Approximately half of the harvested embryos were randomly transferred to a fresh drop of KSOM-BE2 containing 100 ng/ml of IGF-I while the other half were cultured in a fresh drop of KSOM-BE2 containing an equivalent amount of acetic acid as for IGF-I-treated embryos. The experiment was designed as a 2 x 2 x 2 factorial arrangement of treatments to determine the role of IGF-I in preventing the induction of apoptosis caused by heat shock. Embryos were maintained at 38.5°C for 24 h or were heat-shocked at 41°C for 15 h followed by culture at 38.5°C for 9 h while being cultured with 0.1% (v/v) DMSO (vehicle) or 100 nM of wortmannin reconstituted in 0.1% (v/v) DMSO. Heat shock, IGF-I and inhibitor treatments were initiated nearly simultaneously. Following culture, embryos were fixed in 4% (v/v) paraformaldehyde on d 6 and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated 6 times using 31 to 47 embryos per treatment.

**Inhibition of Akt**

Embryos ≥16-cells were collected on d 5 after insemination. Approximately half of the harvested embryos were randomly transferred to a fresh drop of KSOM-BE2 containing 100 ng/ml of IGF-I while the other half were cultured in a fresh drop of KSOM-BE2 containing an equivalent amount of acetic acid as for IGF-I-treated embryos. The experiment was designed as a 2 x 2 x 2 factorial arrangement of treatments to determine the role of IGF-I in preventing the induction of apoptosis caused by heat shock. Embryos were maintained at 38.5°C for 24 h or were heat-shocked at 41°C for 15 h followed by culture at 38.5°C for 9 h while being cultured with 0.1% (v/v) DMSO

(vehicle) or 10 μM of HIMO reconstituted in 0.1% (v/v) DMSO. Heat shock, IGF-I and inhibitor treatments were initiated nearly simultaneously. After culture, embryos were fixed in 4% (v/v) paraformaldehyde on d 6 and stored in PBS-PVP at 4°C until analysis by TUNEL and Hoechst 33342 labeling. The experiment was replicated 4 times using 24 to 31 embryos per treatment.

**Phosphorylation of Akt**

Embryos ≥16-cells were collected on d 5 after insemination. Approximately half of the harvested embryos were randomly transferred to a fresh drop of KSOM-BE2 containing 100 ng/ml of IGF-I while the other half were cultured in a fresh drop of KSOM-BE2 containing an equivalent amount of acetic acid as for IGF-I-treated embryos. Embryos were cultured at 38.5°C for 10 min or 1 hr in humidified air, fixed in 4% (v/v) paraformaldehyde, and stored in PBS-PVP at 4°C until immunofluorescent analysis using an antibody to phosphorylated Akt1 (S473). The staining was replicated 3 times using approximately 20 embryos per treatment.

The procedure for immunofluorescence was performed as follows. A rabbit antibody against phosphorylated Akt1 (at amino acid S473; initial concentration of 900 μg/ml) was diluted to ~ 135 μg/ml and then labeled with Fab fragments against rabbit IgG conjugated to Alexa Flour 488 using the Zenon Alexa Fluor 488 rabbit IgG labeling reagent as per manufacturer's instructions. The labeled antibody complex was then diluted in antibody buffer at a final concentration of 45 μg/ml. All immunostaining steps were performed using microdrops covered with mineral oil in a humidified chamber in the dark. The blocking buffer consisted of PBS containing 20% (v/v) normal goat serum and the antibody buffer contained PBS + 2% (w/v) bovine serum albumin. Fixed

embryos were permeabilized (0.5% (v/v) Triton-X, 0.1% (w/v sodium citrate) for 15 min at room temperature and then washed two times in antibody buffer. Embryos were blocked for 2 hr in 50 μl drops of the blocking buffer and then incubated for 2 hr in 45 μg/ml of the Zenon-labeled Akt1 antibody. As a negative control, rabbit IgG labeled similarly to the anti-Akt was used. Embryos were washed two times in antibody buffer and fixed a second time in 4% (v/v) paraformaldehyde for 15 min at room temperature. Embryos were washed two times in antibody buffer and mounted on 10% (w/v) poly-L-lysine coated slides using 4- to 5-μl drops of glycerol, and coverslips were placed on the slides. Labeling of embryos was observed using a Zeiss Axioplan 2 epifluorescence microscope. Each embryo was analyzed for phosphorylated Akt1 (green staining) with a FITC filter using a 40x objective. Digital images were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera.

**Statistical Analysis**

Data were analyzed by least-squares analysis of variance using the GLM procedure of SAS. Percentage data were transformed by arcsin transformation before analysis. Independent variables included IGF-I, temperature, inhibitor (except for Exp. 1 where no inhibitor was used), and replicate. The mathematical model included main effects and all interactions. Tests of significance were made using errors terms based on replicate being a random effect and other main effects being fixed. For Exp. 2 and 3, additional analyses were performed for each inhibitor subset (wortmannin and HIMO). Thus, two analyses were conducted for Exp. 2 using vehicle or wortmannin-treated embryos and Exp. 3 using vehicle or HIMO-treated embryos, with IGF-I, temperature, and replicate as main

effects. Unless otherwise mentioned, *P*-values reported for these experiments are based on analysis of these subsets, and level of significance was set a $P \leq 0.05$. All values reported are least-squares means ± SEM. Probability values for percentage data are based on analysis of arcsin-transformed data while least-squares means are from analysis of untransformed data.

## Results

### Acute Exposure to IGF-I Blocks Apoptosis Caused by Heat Shock

Culture of embryos with IGF-I starting at the initiation of heat shock blocked the induction of apoptosis caused by heat shock (Figure 5-1A). While heat shock increased the number of apoptotic blastomeres ($P = 0.06$), IGF-I treatment prevented the increase in apoptosis as a result of heat shock (IGF-I x heat shock, $P < 0.001$). Heat shock reduced total cell number ($P < 0.01$), but there was no effect of IGF-I or IGF-I x heat shock (Figure 5-1B).

### Inhibition of PI3K Eliminates the Anti-apoptotic Actions of IGF-I

For embryos cultured in the DMSO vehicle, heat shock increased the percentage of blastomeres that were TUNEL-positive ($P = 0.06$) and IGF-I blocked induction of apoptosis caused by heat shock (IGF-I x heat shock, $P < 0.01$; Figure 5-2A). For embryos cultured with wortmannin, heat shock increased the number of TUNEL-positive nuclei ($P < 0.05$), but IGF-I was no longer effective in blocking the induction of apoptosis caused by heat shock (IGF-I x heat shock, $P = 0.73$). Analysis of the entire dataset revealed that treatment with wortmannin increased the number of TUNEL-positive nuclei ($P < 0.001$).

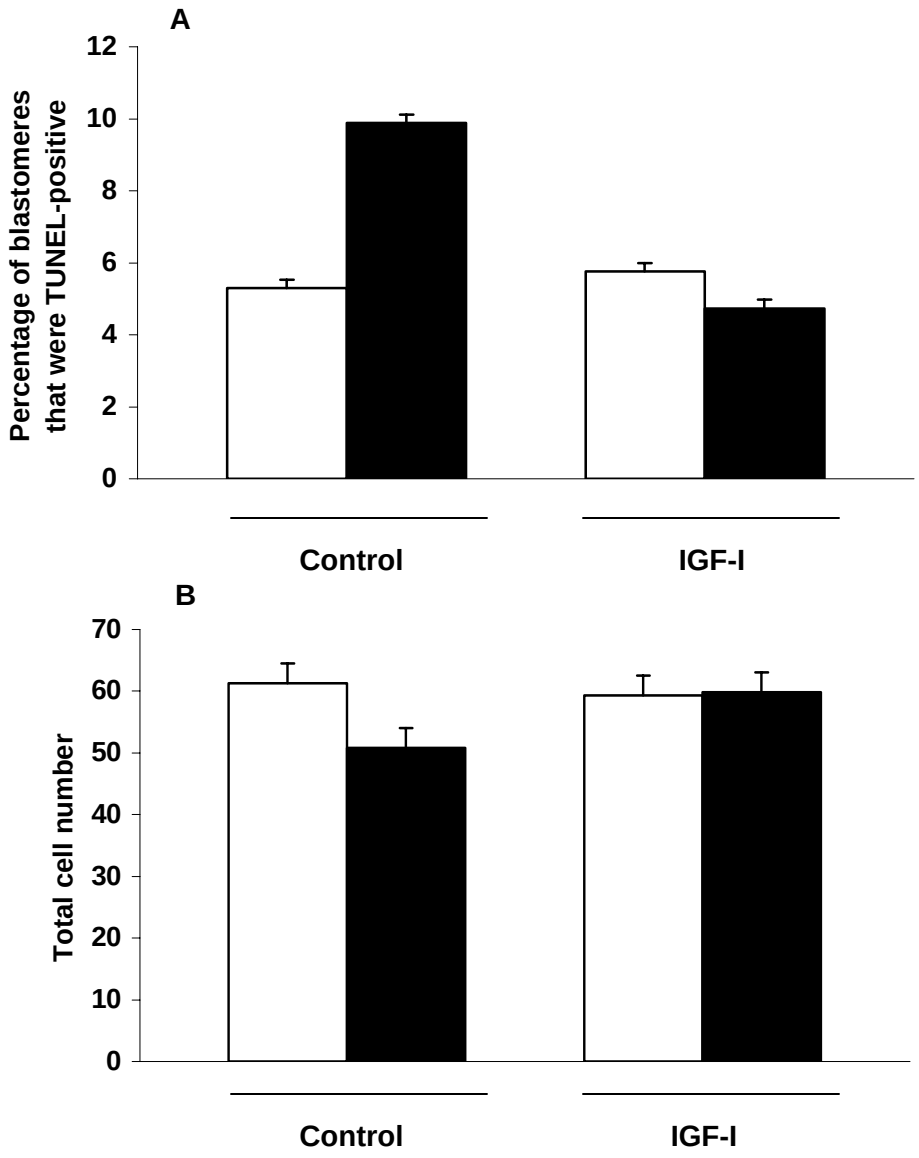


Figure 5-1. Short-term exposure of day 5 bovine preimplantation embryos to IGF-I blocks heat shock-induced apoptosis. Open bars represent data for embryos maintained at 38.5°C for 24 h while filled bars represent data for embryos subjected to 41°C for 15 h followed by culture at 38.5°C for 9 h. Results are least-squares means ± SEM. In Panel A, heat shock increased the percentage of TUNEL-positive blastomeres ($P = 0.06$) and IGF-I prevented the increase in apoptosis caused by heat shock (IGF-I x heat shock, $P < 0.001$). In Panel B, heat shock reduced total cell number ($P < 0.01$). There was no IGF-I or IGF-I x heat shock interaction for total cell number.

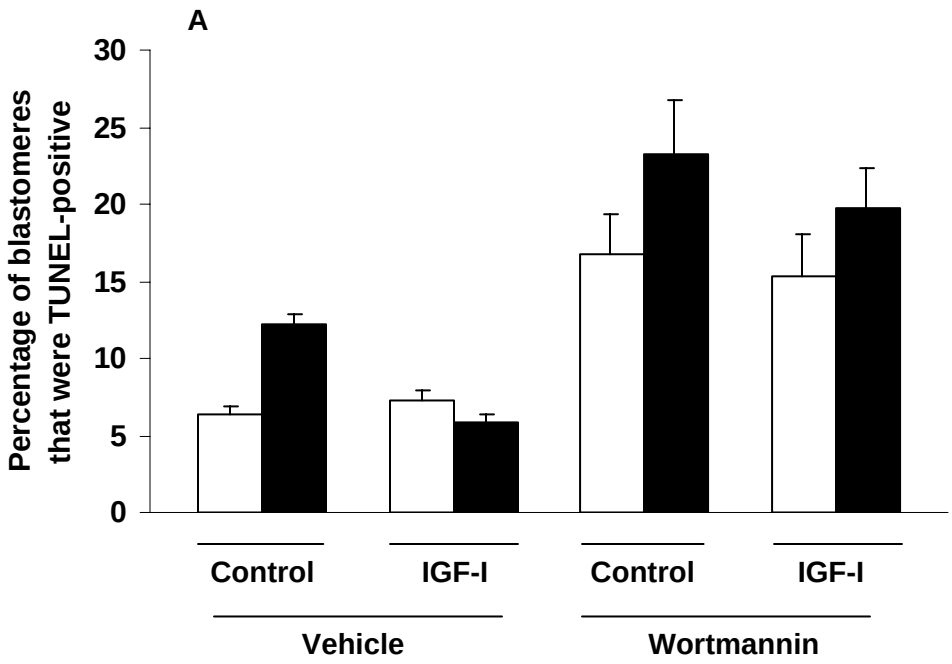


Figure 5-2. The phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin blocks the anti-apoptotic actions of IGF-I. Open bars represent data for embryos maintained at 38.5°C for 24 h while filled bars represent data for embryos subjected to 41°C for 15 h followed by culture at 38.5°C for 9 h. Results are least-squares means ± SEM. In Panel A, heat shock increased the percentage of blastomeres that were TUNEL-positive ($P = 0.06$) and IGF-I blocked induction of apoptosis caused by heat shock (IGF-I x heat shock, $P < 0.01$). Heat shock treatment also increased the number of TUNEL-positive nuclei ($P < 0.05$) for wortmannin-treated embryos, but IGF-I was not able to block the induction of apoptosis caused by heat shock (no IGF-I x heat shock interaction). Combined analysis revealed that wortmannin increased the number of TUNEL-positive nuclei ($P < 0.001$). In Panel B, combined analysis revealed that IGF-I blocked the reduction in embryo total cell number caused by heat shock (IGF-I x heat shock, $P = 0.07$) and wortmannin decreased total cell number ($P < 0.01$).

The other endpoint determined was total cell number at d 6. There were no differences in total cell number for embryos cultured in the DMSO vehicle or wortmannin treatment. However, analysis of combined data revealed that IGF-I blocked the reduction in embryo total cell number caused by heat shock (IGF-I x heat shock, $P = 0.07$; Figure 5-2B) and culture of embryos with wortmannin decreased total cell number ($P < 0.01$).

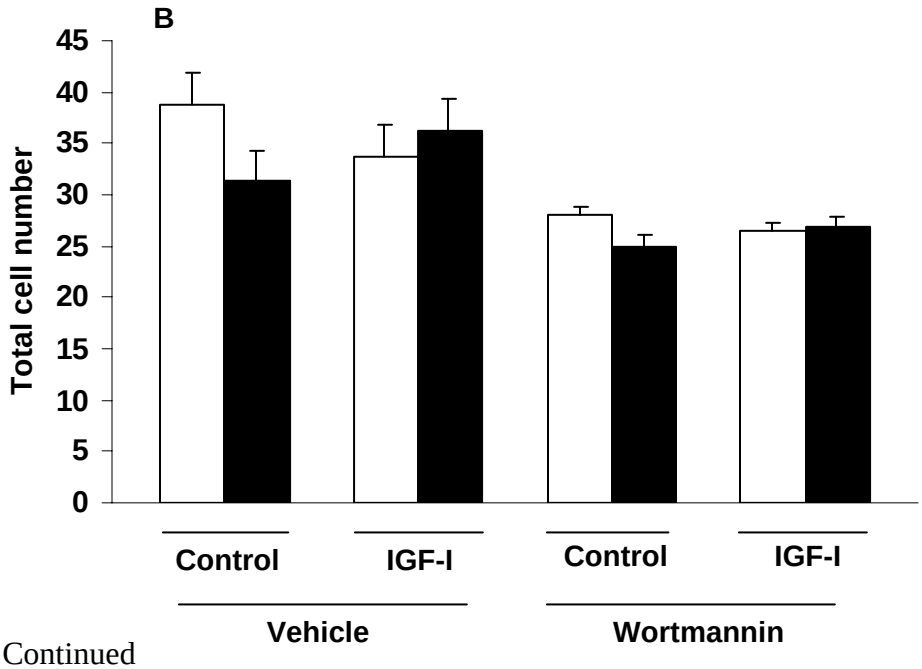


Figure 5-2 Continued

**Inhibition of Akt Eliminates the Anti-apoptotic Actions of IGF-I**

For embryos cultured in the DMSO vehicle, heat shock increased the percentage of blastomeres that were TUNEL-positive ($P < 0.01$) and IGF-I was effective in blocking induction of apoptosis caused by heat shock (IGF-I x heat shock, $P < 0.001$; Figure 5-3A). For embryos cultured with HIMO, heat shock increased the number of TUNEL-positive nuclei ($P < 0.05$), but IGF-I did not block this effect (IGF-I x heat shock, $P = 0.71$). For the combined dataset, heat shock ($P < 0.05$) and culture of embryos with HIMO ($P < 0.05$) increased the number of TUNEL-positive nuclei, but IGF-I was not able to block the induction on TUNEL-positive nuclei in the presence of HIMO (IGF-I x heat shock x inhibitor, $P < 0.01$).

The other endpoint determined was total cell number at d 6. For the subset of embryos cultured in the DMSO vehicle, IGF-I was able to block the reduction in total cell number caused by heat shock (IGF-I x heat shock, $P < 0.05$; Figure 5-3B). There were

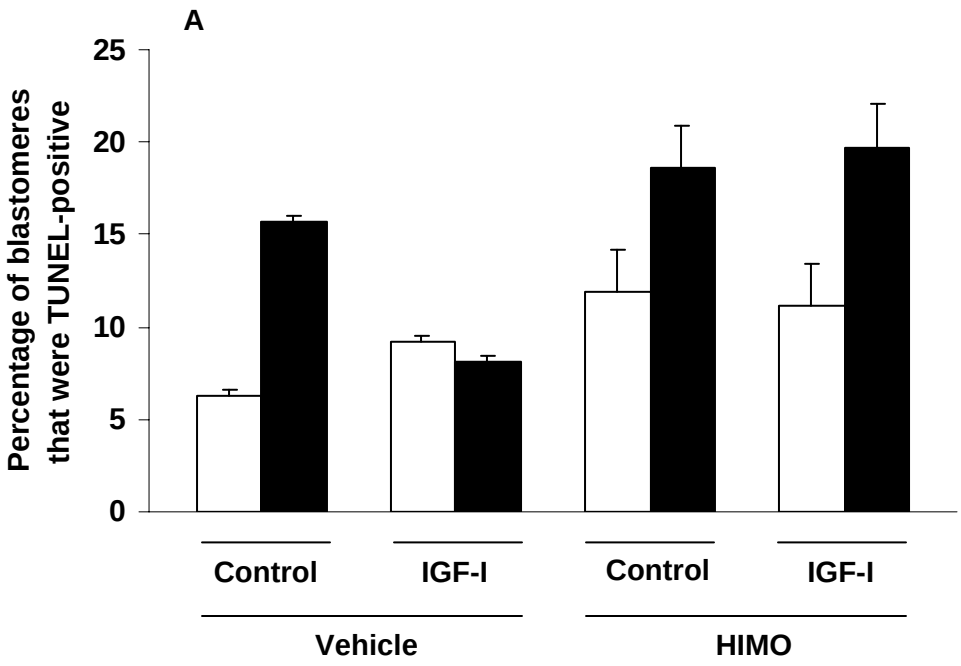


Figure 5-3. Inhibition of Akt blocks the anti-apoptotic actions of IGF-I. Open bars represent data for embryos maintained at 38.5°C for 24 h while filled bars represent embryos subjected to heat shock at 41°C for 15 h followed by culture at 38.5°C for 9 h. Results are least-squares means ± SEM. In Panel A, heat shock increased the percentage of TUNEL-positive blastomeres ($P < 0.01$) and IGF-I blocked induction of apoptosis caused by heat shock (IGF-I x heat shock, $P < 0.001$). Heat shock increased the percentage of TUNEL-positive nuclei when cultured with the Akt inhibitor 1L-6-Hydroxymethyl-*chiro*-inositol 2-(R)-2-O-methyl-3-O-octadecylcarbonate (HIMO; ($P < 0.05$). Combined analysis revealed that heat shock ($P < 0.05$) and HIMO ($P < 0.05$) increased the percentage of TUNEL-positive nuclei, but IGF-I was unable to block the induction of TUNEL-positive nuclei in the presence of HIMO (IGF-I x heat shock x inhibitor, $P < 0.01$). In Panel B, IGF-I blocked the reduction in total cell number caused by heat shock for DMSO-treated embryos (IGF-I x heat shock, $P < 0.05$), as well as for the combined analysis (IGF-I x heat shock; $P < 0.05$).

no effects of heat shock, IGF-I, or IGF-I x heat shock for embryos cultured in the presence of HIMO. Analysis of the entire dataset revealed that IGF-I blocked the reduction in total cell number caused by heat shock (IGF-I x heat shock; $P < 0.05$).

**Phosphorylation of Akt after IGF-I Treatment**

Specific immunolabeling was detected for all embryos labeled with an antibody that recognized Akt1 phosphorylated at S473. (Compare Figure 5-4B and 5-4D labeled

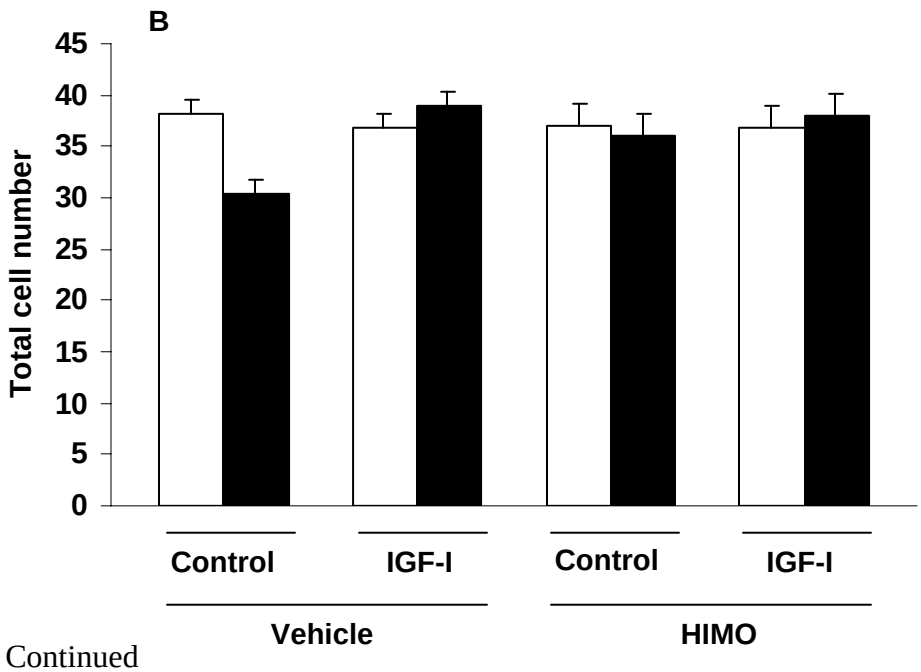


Figure 5-3 Continued

with antibody to Figure 5-4A and 5-4C labeled with rabbit IgG). Differences between control and IGF-I-treated embryos were not apparent (Figure 5-4A and 5-4B for control embryos and Figure 5-4C and 5-4D for IGF-I-treated embryos) at either 10 min or 1 hr after IGF-I treatment.

## Discussion

Results of the current study confirm the cytoprotective actions of IGF-I in the preimplantation bovine embryo seen earlier (Chapters 3 and 4). In particular, IGF-I blocked the increase in apoptosis caused by heat shock. Moreover, this cytoprotective effect, which in earlier studies was tested in embryos exposed to IGF-I for several days, was initiated upon short-term exposure to IGF-I. Additionally, short-term IGF-I treatment was able to prevent the reduction in embryo total cell number caused by heat shock. Thus, the cytoprotective effects of IGF-I in the current study were not the result of some differentiation or other long-term effect of IGF-I (for example, on increased

proliferation), but were rather the result of activation of signal transduction systems acting within minutes to hours after IGF-I treatment.

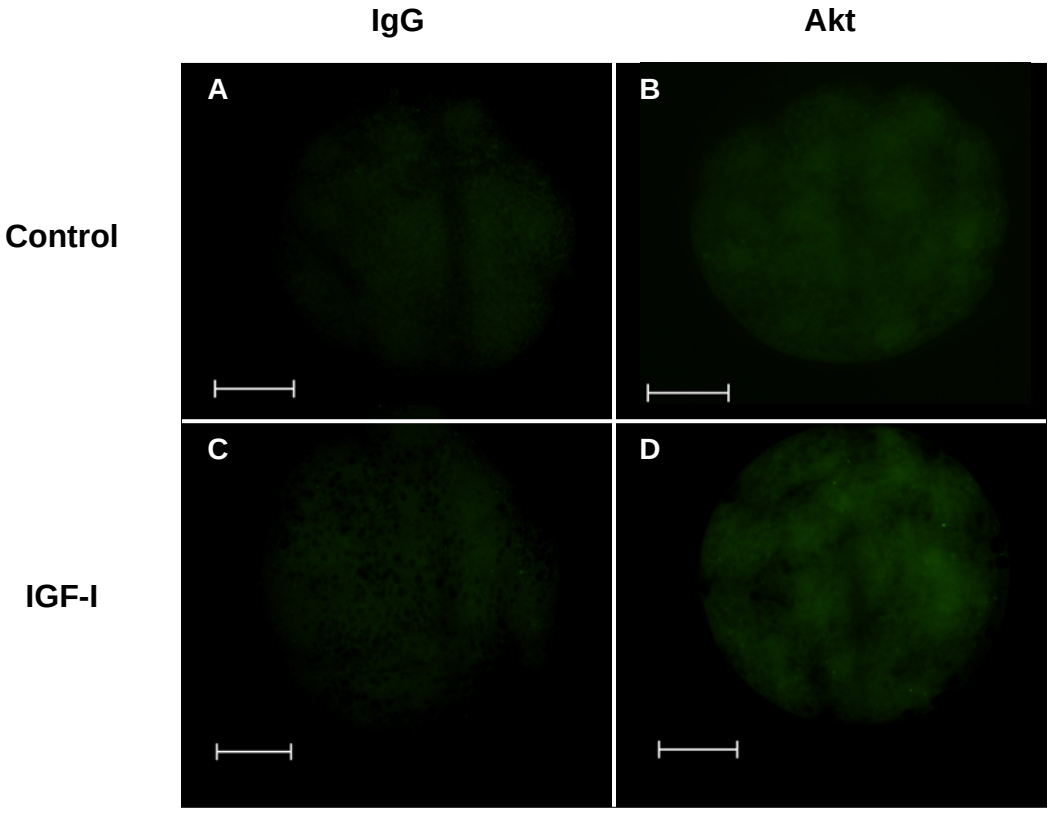


Figure 5-4. Representative fluorescent images of control and IGF-I treated embryos that were immunolabeled with antibody against phosphorylated Akt1. Embryos were cultured for 1 hr before labeling – images appeared similar for embryos cultured for 10 min. The rabbit antibody used recognized Akt1 phosphorylated at Ser 473 (Panels B and D) while controls were rabbit IgG (Panels A and C). Examples of control embryos are depicted in Panels A and B, while IGF-I-treated embryos are depicted in Panels C and D. Embryos were analyzed at 40x and the bars depict 50 μM.

Embryos at d 5 clearly contain activated Akt as revealed by immunofluorescence using the antibody against phosphorylated Akt1. In addition, inhibition of the pathway with either wortmannin or HIMO increased the number of TUNEL-positive cells even in the absence of IGF-I. Also, anti-apoptotic effects of IGF-I resulted from activation of the PI3K/Akt pathway because anti-apoptotic actions of IGF-I were eliminated when PI3K

was inhibited by wortmannin and when an inhibitor of Akt (HIMO) was added. Thus, the bovine preimplantation embryo uses the same pathway for anti-apoptotic effects of IGF-I as other cell types (Nitta et al., 2004; Zheng and Quirion, 2004; Bridgewater et al., 2005; Scott et al., 2005; Zaka et al., 2005). There was no clear difference in intensity of immunolabeling for phosphorylated Akt between control and IGF-I-treated embryos. This result is interpreted to mean that the immunofluoresence is not precise enough to detect subtle changes in amounts of phosphorylated Akt. However, further studies are needed to determine if IGF-I increases the amount of phosphorylated Akt following heat shock.

Akt can phosphorylate and inhibit pro-apoptotic proteins such as Bad (del Peso et al., 1997) and caspase-9 (Cardone et al., 1998) and phosphorylate and stabilize X-linked inhibitor of apoptosis proteins involved in cell survival (Cheng et al., 2002; Dan et al., 2004). In addition, Akt can phosphorylate and inhibit the forkhead transcription factors, thereby preventing the induction of genes involved in pro-apoptotic processes such as Bim and Bax (Brunet et al., 2001; Nitta et al., 2004). Due to the inhibition of pro-apoptotic proteins by Akt, anti-apoptotic proteins such as Bcl-2 and Bcl-x are able to prevent apoptosis (Leverrier et al., 1999; Chrysis et al., 2001).

In addition to effects of IGF-I to block the induction of apoptosis in bovine embryos subjected to heat shock, IGF-I was able to prevent the reduction in embryo total cell number. This pro-proliferative effect requires signaling through the MAPKK pathway (Jones and Clemmons, 1995; Rubinfeld and Seger, 2005) and treatment of bovine embryos subjected to heat shock in the presence of the MAPKK inhibitor PD 98059 abolished the ability of IGF-I to block the reduction in total cell number caused by

heat shock (Chapter 4). Based on results of the current study, the ability of IGF-I to promote an increase in cell proliferation does not involve signaling through the PI3K pathway as IGF-I was still effective in blocking the reduction in total cell number caused by heat shock in the combined analysis of datasets for the DMSO vehicle and wortmannin or HIMO.

It is not obvious that IGF-I will use the same signaling pathways for IGF-I actions in preimplantation embryos. While the onset of transcription has been detected as early as the 2-cell stage of bovine embryonic development (Memili et al., 1998), it takes several rounds of cell division for transcription to be increased that is needed for the continuation of embryonic development (Barnes and First, 1991). However, the PI3K/Akt pathway seems to be intact from the earliest stages of development. Expression of the p85 and p110 subunits of PI3K and Akt occurs in mouse embryos at all stages from the 1-cell through blastocyst stage (Riley et al., 2005). Moreover, PI3K activity is important for glucose metabolism and embryo survival (Riley et al., 2006).

The cytoprotective actions of IGF-I may be beneficial to the bovine embryo during early development. Maternal hyperthermia experienced by pregnant females as a consequence of elevated temperatures can impede continued embryonic development and possibly terminate the pregnancy (Ealy et al., 1993; García-Ispierto et al., 2005). Concentration of IGF-I is highest in peripheral blood and is also produced by the oviduct (Pushpakumara et al., 2002) and endometrium (Geisert et al., 1991), as well as by the embryo itself (Lonergan et al., 2000). It is possible that IGF-I in the reproductive tract, from local and systemic sources, creates a cytoprotective environment that limits deleterious effects of heat shock. Given that IGF-I protects embryos from various

inducers of apoptosis, such as hydrogen peroxide (Kurzawa et al., 2002), ultraviolet irradiation (Herrler et al., 1998), camptothecin and actinomycin D (Fabian et al., 2004) and TNF-α (Kurzawa et al., 2001; Byrne et al., 2002a), it is likely that IGF-I has protective effects against a variety of adverse environmental agents.

One question is whether it is possible to regulate the IGF-I milieu in the reproductive tract. One way to do this would be to increase circulating concentrations of IGF-I through administration of bST (Sharma et al., 1994; Newbold et al., 1997) that likely results from increased hepatic IGF-I mRNA (Sharma et al., 1994; Vanderkooi et al., 1995). Treatment with ST can create a cytoprotective environment for at least some cells because peripheral blood lymphocytes harvested from heifers treated with ST were more resistant to heat shock in vitro than lymphocytes from control heifers (Elvinger et al., 1991a).

In conclusion, short-term treatment of the bovine preimplantation embryo with IGF-I can block induction of heat shock-induced apoptosis through the PI3K/Akt pathway and prevent the reduction in total cell number caused by heat shock. Studies are needed to determine which genes are regulated by IGF-I in the preimplantation embryo to exert its anti-apoptotic actions and to determine if IGF-I increases the amount of phosphorylated Akt.

# CHAPTER 6
# FERTILITY OF LACTATING DAIRY COWS ADMINISTERED RECOMBINANT BOVINE SOMATOTROPIN DURING HEAT STRESS

## Introduction

Treatment of lactating cows exposed to heat stress with bST can increase milk yield (Mohammed and Johnson, 1985; Staples et al., 1988; West et al., 1990b; Johnson et al., 1991; Elvinger et al., 1992; Tarazón-Herrera et al., 1999). One consequence of bST treatment during heat stress is an increase in body temperature (Zoa-Mboe et al., 1989; West et al., 1990b; West et al., 1991; Elvinger et al., 1992; Cole and Hansen, 1993) and some of this increase in body temperature may be independent of a lactational effect (Elvinger et al., 1992; Cole et al., 1993). Given the fact that elevated body temperature compromises fertility in lactating cows (Vasconcelos et al., 2006), it is possible that bST treatment during heat stress could compromise fertility. However, fertility-promoting effects of bST may overcome adverse effects of increased hyperthermia on fertility. Short-term treatment with bST at detected estrus increased the fertility of repeat-breeding dairy cows (Morales-Roura et al., 2001), and bST treatment increased fertility of cows bred via TAI (Moreira et al., 2000b; Moreira et al., 2001; Santos et al., 2004).

Treatment with bST may also increase cellular resistance to elevated temperature, either directly or indirectly. Lymphocytes harvested from heifers treated with bST were more resistant to heat shock in vitro as compared to lymphocytes from non-treated heifers (Elvinger et al., 1991). Insulin-like growth factor-I, whose secretion is induced by bST (Cohick et al., 1989; Newbold et al., 1997; Bilby et al., 1999), reduced the effects of heat

shock on development and apoptosis in preimplantation bovine embryos (Chapters 3 through 5).

Given the fertility-promoting and cytoprotective actions of bST, the hypothesis of this study was that administration of bST to lactating Holstein cows during the summer months would increase pregnancy rates as compared to lactating non-treated Holstein cows.

## Materials and Methods

### Animals, Housing, and Feeding

The experiment was conducted from July 4 to November 7, 2005 at a commercial dairy farm near Quitman, GA (30°47'5" N, 83°33'39" W) utilizing 285 primiparous and multiparous lactating Holstein cows. During the study period, cows were housed in a free-stall barn equipped with fans and a sprinkler system. Hourly dry bulb temperature and relative humidity measurements were recorded inside the free-stall barn from July 18 to November 7, 2005, utilizing a data logger (HOBO® Pro RH/Temp Data Logger, Onset Computer Co., Bourne, MA); results are depicted in Figure 6-1.

All cows were fed as a group two times daily. The diet was a total mixed ration formulated to meet the nutrient requirements for lactating Holstein cows weighing approximately 590 kg. Cows were milked three times daily (0000, 0800, and 1600 h) and milk yields were recorded for individual cows once monthly during the official Georgia DHIA test.

### Experimental Design

A diagram of the experimental protocol is displayed in Figure 6-2. For first service TAI, cows at 44 ± 3.5 d postpartum were presynchronized with two injections of 25 mg of $PGF_{2\alpha}$, i.m. 14 d apart, followed 14 d later by the initiation of a modified Ovsynch

protocol (100 μg of GnRH, i.m. followed 7 d later with 25 mg $PGF_{2\alpha}$, i.m., and a second injection of GnRH (100 μg) given 72 h following $PGF_{2\alpha}$ coincident with TAI; Portaluppi and Stevenson, 2005). All cows received an additional injection of 100 μg of GnRH, i.m., 1 wk prior to pregnancy diagnosis using ultrasonography on d 29 post-TAI. Cows diagnosed as non-pregnant were resynchronized with the continuation of the modified

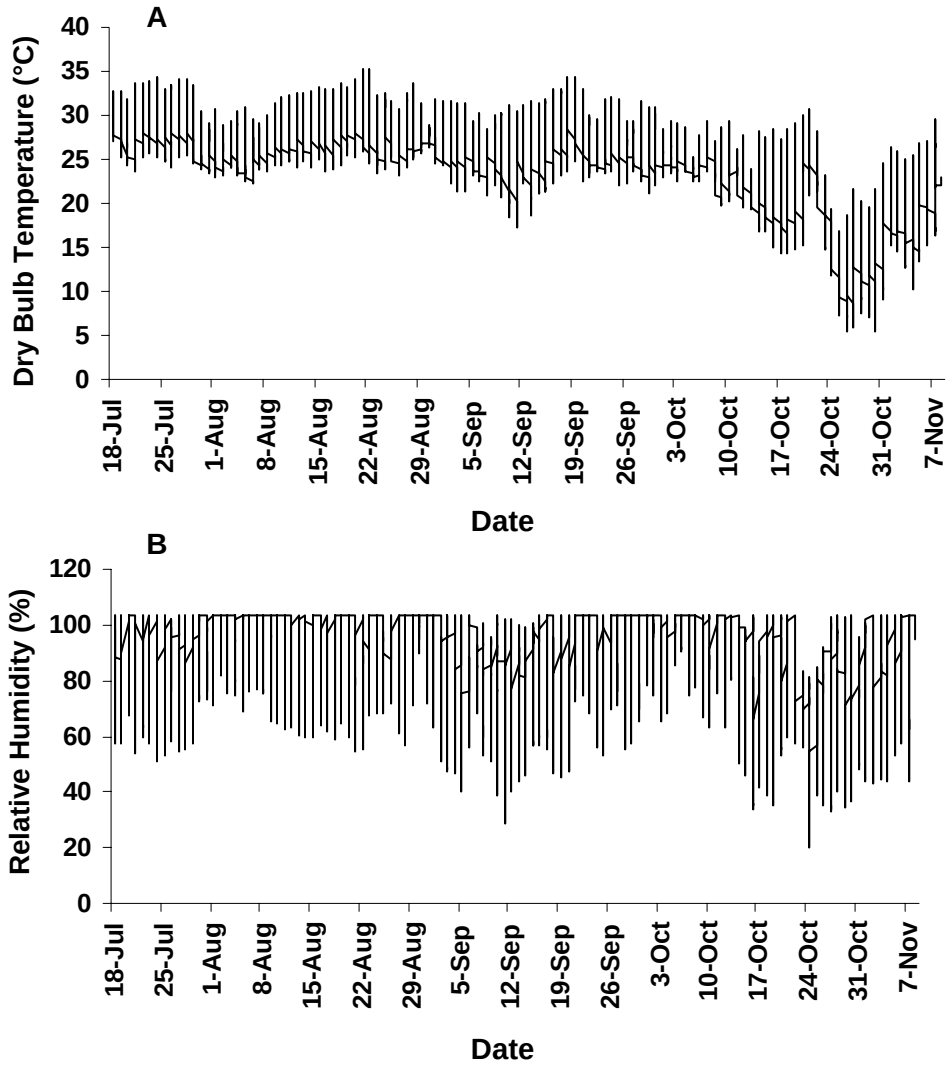


Figure 6-1. Daily dry bulb temperature (Panel A) and relative humidity (Panel B) at the experiment site from July 18 to November 7, 2005. Data represent hourly averages of measurements taken every 15 min.

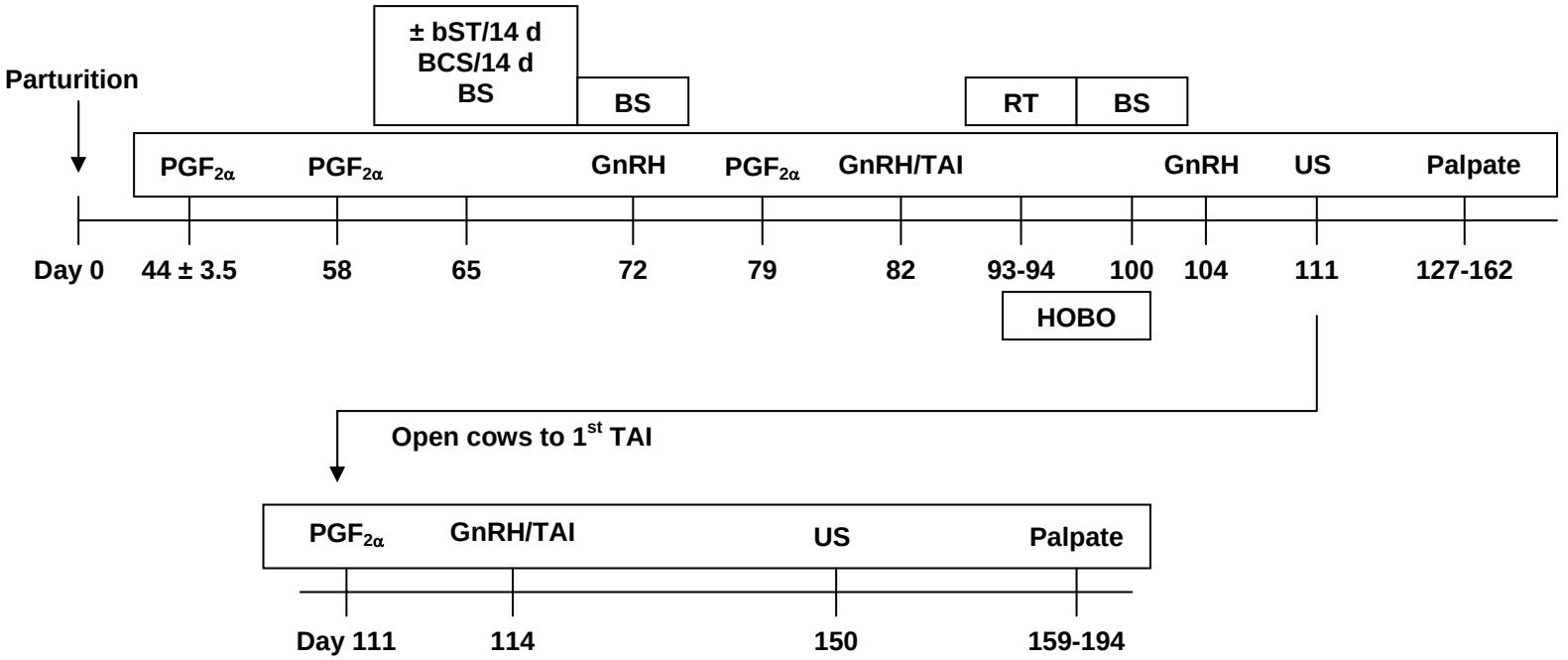


Figure 6-2. Schematic diagram illustrating the time course of the experiment. bST = bovine somatotropin, BCS = body condition score, BS = blood sample, TAI = timed artificial insemination, RT = rectal temperature, HOBO = HOBO device for measuring vaginal temperature, US = ultrasound.

Ovsynch protocol and received TAI. Pregnancy for first- and second-service was confirmed by rectal palpation between d 45 to 80 post-TAI.

Cows entering the experiment each week were randomly assigned to bST or control treatments. Treatment with bST (500 mg, s.c.; Posilac, Monsanto Co., St. Louis, MO), which was administered initially in the depression on the right side of the tailhead with subsequent injections being alternated between the cow's left and right side tailhead depression, was initiated 1 wk prior to the start of the modified Ovsynch protocol and continued at 2-wk intervals throughout the experiment. Control cows received no injection.

Body condition score using a scale of 1 to 5 in 0.25 increments (Edmonson et al., 1989) was recorded for bST-treated and control cows at each bST treatment. A subset of cows (n = 41 cows; 21 bST and 20 control cows) were fitted with a temperature data logger (HOBO® Water Temp Pro v1, Onset Computer Co.) attached to a blank CIDR (Pfizer Animal Health, New York, NY) that was inserted into the vagina. The device recorded vaginal temperature at 15 min intervals beginning at 1200 h on d 10 post-TAI for 1 wk (d 28 of bST treatment). In addition, rectal temperatures were taken between 1600 and 2000 h on d 11 post-TAI (d 29 of bST treatment) for all cows using a digital thermometer (M525/550 Hi-Performance Digital Thermometer; GLA Agricultural Products, San Luis Obispo, CA).

**Blood Sample Collection and IGF-I Immunoradiometric Assay**

A subset of cows (n = 27 cows) were bled to determine IGF-I profiles immediately prior to the first bST injection, 1 wk later, and at d 35 of bST treatment. Blood samples were collected by coccygeal venipuncture into evacuated heparinized tubes (Becton

Dickinson, Franklin Lakes, NJ). Following collection, blood samples were placed in an ice chest until further processing at the laboratory (within approximately 4 to 6 h). Blood samples were centrifuged at 2,000 x *g* for 20 min at 4°C. Plasma was separated and stored at -20°C until assayed for IGF-I concentration which was determined using the ACTIVE® Non-Extraction IGF-I IRMA kit (DSL-2800; Diagnostic Systems Laboratories, Inc., Webster, TX) with a sensitivity of 2.06 ng/ml. The assay was validated with parallelism by using different dilutions of a plasma sample. This was a non-competitive assay in which the analyte to be measured was "sandwiched" between two antibodies, with the first antibody immobilized to the inside wall of the tubes and the second antibody radiolabeled for detection. The analyte present in the plasma samples was bound by both of the antibodies to form a "sandwich" complex and unbound materials were removed by decanting and washing the tubes. All samples were assayed at the same time and the intrassay coefficient of variation was 7%.

**Statistical Analysis**

Nine cows were excluded from the statistical analysis (three control cows - 2.2% and six bST cows - 4.0%) because they were removed from the herd prior to the determination of pregnancy status by ultrasonography at d 29 post-TAI, thereby leaving 276 total cows (n = 132 control cows and n = 144 bST cows) used for analysis of pregnancy rate.

Statistical analysis was performed to determine the effects of bST and other dependent variables. Days in milk class (DIMC) divided cows based on days in milk: <63 d or ≥63 d at the start of bST treatment. Milk yield class (MYC) categorized cows based on milk yield at the monthly DHIA test date immediately prior to the start of bST

treatment. Classes, which were based on quartiles derived from the Univariate procedure of SAS (SAS 9.0, SAS Inst., Inc., Cary, NC), were <31.66 kg/d, 31.66 kg/d to <39.05 kg/d, 39.05 kg/d to <45.86 kg/d, or ≥45.86 kg/d. Finally, cows were categorized according to BCS class (BCSC) into classes of BCS <2.5, equal to 2.5, or >2.5 at the start of bST treatment. All cows were separated based on parity into first-lactation heifers and cows with ≥2 lactations.

First- and second-service TAI pregnancy rates and pregnancy loss were analyzed with the LOGISTIC procedure of SAS using a backward stepwise logistic model. Variables were continuously removed from the model by the Wald statistic criterion if the significance was greater than 0.20. The mathematical model for analysis of first- and second-service TAI pregnancy rates and pregnancy loss included the effects of bST, sire, technician, week of insemination, parity, MYC, DIMC, BCSC, and bST x parity, bST x MYC, bST x DIMC, and bST x BCSC. No significant interactions were found, however, and the final model included only main effects. The adjusted odds ratio (AOR) estimates and the 95% Wald confidence intervals (CI) from logistic regression were obtained for each variable that remained in the final statistical model following the backward elimination. The Wald chi-square statistic was used to determine the probability value for each main effect that remained in the reduced model (significance was considered as $P < 0.05$). In addition, differences between levels of variables having more than 1 degree of freedom were determined using the GENMOD procedure of SAS.

Treatment effects on concentration of IGF-I, milk yield, BCS, rectal temperature, and vaginal temperature were analyzed using least squares ANOVA with the GLM procedure of SAS. Tests of significance were made using the appropriate error terms

based on calculation of expected means squares. Cow was a random effect and other main effects were considered fixed.

Because only a subset of cows were bled to determine the concentration of plasma IGF-I, the model included main effects of bST, time, cow nested within bST, and the interaction of bST x time.

The initial analysis of milk yield used data for the three monthly test dates prior to the start of bST treatment and the four monthly test dates following the start of bST treatment. The model included main effects of bST, parity, time, BCSC, cow nested within bST, parity, and BCSC, and interactions between main effects. Other analyses were performed using datasets consisting for each test date separately for the four monthly test dates following the start of bST treatment. In this case, DIM and milk yield at the test date immediately prior to the start of bST treatment as covariates. The model also included effects of bST, parity, BCSC, bST x parity, and bST x BCSC.

Body condition score was analyzed using the main effects of bST, parity, time, cow nested within bST and parity and interactions between main effects. The change in BCS from the start of bST treatment until the end of the experiment was calculated as the final BCS at wk 12 minus the initial BCS at wk 0. This variable was analyzed using least-squares ANOVA to determine the effects of bST, parity, and bST x parity. In addition, BCS at each measurement date were further analyzed using the CATMOD procedure of SAS with the main effect of bST treatment.

Rectal temperature data were analyzed using the main effects of bST, parity, MYC, BCSC, and interactions between main effects. Because only a subset of cows were fitted vaginally with a HOBO digital temperature recorder, the statistical model to determine

effects on vaginal temperature included effects of bST, cow nested within bST, time, and the interaction of bST x time. Unless otherwise mentioned, all values reported for IGF-I concentration, milk yield, BCS, rectal temperature, and vaginal temperature are least-squares means ± SEM.

## Results

Plasma IGF-I concentrations were similar for bST and control cows prior to the start of bST treatment (Figure 6-3). Concentrations remained constant thereafter for control cows, but increased approximately four-fold at d 7 and 35 after treatment in the bST-treated group (bST x time: $P < 0.001$).

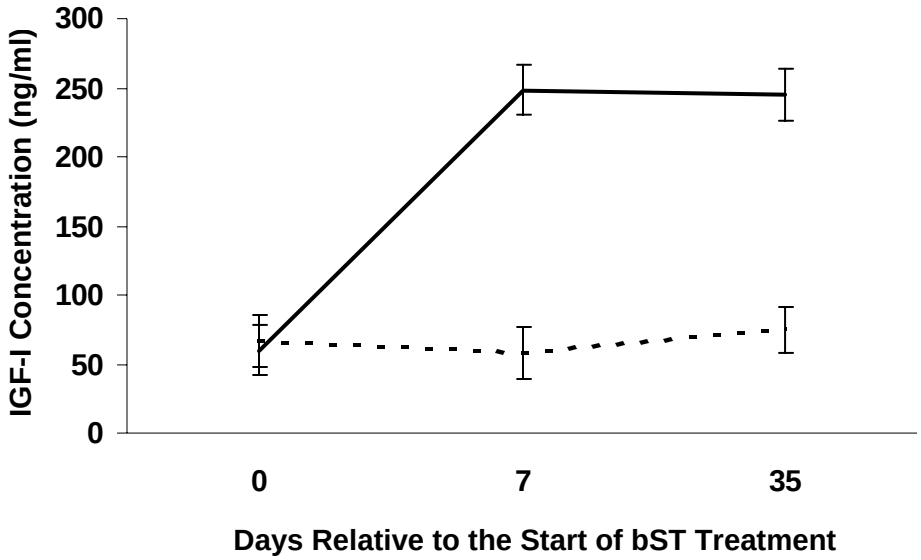


Figure 6-3. Plasma insulin-like growth factor (IGF-I) response to bovine somatotropin (bST). Depicted are the concentrations of plasma IGF-I for bST (solid line) and control cows (dashed line) at day 0 (initiation of bST treatment), 7, and 35 relative to the start of bST treatment. Data represent least-squares means ± SEM. Cows treated with bST had increased concentrations of plasma IGF-I at day 7 and 35 as compared to control cows (bST x time, $P < 0.001$).

Milk yields were similar between bST and control cows prior to the start of bST treatment, but bST-treated cows had higher milk yields than control cows after the initiation of bST treatment (Figure 6-4A; bST x time, $P < 0.05$). The increase in milk

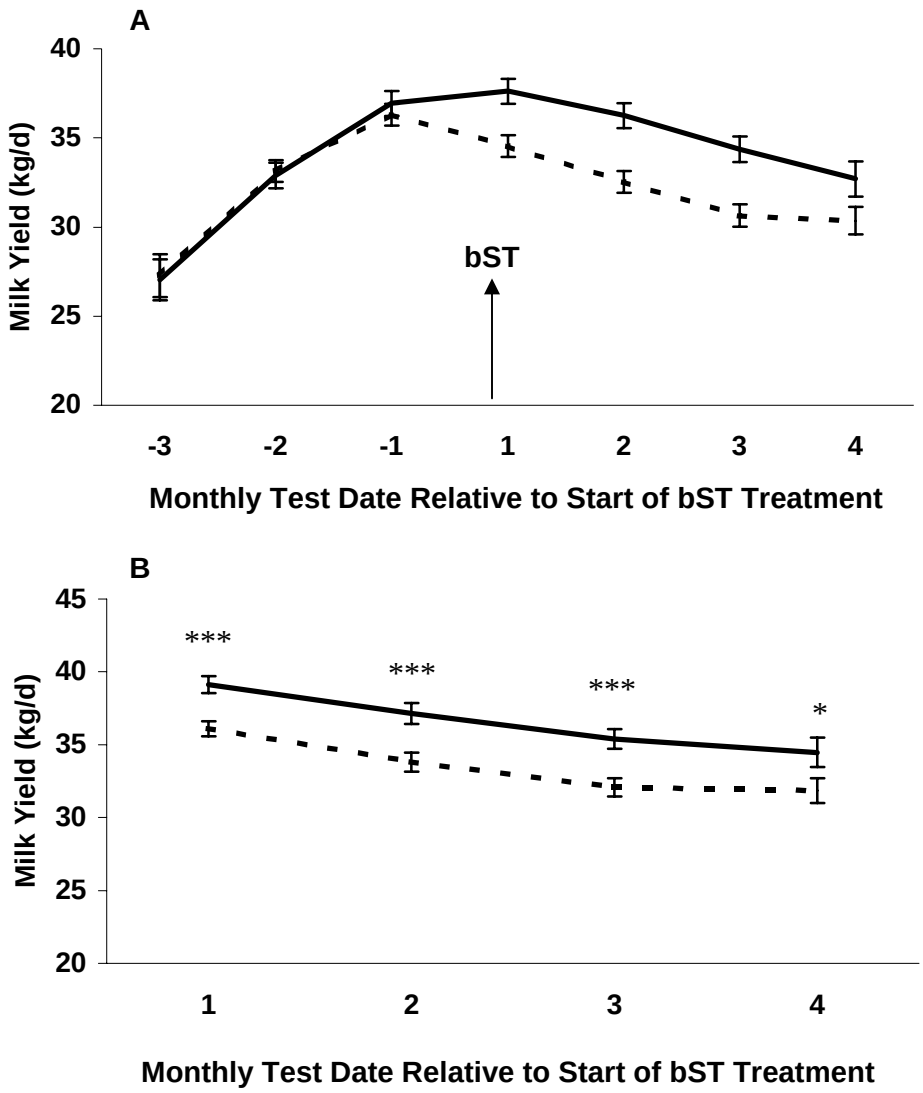


Figure 6-4. Milk yield response to bovine somatotropin (bST). Panel A depicts milk yield collected at each monthly DHIA test date for bST (solid line) and control cows (dashed line) throughout the experiment. Panel B represents milk yield responses for bST (solid line) and control cows (dashed line) after the start of bST treatment using differences in the number of days in milk and the test date milk yield at the test date immediately prior to the start of bST treatment as covariates. Data represent least-squares means ± SEM. In Panel A, cows treated with bST had an increased milk yield as compared to control cows following the start of bST treatment (bST x time, $P < 0.01$). For Panel B, three asterisks (***; $P < 0.001$) or one asterisk (*; $P < 0.05$) represent an increase in milk yield for bST-treated cows over control cows.

yield at each test date due to bST ranged from 8 to 12% and 2.4 to 3.7 kg/d. Cows in their second or greater lactation had a higher milk yield (37.2 ± 0.6 kg; $P < 0.001$) than

lactating heifers in their first lactation (28.5 ± 0.9 kg), but there was no interaction between bST x parity.

A separate analysis of milk yield following the start of bST treatment was conducted in which DIM and milk yield at the test date immediately prior to the start of bST treatment were covariates. Cows treated with bST had significantly higher milk yield when compared to control cows at each of the monthly test dates following the start of bST (Figure 6-4B).

Rectal temperature on d 29 of bST treatment was higher for bST cows as compared to control cows (Figure 6-5A; $P < 0.05$). In addition, vaginal temperature recorded every 15 min from d 28 to 35 of bST treatment was higher for bST cows as compared to control cows (Figure 6-5B; $P < 0.05$). The time of day x bST treatment interaction was not significant.

The BCS was affected by an interaction of bST x time (Figure 6-6A; $P < 0.05$). Before initiation of bST treatment, BCS were similar for control and bST cows and increased over time. Following initiation of bST, BCS declined in both groups of cows and the loss in BCS was more pronounced for bST cows. There was also an effect of bST treatment ($P = 0.05$) on the net change in BCS from the initial to final BCS measurement (+0.05 and +0.14 for bST-treated and control cows, respectively, SEM = 0.03). As an additional test of effects of bST on BCS, data at each measurement were analyzed separately by CATMOD analysis to determine whether bST affected the distribution of BCS. A representative example of the effects of bST on distribution of BCS is shown for wk 10 after bST (Figure 6-6B). Treatment with bST affected distribution of BCS at wk 8 ($P < 0.001$), 10 ($P < 0.001$), and 12 ($P < 0.05$) after bST.

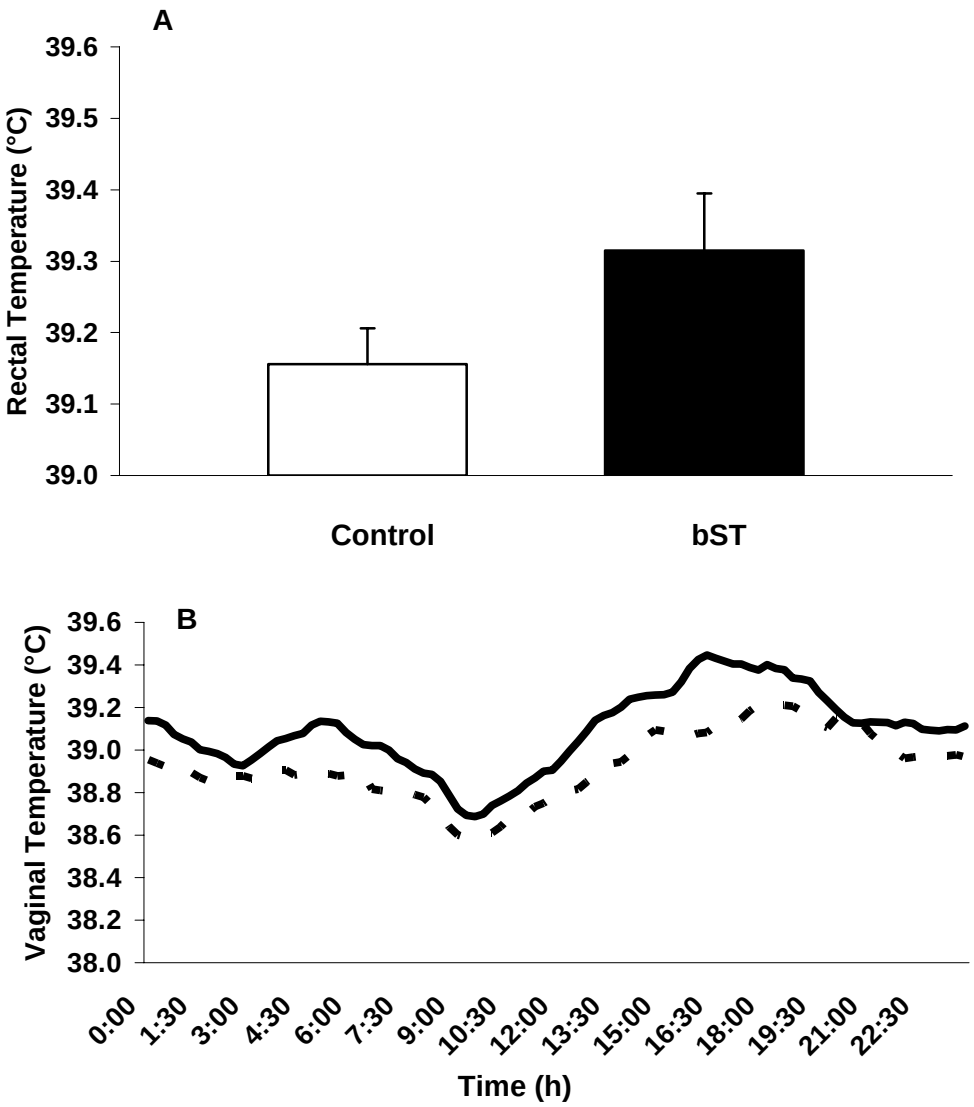


Figure 6-5. Rectal and vaginal temperatures as affected by bovine somatotropin (bST). Panel A represents the rectal temperature for bST (filled bar) and control cows (open bar) taken between 1600 and 2000 h on d 29 of bST treatment. Panel B depicts the vaginal temperature for bST (solid line) and control cows (dashed line) recorded every 15 min between d 28 and 35 of bST treatment. Data in both panels represent least-squares means ± SEM. Treatment of cows with bST increased rectal (Panel A; $P < 0.05$) and vaginal temperature (Panel B; $P < 0.05$) as compared to control cows. There was no time x bST treatment interaction for vaginal temperature.

Pregnancy rates following the first- and second-service TAI for bST and control cows are depicted in Figure 6-7. For first-service TAI (Figure 6-7A), there was no significant difference in pregnancy rate on d 29 post-TAI between bST-treated (18.8%;

27 cows pregnant/144 cows total) vs control cows (17.4%; 23/132). Similarly, pregnancy rate based on rectal palpation at d 45 to 80 post-TAI was not significantly difference between bST-treated cows (16.7%; 24/144) and control cows (15.2%; 20/132). In addition, there was no effect of bST on pregnancy loss between the two diagnoses of

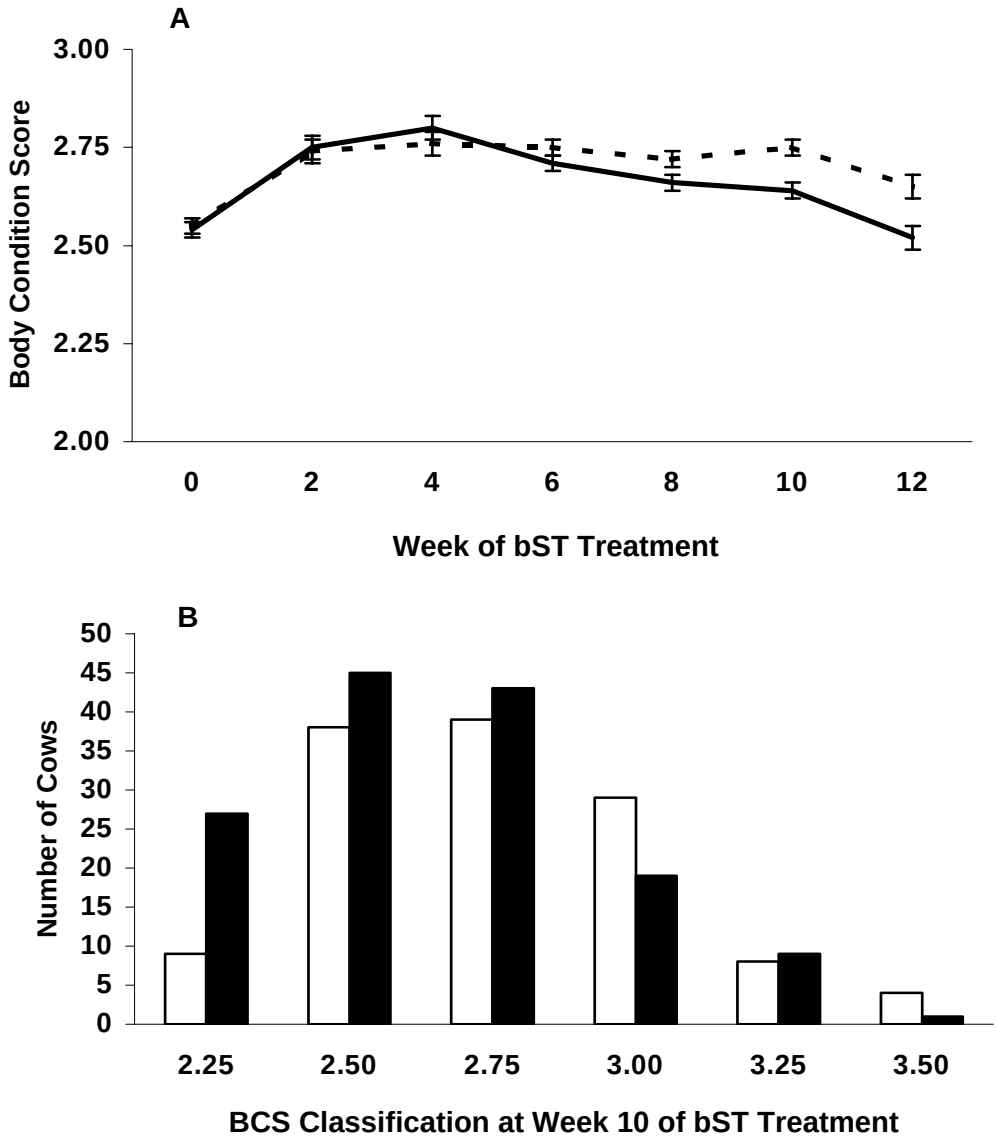


Figure 6-6. Effect of bovine somatotropin (bST) on body condition score (BCS). Panel A depicts BCS for bST (solid line) and control cows (dashed line) throughout the experiment. Data represent least-squares means ± SEM. In Panel A, bST-treated cows lost more body condition over time as compared to the control cows ($P < 0.05$). Panel B represents the distribution of BCS at wk 10 after initiation of bST for bST-treated (filled bar) and control cows (open bar). Treatment with bST affected distribution of BCS ($P < 0.001$).

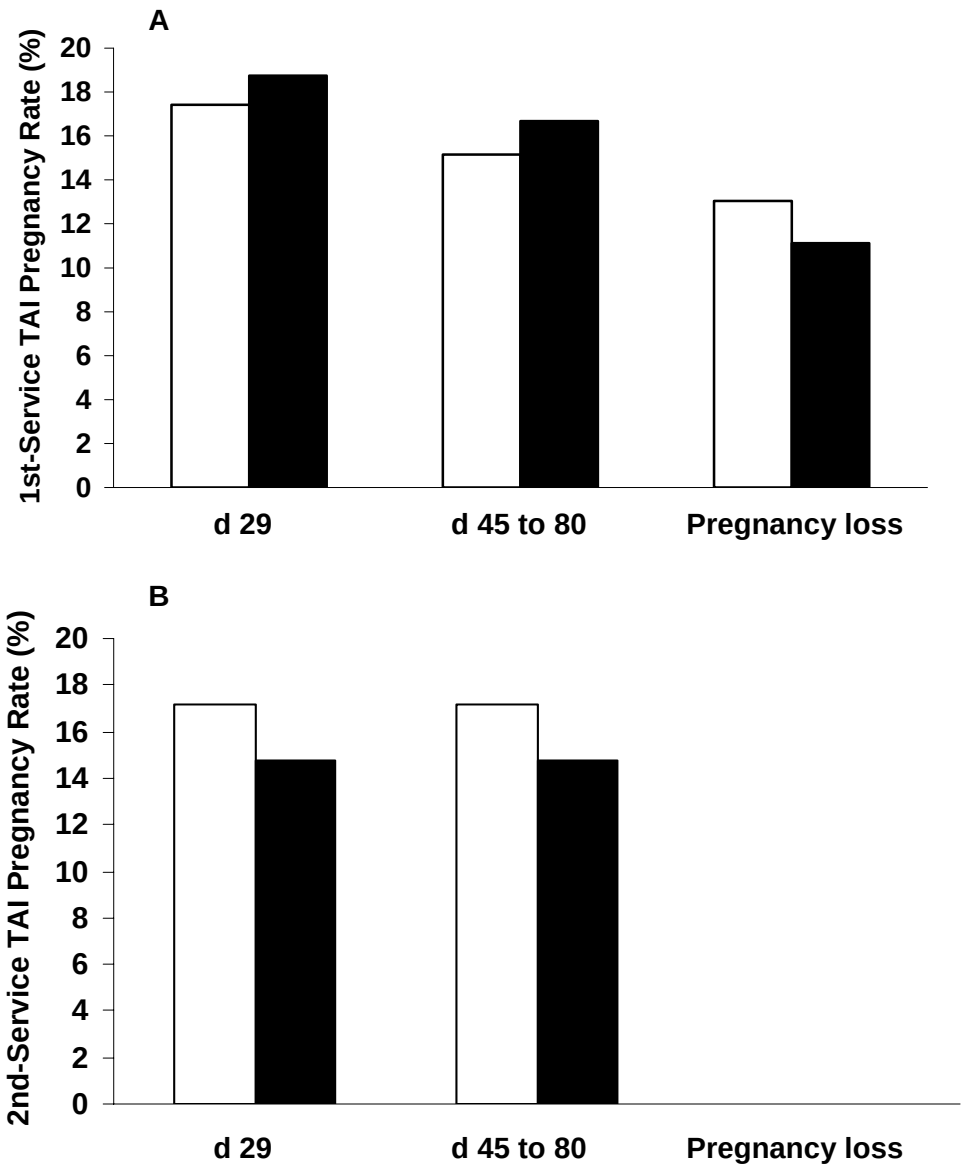


Figure 6-7. Pregnancy rates and losses for bovine somatotropin (bST)-treated and control cows following first- and second-service TAI. Panel A represents the first-service TAI pregnancy rates for bST (filled bar) and control cows (open bar) at day 29 post-TAI as determined by ultrasonography, pregnancy rate at day 45 to 80 post-TAI as determined by rectal palpation, and the percentage of pregnancies lost between day 29 and the second pregnancy diagnosis. Panel B represents the second-service TAI pregnancy rates for bST (filled bar) and control cows (open bar) at day 29 post-TAI as determined by ultrasonography, pregnancy rate at day 45 to 80 post-TAI as determined by rectal palpation, and the percentage of pregnancies lost between day 29 and the second pregnancy diagnosis. Note that no pregnancies were lost.

pregnancy [11.1% (3/27) for bST vs 13.0% (3/23) for control). For the second-service TAI (Figure 6-7B), there was no pregnancy loss between the two pregnancy diagnoses. Thus, pregnancy rates are the same at both times and were 14.8% (9/61) for bST cows and 17.2% (11/64) for control cows. The effect of bST was not significant.

Body condition score class influenced first-service pregnancy rate based on rectal palpation ($P < 0.05$). Those cows categorized as having a BCS of <2.5, 2.5, or >2.5 had a first-service pregnancy rate of 9.6% (5/52), 12.8% (18/141), and 25.3% (21/83), respectively. The adjusted odds ratio for first-service pregnancy rate for cows categorized with a BCS of >2.5 compared to cows with a BCS of <2.5 was 3.37 (95% Wald confidence interval: 1.18, 9.66; $P < 0.05$), whereas the adjusted odds ratio for first-service pregnancy rate for cows categorized with a BCS of >2.5 compared to cows with a BCS of 2.5 was 2.21 (95% Wald confidence interval: 1.09, 4.47; $P < 0.05$). For the second-service TAI, there was no effect of BCSC on pregnancy rate at d 45 to 80 post-TAI (pregnancy rates were 15.2% (5/33), 16.7% (11/66), and 15.4% (4/26) for cows with a BCS of <2.5, 2.5, or >2.5, respectively).

## Discussion

Results from this study indicate that bST can be administered to lactating Holstein cows during summer heat stress to increase milk yield without compromising first- or second-service TAI pregnancy rates. Thus, despite the increased strain expressed as increased milk yield and body temperature and reduced BCS, cows treated with bST remained as fertile as control cows. The pregnancy rates achieved by TAI were similar to what has been reported for other lactating cows exposed to heat stress (de la Sota et al., 1998; Aréchiga et al., 1998; Franco et al., 2006). That bST did not reduce pregnancy rates despite increasing body temperature may be the result of the fertility-promoting

effects of this hormone (Morales-Roura et al., 2001; Moreira et al., 2000b; Moreira et al., 2001; Santos et al., 2004) and the actions of bST and IGF-I to increase cellular resistance to elevated temperature (Elvinger et al., 1991; Chapters 3 through 5).

As reported previously (Cohick et al., 1989; Newbold et al., 1997; Bilby et al., 1999), bST caused a large rise in plasma IGF-I concentrations, indicating that heat stress does not block the IGF-I response to bST. The increase in milk yield in response to 500 mg of bST administered biweekly, whether expressed on an absolute basis (2.4-3.7 kg/d) or a percentage basis (8-12%), was similar to what has been observed for non-heat stressed cows (Hartnell et al., 1991; Huber et al., 1997; Bauman et al., 1999; Santos et al., 2004; VanBaale et al., 2005). Indeed, several studies have shown that bST increases milk yield of cows under conditions of heat stress (West et al., 1990b; Johnson et al., 1991; Elvinger et al., 1992; Lotan et al., 1993; Tarazón-Herrera et al., 1999).

As has been reported previously (West et al., 1990b; Elvinger et al., 1991; Elvinger et al., 1992; Cole and Hansen, 1993), cows treated with bST experienced higher body temperatures than control cows. Milk temperatures in heat stressed cows have also been reported to be increased by bST treatment (West et al., 1991). Data obtained on vaginal temperatures indicated that this difference persisted throughout the day. Indeed, cows were hyperthermic nearly continuously. The increase in body temperature caused by bST could be due to the increase in milk yield caused by bST treatment. The possibilities for effects independent of lactational actions of bST exist, however. Elvinger et al. (1982) found that treatment of cows in Florida with bST during the summer increased rectal temperature even though there were little absolute differences in milk yield between bST

and control cows. Additionally, bST increased body temperature and rate of open-mouthed panting for non-lactating cows exposed to heat stress (Cole and Hansen, 1993).

Effects of bST on body temperature could be expected to decrease fertility because fertility declines as body temperature rises (Vasconcelos et al., 2006). That such a negative effect of bST on fertility was not seen implies that other effects of bST either compensate for the increased body temperature. One possibility is that bST or IGF-I increases resistance of embryos to elevated temperature. Lymphocytes from bST-treated heifers were more resistant to the effects of heat shock in culture than lymphocytes from control heifers (Elvinger et al., 1991). Also, IGF-I can protect embryos from effects of heat shock on development and apoptosis (Chapters 3 through 5). Bovine somatotropin can also have fertility-enhancing effects in lactating cows subjected to TAI independent of heat stress (Morales-Roura et al., 2001; Moreira et al., 2000b; Moreira et al., 2001; Santos et al., 2004). The mechanisms responsible for this effect of bST are not clear but could involve increased weight of the CL (Lucy et al., 1995), number of follicles 10 to 15 mm in diameter (Lucy et al., 1995), fertilization rates (Moreira et al., 2002a), IGF-I-mediated changes in rate of early embryonic development (Makarevich and Markkula, 2002; Moreira et al., 2002b; Block et al., 2003; Sirisathien et al., 2003) and embryonic apoptosis (Byrne et al., 2002; Makarevich and Markkula, 2002; Chapters 3 through 5), modulation of $PGF_{2\alpha}$ synthesis (Badinga et al., 2002), and effects on the oviduct and uterus (Pershing et al., 2002).

In the current study, cows classified as having a BCS of <2.5 or equal to 2.5 had a reduced first-service TAI pregnancy rate (d 45 to 80 post-TAI) as compared to cows with a BCS of >2.5 (9.6, 12.8, and 25.3% first-service TAI pregnancy rate for cows classified

as having a BCS of <2.5, equal to 2.5, and >2.5, respectively). A reduction in fertility associated with low BCS has been reported previously (Moreira et al., 2000a; Buckley et al., 2003). Some of the low pregnancy rate in cows with low BCS is likely to have been caused by failure of cows to resume ovarian cyclicity at first service (Shrestha et al., 2005). The fact that BCS did not affect second-service TAI pregnancy rate could indicate that the major effect of BCS is on resumption of cyclicity, assuming most cows had resumed estrous cycles by second insemination.

In the current study, bST reduced BCS but the magnitude of the effect was probably too small to compromise reproductive function. The reduction in BCS caused by bST was likely caused by mobilization of nonesterified fatty acids from adipose tissue (Tarazón-Herrera et al., 1999). Other studies have found that cows administered bST lost body weight and body condition (West et al., 1990a; West et al., 1990b; Lotan et al., 1993; Moallem et al., 2000) although Johnson et al. (1991) reported no change in body weight for bST-treated cows.

In conclusion, this study demonstrated that treatment of lactating Holstein cows with bST during heat stress increased concentrations of IGF-I and milk yield. In addition, bST-treated cows had increased rectal and vaginal temperatures and lost slightly more body condition over time as compared to control cows. Despite these changes, first- and second-service TAI pregnancy rates and pregnancy losses were not reduced, indicating that, at least under certain housing conditions, bST can be administered to lactating cows during heat stress without compromising fertility.

# CHAPTER 7
# PREDICTION OF DEVELOPMENTAL POTENTIAL OF PREIMPLANTATION EMBRYOS THROUGH MEASUREMENT OF GROUP II CASPASE ACTIVITY

## Introduction

Selection of the most viable embryo(s) from within a given group can reduce the number of embryos transferred during a given IVF cycle and facilitate single embryo transfer. Approaches taken to assess embryonic viability include development of morphological criteria, measurement of rates of development, analysis of metabolism, and proteomic analysis to identify protein markers of embryo quality (Van Soom et al., 2001, 2003; Ebner et al., 2003; Gardner and Sakkas, 2003, Sakkas and Gardner, 2005; Katz-Jaffe et al., 2006).

One potentially valuable approach to assess embryo competence for development is to evaluate the degree of blastomere apoptosis. Apoptosis has been related to occurrence of embryo fragmentation at certain stages of development (Jurisicova et al., 2003; Honda et al., 2005) and fragmentation is related to reduced competence of embryos to develop to the blastocyst stage and pregnancy rate following transfer (Ebner et al., 2001; Stone et al., 2005). Embryos treated with molecules that block apoptosis, such as IGF-I (Byrne et al., 2002b; Chapters 3 through 5) and granulocyte colony stimulating factor (Behr et al., 2005), can achieve higher pregnancy rates when transferred to recipients (Block et al., 2003; Sjöblom et al., 2005).

While definitive determination of apoptosis is an invasive procedure, biochemical correlates of apoptosis can be measured using assays that do not lead to destruction of all

or a portion of the embryo. One of the simplest assays is measurement of caspase enzymatic activity using fluorogenic substrates. A commercially available substrate for group II caspases (those that have substrate specificity for the amino acid motif DEXD; i.e. caspases-2, -3 and -7) has been used to assess caspase activity in bovine oocytes and preimplantation embryos (Krininger et al., 2002; Paula-Lopes and Hansen, 2002a; Roth and Hansen, 2004a). Inhibition of group II caspases blocks heat shock-induced apoptosis in the oocyte (Roth and Hansen, 2004a) and embryo (Paula-Lopes and Hansen, 2002a). The hypothesis tested in this study was that measurement of group II caspase activity among blastocysts having good morphology could distinguish between those with a high potential for subsequent development from those with a low potential for subsequent development.

## Materials and Methods

### Materials

The materials used for these experiments were as described in Chapter 3 with the following addition. The PhiPhiLux-$G_1D_2$ reagent was from OncoImmunin Inc. (Gaithersburg, MD).

### In Vitro Production of Embryos

Embryo production was performed using in vitro maturation of oocytes and in vitro fertilization as described in Chapter 3. Putative zygotes were cultured in KSOM-BE2 overlaid with mineral oil. Blastocysts classified as quality grade 1 (IETS, 1998; those with excellent or good morphology) were selected from drops at d 7 (Exp. 2) or 8 (Exp. 1) after insemination and used for the experiment.

**Assay for Group II Caspase Activity**

Groups of equal numbers of blastocysts (not exceeding 10) were incubated in 25-μl drops of Hepes-TALP containing 5 μM PhiPhiLux-$G_1D_2$ at 39°C on a stage warmer for 40 min in the dark. Following incubation, blastocysts were washed three times in 50-μl drops of Hepes-TALP and placed individually on 2-well slides (Fisher, Pittsburgh, PA) containing 125 μl of pre-warmed Hepes-TALP. Caspase activity was measured using a Zeiss Axioplan 2 epifluorescence microscope (Zeiss, Göttingen, Germany) with a FITC filter and a 20x objective. Digital images were acquired using AxioVision software (Zeiss) and a high-resolution black and white Zeiss AxioCam MRm digital camera set to an exposure length of 2000 milliseconds. Using the computer mouse, a circular draw function was manually performed around the internal side of the zona pellucida and intensity per unit area was determined. Embryos were separated on the basis of group II caspase activity into three categories based on quantification of fluorescent pixel intensity (low activity: <15.0 pixels; medium activity: 15.0 to <30.0 pixels; high activity: ≥30.0 pixels). Embryos were then returned to culture as described in the experiments below.

**Experimental Design**

In Exp. 1, blastocysts cultured in KSOM-BE2 at 38.5°C in an atmosphere of 5% $CO_2$ in humidified air were removed from culture at d 8 after insemination and subjected to the group II caspase activity assay. Embryos were classified according to caspase activity into low, medium and high groups and cultured within classification in groups of no more than 15 embryos in fresh 25-μl drops of KSOM-BE2 at 38.5°C in an atmosphere of 5% $CO_2$ in humidified air. Embryos were evaluated at 24 h intervals and stage of development was recorded for each embryo until d 10 post-insemination when the

proportion of blastocysts that hatched (i.e. emerged from the zona pellucida) based on caspase activity was assessed. A total of 264 blastocysts were used in this experiment.

The second experiment was conducted in a similar manner except that embryos were also grouped by blastocyst stage (non-expanded blastocysts vs expanded blastocysts) to avoid potential confounding effects of caspase activity with whether or not embryos were more developmentally advanced at the time of collection. Blastocyst stage embryos cultured in KSOM-BE2 at 38.5°C in an atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$ were removed from culture at d 7 after insemination and subjected to the group II caspase assay. Following determination of caspase activity, embryos were cultured individually in fresh 10-μl drops of KSOM-BE2 at 38.5°C in an atmosphere of 5% $CO_2$, 5% $O_2$, and 90% $N_2$. An additional group of d 7 blastocysts not subjected to the caspase assay and serving as controls were also cultured individually in fresh 10-μl drops of KSOM-BE2. Embryos were evaluated at 24 h intervals and stage of development was recorded for each embryo until d 10 post-insemination when the proportion of blastocysts that hatched was assessed. A total of 243 blastocysts were used in this experiment.

**Statistical Analysis**

Embryo hatching rates at d 10 post-insemination were analyzed using the CATMOD procedure of SAS. Independent variables included caspase activity and blastocyst stage (Exp. 2 only). The mathematical model for Exp. 1 included the main effect of caspase activity while the model for Exp. 2 included caspase activity, blastocyst stage, and the interaction of caspase activity x blastocyst stage. Level of significance was set at $P < 0.05$. The CATMOD procedure was also used to determine effect of embryo stage (non-expanded vs expanded) on caspase activity (low, medium, or high).

## Results

Representative images of embryos exhibiting low, medium, and high group II caspase activity are depicted in Figure 7-1. Differences in caspase activity are readily apparent through visual observation as low group II caspase embryos have a faint green fluorescent intensity indicative of low caspase activity while the high group II caspase embryos have a more intense green expression indicative of high caspase activity.

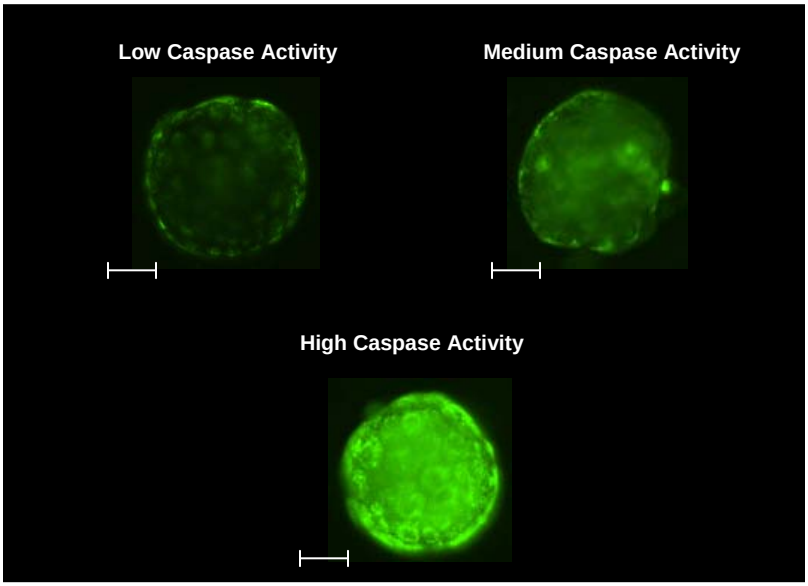


Figure 7-1. Representative examples of grade 1 bovine blastocysts classified as having low, medium, or high caspase expression based on group II caspase activity. Bars = 50 µM.

In Exp. 1, using grade 1 blastocysts collected at d 8 after insemination, 88 of 264 (33.3%) were classified as having low caspase activity, 123 of 264 (46.6%) had medium caspase activity, and 53 of 264 (20.1%) had high caspase activity. Caspase classification had a significant effect ($P < 0.05$) on the proportion of embryos that hatched from the zona pellucida by d 10 post-insemination (Table 7-1). Rate of hatching was highest for embryos with low caspase activity, intermediate for embryos with medium caspase activity, and lowest for embryos with high caspase activity.

Table 7-1. Hatching rates of grade 1 blastocysts as affected by group II caspase activity and embryo stage of development at the initiation of the caspase assay for quality grade 1 blastocysts selected on day 8 post-insemination.

| Caspase Activity | Hatching Rate (n) | Percent Hatched [a] |
|---|---|---|
| Low | 40/88 | 45.5 |
| Medium | 45/123 | 36.6 |
| High | 13/53 | 24.5 |

[a] Caspase classification affected the proportion of embryos that hatched from the zona pellucida by day 10 post-insemination ($P < 0.05$).

One possible explanation for the results in Exp. 1 was that embryos with low caspase activity were more likely to be expanded when selected at d 8 and therefore more likely to hatch from the zona pellucida within the subsequent 48 h of culture. Experiment 2 was then conducted with d 7 blastocysts to determine whether the caspase assay could distinguish embryo competence for hatching among a group of grade 1 blastocysts at the same stage of blastocyst development (Table 7-2). As expected, stage of development affected caspase classification ($P < 0.05$); a greater proportion of expanded blastocysts were classified as having low caspase activity (40/57; 70.2%) as compared to non-expanded blastocysts (52/110; 47.3%). Also, a greater ($P < 0.05$) proportion of expanded blastocysts hatched (50/57; 87.7%) as compared to non-expanded blastocysts (70/110; 63.6%). Caspase classification affected hatching rate ($P < 0.05$), as low caspase embryos were more likely to hatch from the zona pellucida than medium or high caspase embryos. There was no interaction between the main effects of group II caspase activity and blastocyst stage, indicating that caspase activity affected hatching rate for both expanded and non-expanded blastocysts.

Table 7-2. Hatching rates of grade 1 blastocysts as affected by group II caspase activity and embryo stage of development at the initiation of the caspase assay for quality grade 1 blastocysts selected on day 7 post-insemination.

| | Stage of Development at Time of Caspase Assay | | | |
|---|---|---|---|---|
| | Non-expanded Blastocysts [a] | | Expanded Blastocysts [a] | |
| Caspase Activity | Hatching Rate (n) | Percent Hatched [b] | Hatching Rate (n) | Percent Hatched [b] |
| Control | 31/47 | 66.0 | 26/29 | 89.7 |
| Low | 39/52 | 75.0 | 36/40 | 90.0 |
| Medium | 8/21 | 38.1 | 7/8 | 87.5 |
| High | 23/37 | 62.2 | 7/9 | 77.8 |

[a] A greater proportion of expanded blastocysts (40/57; 70.2%) were classified as having low caspase activity as compared to non-expanded blastocysts (52/110; 47.3%; $P < 0.05$).
[b] Caspase classification affected the proportion of embryos that hatched from the zona pellucida by day 10 post-insemination ($P < 0.05$).

Hatching rate in embryos subjected to the caspase assay was compared to embryos not assayed to determine the usefulness of the assay for identifying embryos with a high competence for subsequent development. While not significant, non-expanded blastocysts selected on the basis of low caspase activity tended to have higher hatching rates than embryos not subjected to the caspase assay (75.0 vs 66.0%). For expanded blastocysts, which had a higher hatching rate as compared to non-expanded blastocysts, there were no differences in hatching rate between those identified as having low caspase activity versus those not subjected to the caspase assay (90.0 vs 89.7%).

## Discussion

This study demonstrates that determination of group II caspase activity can be used as an indicator of continued embryonic development when performed at the blastocyst stage of development. Group II caspases include two caspases, caspase-3 and -7, that serve as execution caspases during apoptosis (Chang and Yang, 2000), as well as caspase-2, an upstream initiator of mitochondrial permeability (Troy and Shelanski, 2002). The rationale for using group II caspase enzymatic activity as a tool for predicting embryo survival was based on the assumption that an embryo undergoing extensive apoptosis has lower probability of continued development, that group II caspase activity is required for induced apoptosis in bovine embryos (Paula-Lopes and Hansen, 2002a) and that the assay is a rapid, simple, and non-invasive procedure.

The usefulness of the group II caspase assay for identifying embryos with superior competence for continued development is highlighted by the fact that all of the blastocysts used in this study were classified as grade 1 (i.e. had morphological characteristics of an excellent or good embryo according to the IETS, 1998) and are routinely transferred in commercial bovine ET systems. Even among these “excellent or

good embryos", however, there were clear differences in developmental competence as revealed by the caspase assay. Thus, in Exp. 1, in which blastocysts were produced in 5% $CO_2$ in humidified air (20.95% oxygen, v/v) and collected at d 8 after fertilization, only 24.5% of the blastocysts with high caspase activity hatched compared to 45.5% of the blastocysts with low caspase activity. In Exp. 2, embryos used were blastocysts produced in a low oxygen environment (5%, v/v) and collected at d 7 after insemination. Among the subset of d 7 blastocysts that were non-expanded, hatching rate was lower for embryos classified as having medium or high caspase activity as compared to those having low caspase activity. A similar trend was apparent for expanded blastocysts at d 7 although the fact that only a small proportion of these embryos were classified as having high caspase activity limits conclusions.

Blastocysts collected at d 7 have a higher capacity for establishing pregnancies when transferred into recipients than those collected at d 8 (Hasler et al., 1995). Consistent with this superior quality, a larger proportion of blastocysts were classified as having low caspase activity at d 7 (55.1%; Exp. 2) than d 8 (33.3%; Exp. 1). It is also possible that oxygen environment altered the degree of caspase activity.

In the present study, expanded blastocysts had a higher percentage of low caspase activity as compared to non-expanded blastocysts (70.2 vs 47.3%) and a lower percentage of high caspase activity as compared to non-expanded blastocysts (15.8 vs 33.6%). Consistent with this finding implying that expanded blastocysts have a higher potential for development is the finding that pregnancy rates in cattle were higher following transfer of expanded blastocysts as compared to transfer of early blastocyst or blastocyst stage embryos (Kubisch et al., 2004).

The caspase assay has practical implications for selecting embryos for transfer that appear to be morphologically good or excellent. It is likely that the pregnancy rate following the transfer of high-quality embryos could be improved by selection using the caspase assay. Thus, utilization of the group II caspase assay allows for increased discrimination between morphologically acceptable embryos in predicting their developmental potential. The assay might be best utilized for developmentally advanced embryos that are capable of undergoing apoptosis in contrast to early cleavage stage embryos when spontaneous apoptosis does not occur before embryonic genome activation (between the 4- and 8-cell stage for human embryos and the 8- and 16-cell stage for bovine embryos; reviewed by Fabian et al., 2005). It is likely that combining the group II caspase assay with morphological assessment of embryos could be of value in selecting the most viable embryo to be transferred and reduce the need for transfer of multiple embryos.

# CHAPTER 8
# GENERAL DISCUSSION

The long-term goal of this research is to develop strategies for improving fertility during heat stress. Embryo transfer has already been identified as a useful approach for increasing pregnancy rates of lactating dairy cows during heat stress (Putney et al., 1989a; Ambrose et al., 1999; Drost et al., 1999; Al-Katanani et al., 2002a). Treatment of embryos for transfer with cytoprotective molecules might further improve the pregnancy rate achieved with ET in the summer, as has been demonstrated with IGF-I (Block et al., 2003; Block and Hansen, 2006). Cytoprotective molecules might also be candidate molecules for improving fertility in inseminated cows if strategies are found to increase concentrations of the thermoprotectant in the oviduct and uterus. Another way to improve pregnancy rate to embryo transfer is to identify those embryos most likely to survive following transfer. Identification of markers of embryonic survival would also be useful for improving pregnancy rates of cows.

As stated in the Introduction, the overall goal of the dissertation has been to understand determinants of preimplantation bovine embryonic survival, particularly following heat shock. There were two main areas of focus – the role of IGF-I as a cytoprotective molecule, and the impact of apoptosis as a determinant of embryonic survival following heat shock as well as a predictor of embryonic potential for continued development. Results of the dissertation indicate that both areas of focus offer opportunities for increasing fertility. Insulin-like growth factor-I was shown to protect embryos from deleterious effects of heat shock. The role of apoptosis was shown to be

more complex. The anti-apoptotic actions of IGF-I does not appear to be the reason whereby IGF-I was able to prevent the reduction in blastocyst development caused by heat shock, yet the total amount of apoptosis in blastocysts, as determined by assay of group II caspase activity, was predictive of embryonic competence for continued development in vitro. Implications of these findings and suggestions for further study will be the topic of this general discussion.

A model that summarizes findings of pathways by which IGF-I can protect embryos from detrimental effects of heat shock is depicted in Figure 8-1. Results from Chapter 3 demonstrated that IGF-I was effective in blocking the effects of heat shock on reducing total cell number and development to the blastocyst stage and increasing the induction of apoptosis in d 5 bovine embryos. This cytoprotective action of IGF-I is consistent with other studies in preimplantation embryos, as IGF-I blocked apoptosis caused by ultraviolet irradiation (Herrler et al., 1998), camptothecin and actinomycin D (Fabian et al., 2004), and TNF-$\alpha$ (Byrne et al., 2002a), as well as the detrimental effect of hydrogen peroxide on embryonic development (Kurzawa et al., 2002). As shown in Chapter 5, treatment with IGF-I immediately before heat shock was sufficient to induce thermotolerance. Therefore, IGF-I can protect the embryo from heat shock through relatively rapid changes in cell function. What was surprising was that the anti-apoptotic effects of IGF-I appear unrelated to the ability of IGF-I to block the reduction in blastocyst development caused by heat shock.

Experiments in Chapters 4 and 5 indicated that, in the same manner as for other cells (Nitta et al., 2004; Zheng and Quirion, 2004; Bridgewater et al., 2005; Scott et al., 2005; Zaka et al., 2005), IGF-I exerted its anti-apoptotic effects in embryos through the

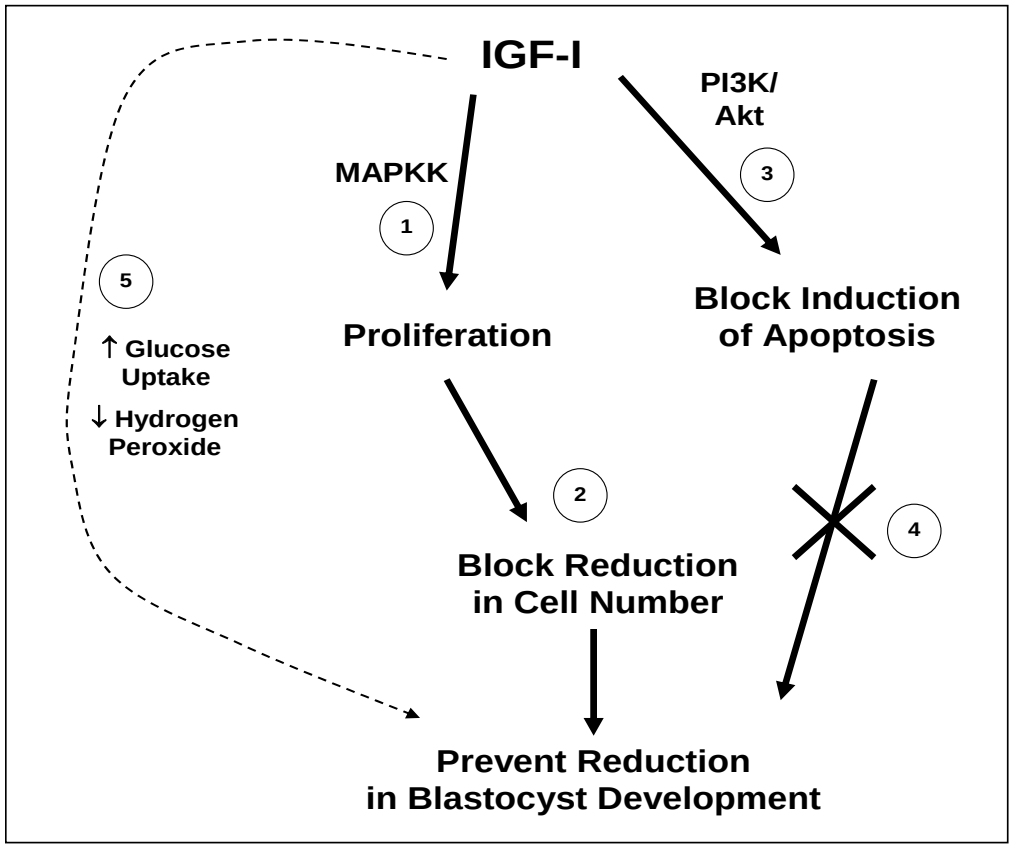


Figure 8-1. Summary of potential pathways whereby IGF-I confers cytoprotective effects during preimplantation bovine embryonic development. Solid arrows represent demonstrated pathways whereas dashed arrows represent speculative pathways. Treatment of embryos with IGF-I prevents the reduction in cell number and blastocyst development caused by heat shock. The proliferative actions of IGF-I requires the MAPKK pathway, as the MAPKK inhibitor PD 98059 prevented the actions of IGF-I to block the reduction in cell number (1). It is possible that the reason that IGF-I prevents the reduction in blastocyst development caused by heat shock is because it prevents the reduction in total cell number caused by heat shock (2). Insulin-like growth factor-I also blocks the increase in apoptosis caused by heat shock; this action requires the PI3K/Akt pathway because the anti-apoptotic actions of IGF-I were prevented by PI3K inhibitors (LY 294002 or wortmannin) and an inhibitor of Akt (HIMO; 3). The anti-apoptotic actions of IGF-I appear to be independent of the action of IGF-I to block the reduction in blastocyst development caused by heat shock (4). This is so because LY 294002 treatment did not reduce ability of IGF-I to block heat shock effects on blastocyst development. Mechanisms of IGF-I other than those that are pro-proliferation and anti-apoptotic could also be involved in the protective effects of IGF-I on development, including up-regulation of glucose uptake or reduction of effects of hydrogen peroxide or other free radicals caused by heat shock (5).

PI3K pathway. This was so because the PI3K inhibitors LY 294002 and wortmannin and an inhibitor of Akt (HIMO) blocked the effects of IGF-I on heat shock-induced apoptosis. However, IGF-I was still effective in preventing the decrease in blastocyst development of heat-shocked embryos when embryos were cultured with LY 294002. Furthermore, addition of z-DEVD-fmk, which inhibits activity of caspases-2, -3, and -7 and blocks induction of apoptosis caused by heat shock in bovine embryos (Paula-Lopes and Hansen, 2002b), did not mimic the effects of IGF-I on embryonic development and rather exacerbated the effects of heat shock in IGF-I-treated embryos. Additionally, IGF-I could prevent decreased blastocyst development of heat-shocked embryos through other mechanisms, such as up-regulation of glucose uptake (Pantaleon and Kaye, 1996) and reduction of effects of hydrogen peroxide caused by heat shock (Kurzawa et al., 2002).

There are two implications of the finding that the ability of IGF-I to block effects of heat shock on development is independent of apoptosis. The first relates to potential mechanisms by which IGF-I allows for development of heat-shocked embryos and the second relates to the importance of apoptosis as a determinant of embryonic survival. Each of these implications will be discussed in turn.

Experiments in Chapter 4 demonstrated that the action of IGF-I to block the reduction in total cell number required signaling through the MAPKK pathway because the MAPKK inhibitor PD 98059 prevented the ability of IGF-I to block the decrease in total cell number. Signaling through this pathway has been shown to increase cell proliferation in other cell types (reviewed by Jones and Clemmons, 1995; Rubinfeld and Seger, 2005), and in some instances, block the induction of apoptosis (Párrizas et al., 1997). One possibility, therefore, is that IGF-I prevented effects of heat shock on

reduced development by increasing blastomere proliferation. However, there was no effect of IGF-I on the proportion of blastomeres that stained positive for proliferating cell nuclear antigen in bovine embryos (Makarevich and Markkula, 2002). Thus, the role of proliferative pathways in conferring IGF-I induced thermotolerance requires additional study. It is possible that IGF-I prevents the reduction in proliferating cell nuclear antigen caused by heat shock as compared to control embryos. Signaling through the MAPKK pathway was needed for blastocyst development, as PD 98059 prevented blastocyst development at d 6 (data not shown). As mentioned earlier, signaling through the MAPKK pathway can prevent apoptosis, so it is possible that IGF-I-treated embryos cultured with the PI3K inhibitor LY 294002 developed effectively to blastocysts following heat shock due to proliferative and possibly anti-apoptotic actions through the MAPKK pathway. Moreover, the ability of IGF-I to block the reduction in total cell number caused by heat shock could have prevented the reduction in blastocyst development caused by heat shock. In addition, IGF-I could act in other ways to prevent the reduction in blastocyst development caused by heat shock, for example, by up-regulation of glucose uptake (Pantaleon and Kaye, 1996), preventing free radical damage (Kurzawa et al., 2002), or enhanced DNA repair (Yang et al., 2005), and studies are warranted to examine if these actions are mediated by IGF-I. Thus, IGF-I likely induces a multiplicity of cellular changes including those that maintain embryonic competence for development following heat shock.

The second implication that anti-apoptotic effects of IGF-I are not the cause of increased resistance to heat shock with regard to blastocyst development lies with the understanding of the role of apoptosis in preimplantation embryonic development. Heat

shock in the bovine oocyte disrupts the capacity of the oocyte to become a blastocyst after fertilization and increases caspase activity and the proportion of TUNEL-positive oocytes (Roth and Hansen, 2004a, 2005). The 2- and 4-cell bovine embryos are also sensitive to heat shock, but embryos at these early developmental stages are incapable of undergoing apoptosis in response to a variety of pro-apoptotic stimuli, including heat shock (Paula-Lopes and Hansen, 2002b), arsenic (Krininger et al., 2002), and TNF-α (Soto et al., 2003). As embryonic development progresses, the embryo becomes capable of undergoing apoptosis and this action seems to be a survival mechanism for the embryo. For example, in Chapter 4, z-DEVD-fmk reduced blastocyst development of IGF-I-treated embryos, indicating that limited apoptosis experienced by the embryo might be beneficial for continued development following heat shock. Perhaps the degree of apoptosis was so low for IGF-I-treated embryos cultured with z-DEVD-fmk that the inability of the embryo to remove those damaged blastomeres compromised continued development to the blastocyst stage following heat shock. Similar evidence for beneficial effects of limited apoptosis on continued development of d 4 heat-shocked bovine embryos has been shown previously (Paula-Lopes and Hansen, 2002a). By the blastocyst stage, however, the amount of group II caspase activity in blastocysts was predictive of continued development, with embryos with higher caspase activity being less likely to undergo hatching. Therefore, increased potential for apoptosis at the blastocyst stage, based on caspase activity, was detrimental to continued development. These studies depict apoptosis as being beneficial at later stages of embryonic development (after d 4), but only to a limited degree as embryos with increased caspase activity had reduced competency for continued development.

Experiments reported here point the way to possible interventions for enhancing embryonic development during heat stress. Results imply that embryos cultured with IGF-I would be more likely to establish pregnancy during heat stress than control embryos because of increased thermotolerance. Indeed, a study done simultaneously with studies for this dissertation revealed that culture of embryos with IGF-I could increase the probability for pregnancy establishment after transfer in the summer, but not the winter (Block and Hansen, 2006).

One way to increase IGF-I concentrations in cattle is through administration of bST (Cohick et al., 1989; Newbold et al., 1997; Bilby et al., 1999). The experiment in Chapter 6 was conducted to determine if treatment of lactating Holstein cows with bST during heat stress could improve pregnancy rates as compared to lactating Holstein cows not treated with bST. No improvement was seen. Rather, pregnancy rates at first- and second-service were similar for bST-treated and control cows. Nonetheless, the results are interpreted as indicating fertility-promoting effects, as without such effects one would have expected fertility to be lower in bST-treated cows because bST increased milk yield and body temperature and reduced BCS. Effects of bST include general fertility-promoting effects of this hormone (Morales-Roura et al., 2001; Moreira et al., 2000b, 2001; Santos et al., 2004), as well as the actions of bST and IGF-I to increase cellular resistance to elevated temperature (Elvinger et al., 1991a; Chapters 3 to 5). As a practical concern, results from Chapter 6 support the use of bST during heat stress because bST treatment increased milk yield without compromising first- or second-service TAI pregnancy rates.

Another strategy to increase IGF-I to enhance fertility during the summer could be to treat cows with a single injection of bST at insemination, which has been shown to be effective in promoting fertility of lactating dairy cows, but not when applied exclusively during the summer (Moreira et al., 2000b, 2001; Morales-Roura et al., 2001; Santos et al., 2004b). Additionally, a single injection of bST at insemination would avoid anti-fertility effects associated with increased milk yield and body temperature. Manipulation of diet may be another way to regulate IGF-I secretion to improve embryo survival. Energy status of the lactating cow plays a role in responsiveness to bST because the ST/IGF-I axis is uncoupled when in a negative energy balance and increases in concentrations of IGF-I will not occur (Kobayashi et al., 1999; Radcliff et al., 2003; Kim et al., 2004b).

Currently, it appears that use of TET using IGF-I-treated embryos might be the best strategy to increase pregnancy rates of lactating dairy cows during the summer as noted earlier. This strategy offers several advantages, including transfer of an embryo capable of undergoing limited apoptosis that has been made thermotolerant by actions of IGF-I, and use of an ovulation synchronization scheme that avoids the need for estrus detection. The other potentially practical outcome of this dissertation was the finding that group II caspase activity might be useful as a predictor of embryonic potential for further development. This result implies that more extensive apoptosis encountered by the early embryo could be associated with decreased development. As discussed earlier, the ability of the embryo to undergo limited apoptosis seems to be beneficial for development whereas excessive apoptosis might compromise development. Activation of caspases confers death to a cell and examining embryos based on caspase expression may be a useful strategy to distinguish between morphologically normal embryos with varying

potential for continued development. Results of Chapter 7 provide preliminary evidence that caspase activity can be used as a predictor of embryonic survival. The potential usefulness of the assay is based on the fact that it is simple, non-invasive, and can be conducted quickly. Further studies should be conducted to determine whether use of the procedure to select embryos increases pregnancy rates and to verify that the offspring are unaffected by this procedure.

Taken together, research for this dissertation provides a better understanding of the importance of apoptosis during embryonic development, its regulation by the cytoprotective molecule IGF-I, and potential for continued embryonic development based on caspase activity. Not all of the hypotheses regarding apoptosis and embryonic development were supported. In particular, the ability of IGF-I to prevent the reduction in blastocyst development caused by heat shock was independent of its anti-apoptotic actions. Thus, studies to gain additional insight into cytoprotective actions of IGF-I are warranted, including determining whether IGF-I causes up-regulation of glucose uptake or prevention of free radical damage caused by heat shock. Results support the concept that there is a changing role of apoptosis during embryonic development. Limited apoptosis to remove the most damaged cells seems beneficial for continued development at d 5 but extensive apoptosis has negative consequences for continued development at the blastocyst stage. Therefore, apoptosis can be both a positive and negative regulator of continued embryonic development dependent upon embryonic developmental stage.

As an animal scientist, it was important to relate in vitro findings to practical significance, especially since heat stress compromises fertility of lactating dairy cows. Although bST did not increase pregnancy rates of lactating cows during the summer, the

findings are still of practical significance because the increased stress caused by bST-induced increases in milk yield and body temperature might have been offset by increased protection of the embryo by increased concentrations of IGF-I. An additional study to determine if a single bST injection at insemination can increase pregnancy rates of lactating cows during the summer is needed. Treatment of embryos with IGF-I during culture and selection of embryos using caspase activity (after additional studies) in a TET program may provide the best strategy to improve fertility of lactating cows during the summer. Overall, the research points to potential therapies for protecting embryos from heat shock by IGF-I treatment and may lead to additional strategies to provide practical solutions to improving fertility of lactating dairy cows during the summer.

# APPENDIX
# FACTORS ASSOCIATED WITH EARLY AND MID-TO-LATE FETAL LOSS IN LACTATING AND NON-LACTATING HOLSTEIN CATTLE IN A HOT CLIMATE

## Introduction

One contributor to infertility in cattle is pregnancy loss during the fetal period that extends from 42 d of gestation until parturition (Committee on Bovine Reproductive Nomenclature, 1972). Based on a compilation of studies by Santos et al. (2004c), estimation of the incidence of fetal loss ranged from 8.3 to 24.0% for lactating dairy cows and 1.5 to 10.2% for dairy heifers. Identification of the factors contributing to fetal mortality could be useful for identifying strategies to reduce these losses. One possible factor is mastitis, which has been associated with loss of pregnancies during the embryonic (Chebel et al., 2004) and fetal period (Risco et al., 1999; Santos et al., 2004a). Other potential causes of fetal loss are the physiological changes associated with high milk yield. However, a correlation between milk yield and fetal loss in dairy cattle has not always been observed (López-Gatius et al., 2002; Silke et al., 2002). Similarly, there was no relationship between pregnancy loss during the embryonic period and milk yield (Chebel et al., 2004). Heat stress might also contribute to fetal loss because of its actions to reduce placental function and fetal growth (Collier et al., 1982); such a link has not been described.

Only a limited number of studies have evaluated possible causes of fetal mortality in dairy cattle. The particular importance of factors influencing fetal loss could depend upon environment and little information exists for dairy cattle raised in hot climates. The

objective of the present study was to characterize factors associated with rates of early and mid-to-late fetal loss in a herd of dairy cattle maintained in north Florida. It was hypothesized that lactating females would have a higher incidence of early and mid-to-late fetal loss than non-lactating heifers and that early and mid-to-late fetal loss would be higher for females with high milk production, high somatic cell count score (SCCS) at breeding, and inseminated during the hotter months of the year. We also determined whether the number of times a female is inseminated (a measure of the female's fertility; Chebel et al., 2004) or the interval from calving to conception (days open) is related to the frequency of fetal loss.

## Materials and Methods

### Animals

Breeding records for lactating Holstein females (both first parity and multiparous cows) and non-lactating Holstein heifers were obtained from the University of Florida Dairy Research Unit at Hague, FL (29º46'N 82º25'W), for the period of April 2000 through March 2003 using the PCDART Herd Manager software (Version 7; Dairy Records Management Systems, Raleigh, NC). Lactating females were administered bST (Posilac) according to manufacturer's recommendations, milked twice daily, and maintained in free-stall barns equipped with either sprinklers and fans or misters and fans. Non-lactating heifers were maintained in a variety of facilities with either shade trees or free-standing shade structures covered with shade cloth.

The voluntary waiting period from calving to first insemination was approximately 80 d. For the first insemination after calving, TAI was performed using the Ovsynch protocol (Pursley et al., 1997) initiated at approximately d 70 after calving and after cows were presynchronized with two injections of $PGF_{2\alpha}$ 14 d apart at approximately 42 and

56 d after calving. Subsequent inseminations for lactating females were conducted at either standing estrus (spontaneous, after injection of $PGF_{2\alpha}$ or following the SelectSynch procedure (Stevenson et al., 2000) or by TAI using the Ovsynch procedure. Non-lactating heifers were inseminated beginning at an approximate target weight of 340 kg. Inseminations were performed at either standing estrus (spontaneous) or after injection of $PGF_{2\alpha}$.

**Determination of Fetal Loss**

Pregnancy diagnosis was performed between d 40 and 50 after insemination using palpation per rectum, and females determined to be pregnant were reconfirmed for pregnancy status by palpation per rectum between d 70 and 80 after insemination. Females that were pregnant between d 40 and 50, but were not pregnant between d 70 and 80, were classified as having undergone early fetal loss. Those females determined to be pregnant between d 40 and 50 and again between d 70 and 80, but whose calving date occurred four wk or more before their expected calving date (premature parturition), were classified as having undergone mid-to-late fetal loss. The average gestation length for all animals that did not experience fetal loss was 276 d, with a range of 253 to 292 d.

**Description of Potential Factors Associated with Early and Mid-to-Late Fetal Loss for Lactating Females**

The following factors were analyzed for associations with early and mid-to-late fetal loss: SCCS at insemination, average milk yield at the time of insemination (milk yield), lactation number at breeding in which pregnancy was established (lactation number), number of days between the previous calving and the date of breeding that resulted in pregnancy (days open), number of times inseminated until pregnancy was established (times inseminated), and season of the year in which the animal was bred

(season). Each cow's milk yield and SCCS were recorded during a monthly test date. Data corresponding to the test date closest to the breeding date at which the female was determined to be pregnant was used for the determination of effects of milk yield and SCCS at the time of breeding. If the breeding date occurred 7 to 21 d between two test dates, the value used for data analysis for milk yield and SCCS at breeding was an average of the test dates before and after the breeding date.

Each of the above-mentioned factors was categorized as follows. The SCCS was classified as being ≤2.5, 2.6 to 5.0, or >5.0 at the time of breeding. The SCCS is a score calculated by the Dairy Herd Improvement Association based upon the raw somatic cell count (SCC; a count of the white blood cells (WBC) in a milliliter of milk). A SCCS between 0 and 2.5 equates to a SCC range of ≤75,000 WBC/ml of milk, a SCCS between 2.5 and 5.0 equates to a SCC range of >75,000 to ≤400,000 WBC/ml of milk, and a SCCS >5.0 equates to a SCC range >400,000 WBC/ml of milk. Milk yield on the test date closest to the female's breeding date was categorized as <22.7 kg/d, 22.8 to 34.1 kg/d, 34.2 to 45.5 kg/d, or ≥45.6 kg/d at breeding. Lactation number was organized into first lactation, second lactation, or third or greater lactation. Days open was categorized as <75, 76 to 150, 151 to 300, or >300 d. Categories for number of times inseminated until the establishment of pregnancy were one insemination, two or three inseminations, or four or more inseminations. Season of the year of breeding was classified as cool months (October through March) or hot months (April through September).

**Description of Potential Factors Associated with Early and Mid-to-Late Fetal Loss for Non-lactating Heifers**

Factors analyzed were the number of times inseminated until pregnancy was established (times inseminated), the season of the year in which the heifer was

inseminated (season), and the age of the heifer at the breeding that resulted in an established pregnancy (age at pregnancy). Data for number of times inseminated and season of the year of breeding were categorized as described above for lactating females. Age of heifer at establishment of pregnancy was characterized as ≤15 mo or >15 mo of age.

**Statistical Analysis**

Data were analyzed by logistic regression with the LOGISTIC procedure of SAS using a backward stepwise logistic model. Variables were continuously removed from the model by the Wald statistic criterion if the significance was greater than 0.20. The full statistical model included main effects and all interactions except for the days open x times inseminated because these terms are closely correlated; however, no significant interactions were found and the final model included only main effects. The Wald chi-square statistic was used to determine the significance of each main effect that remained in the reduced model. Effects and classes within a variable were considered to be significant at a level of $P \leq 0.05$ using the Wald test statistic for each main effect and by the GENMOD procedure of SAS for differences in classes within a variable. Reported *P*-values were obtained from the analysis using GENMOD. The adjusted odds ratio (AOR) estimates and the 95% Wald confidence intervals from logistic regression were obtained for each variable that remained in the final statistical model following the backward elimination.

The mathematical model for analysis of early and mid-to-late fetal loss for lactating females included the effects of SCCS, milk yield, lactation number, days open, number of times inseminated, and season. For non-lactating heifers, the mathematical model

included number of times inseminated, season, and age at pregnancy. Parity was the only effect in the model to determine differences in early and mid-to-late fetal loss between lactating females and non-lactating females.

## Results

### Differences in Incidence of Early and Mid-to-Late Fetal Loss between Lactating Females versus Non-lactating Heifers

Lactating females had a greater incidence of early and mid-to-late fetal loss than non-lactating heifers (Table A-1). The proportion of animals with early fetal loss was 6.3% for lactating females and 3.6% for non-lactating heifers (AOR = 1.79; Wald $\chi^2$ statistic = 3.70; $P = 0.055$). The proportion of animals with mid-to-late fetal loss was 3.7% for lactating females and 1.1% for non-lactating heifers (AOR = 3.54; Wald $\chi^2$ statistic = 5.63; $P < 0.05$).

### Factors Associated with Early and Mid-to-Late Fetal Loss for Lactating Females

Descriptive statistics for all factors analyzed are shown in Table A-2. The average SCCS for lactating females was 3.02 with a range of 0.01 to 9.25. There was no relationship between SCCS and early fetal loss for lactating females. However, SCCS at insemination was associated with mid-to-late fetal loss (Wald $\chi^2$ statistic = 12.09, $P < 0.01$; Table A-3). Specifically, lactating females with a SCCS >5.0 at the time of insemination had a higher percentage of mid-to-late fetal loss (7.3%) than cows with a SCCS of <2.5 (2.1%) or 2.6 to 5.0 (4.1%; Table A-2).

Average number of days open among lactating females was 164 d, with a range of 53 to 659 d, and days open was associated with both early and mid-to-late fetal loss of lactating females (Wald $\chi^2$ statistic = 8.66 [$P < 0.05$] and 7.60 [$P = 0.055$], respectively; Table A-3]. In particular, lactating females with days open between 151 and 300 d had a

Table A-1. Descriptive statistics, odds ratio (OR) estimates, and 95% Wald confidence intervals (CI) for differences between lactating females versus non-lactating heifers in incidence of early and mid-to-late fetal loss.

| | Early Fetal Loss | | | | | Mid-to-Late Fetal Loss | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Proportion [a] | % | AOR | 95% Wald CI [b] | *P*-value [c] | Proportion [a] | % | AOR | 95% Wald CI [d] | *P*-value [c] |
| Lactating Females | 60/950 | 6.3 | 1.79 | 0.99, 3.25 | 0.055 | 33/890 | 3.7 | 3.54 | 1.25, 10.07 | 0.018 |
| Non-lactating Heifers | 14/386 | 3.6 | 1.00 | | | 4/372 | 1.1 | 1.00 | | |

[a] Data represent the number of females with fetal loss / total number of females.
[b] Wald chi-square statistic = 3.70 ($P = 0.055$).
[c] Derived from PROC GENMOD.
[d] Wald chi-square statistic = 5.63 ($P < 0.05$).

Table A-2. Descriptive statistics for the incidence of early and mid-to-late fetal loss for lactating females.

| | Early Fetal Loss | | Mid-to-Late Fetal Loss | |
|---|---|---|---|---|
| Factor | Proportion [a] | % | Proportion [a] | % |
| SCCS [b] | | | | |
| <2.5 | 23/447 | 5.1 | 9/424 | 2.1 |
| 2.6 to 5.0 | 23/339 | 6.8 | 13/316 | 4.1 |
| >5.0 | 14/164 | 8.5 | 11/150 | 7.3 |
| Milk Yield | | | | |
| <22.7 kg/d | 1/50 | 2.0 | 3/49 | 6.1 |
| 22.8 to 34.1 kg/d | 23/369 | 6.2 | 16/346 | 4.6 |
| 34.2 to 45.5 kg/d | 31/423 | 7.3 | 11/392 | 2.8 |
| >45.5 kg/d | 5/108 | 4.6 | 3/103 | 2.9 |
| Lactation | | | | |
| First | 20/369 | 5.4 | 14/349 | 4.0 |
| Second | 19/259 | 7.3 | 10/240 | 4.2 |
| ≥Third | 21/322 | 6.5 | 9/301 | 3.0 |
| Days Open | | | | |
| <75 d | 5/57 | 8.8 | 1/52 | 1.9 |
| 76 to 150 d | 22/503 | 4.4 | 15/481 | 3.1 |
| 151 to 300 d | 27/273 | 9.9 | 7/246 | 2.8 |
| >300 d | 6/117 | 5.1 | 10/111 | 9.0 |
| No. of Inseminations | | | | |
| One | 16/315 | 5.1 | 10/299 | 3.3 |
| Two to three | 23/336 | 6.8 | 8/313 | 2.6 |
| ≥Four | 21/299 | 7.0 | 15/278 | 5.4 |
| Season | | | | |
| Cool | 34/655 | 5.2 | 18/621 | 2.9 |
| Hot | 21/236 | 8.9 | 10/225 | 4.4 |

[a] Data represent the number of females with fetal loss / total number of females.
[b] Somatic cell count score.

Table A-3. Adjusted odds ratio (AOR) estimates and 95% Wald confidence interval (CI) for factors significantly associated with early and mid-to-late fetal loss: days open and somatic cell count score (SCCS).

| | Early Fetal Loss | | | Mid-to-Late Fetal Loss | | |
|---|---|---|---|---|---|---|
| | AOR | 95% Wald CI | *P*-value [e] | AOR | 95% Wald CI | *P*-value [e] |
| Days Open [a] | | | | | | |
| 76 to 150 d vs. <75 d | 1.90 | 0.63, 5.80 | 0.257 | | | |
| 76 to 150 d vs. 151 to 300 d | 2.44 | 1.33, 4.49 | 0.004 | | | |
| 76 to 150 d vs. >300 d | 1.28 | 0.50, 3.27 | 0.604 | | | |
| SCCS [b, c] | | | | | | |
| <2.5 vs. 2.6 to 5.0 | | | | 1.52 | 0.56, 4.13 | 0.336 |
| <2.5 vs. > 5.0 | | | | 5.43 | 1.97, 14.97 | 0.001 |
| Days Open [d] | | | | | | |
| <75 d vs. 76 to 150 d | | | | 1.54 | 0.19, 12.38 | 0.415 |
| <75 d vs. 151 to 300 d | | | | 1.18 | 0.14, 10.20 | 0.443 |
| <75 d vs. >300 d | | | | 4.41 | 0.53, 36.76 | 0.086 |

[a] Wald chi square statistic = 8.66 ($P < 0.05$).
[b] Somatic cell count score.
[c] Wald chi square statistic = 12.09 ($P < 0.01$).
[d] Wald chi square statistic = 7.60 ($P = 0.055$).
[e] Derived from PROC GENMOD.

higher incidence of early fetal loss (9.9%) than females with days open between 76 and 150 d (4.4%; Table A-2). For mid-to-late fetal loss, lactating females with days open over 300 d experienced a greater percentage of mid-to-late fetal loss (9.0%) as compared to lactating females in the other categories (1.9, 3.1, and 2.8%, respectively, for cows that were <75 d, 76 to 150 d, and 151 to 300 d open; Table A-2).

There were no associations of milk yield, lactation number, times inseminated, or season of breeding with the incidence of early or mid-to-late fetal loss. Average milk yield at insemination was 35.6 kg/d (range = 9.3 to 57.3 kg/d), lactation number was 2.2 (range = 1 to 8 lactations), and number of times inseminated was 3.1 (range = 1 to 16 inseminations).

**Factors Associated with Early and Mid-to-Late Fetal Loss for Non-lactating Heifers**

The descriptive statistics of each factor examined are depicted in Table A-4. There were no associations with number of times inseminated, season of breeding, or age at pregnancy on early or mid-to-late fetal loss.

## Discussion

The overall rate of fetal loss (i.e., the sum of early and mid-to-late fetal losses) in the present study was 10.0% for lactating females and 4.7% for non-lactating heifers. These values are comparable to values compiled from the literature by Santos et al. (2004c). In that review, overall rate of pregnancy loss was 10.7% for lactating females (based on a compilation of 10 studies) and 4.2% for dairy heifers (based on 5 studies). The present conclusion that lactating females are more likely to have increased fetal losses during both the early and mid-to-late periods as compared to non-lactating heifers is thus consistent with the literature.

Table A-4. Descriptive statistics for the incidence of early and mid-to-late fetal loss for non-lactating heifers.

| | Early Fetal Loss | | Mid-to-Late Fetal Loss | |
|---|---|---|---|---|
| Factor | Proportion [a] | % | Proportion [a] | % |
| No. of Inseminations | | | | |
| One | 9/177 | 5.1 | 2/168 | 1.2 |
| Two to three | 3/130 | 2.3 | 2/127 | 1.6 |
| ≥Four | 2/79 | 2.5 | 0/77 | 0.0 |
| Season | | | | |
| Cool | 7/231 | 3.0 | 3/224 | 1.3 |
| Hot | 7/154 | 4.5 | 1/147 | 0.7 |
| Age at Breeding | | | | |
| ≤15 mo | 12/325 | 3.7 | 4/313 | 1.3 |
| >15 mo | 2/61 | 3.3 | 0/59 | 0.0 |

[a] Data represent the number of females with fetal loss / total number of females.

Comparisons of lactating females with non-lactating heifers involved confounding effects of age, lactation status, housing, breeding protocols, and other factors. Of these factors, age and lactation status are the most likely to have caused the difference in fetal loss between cows and heifers. Uterine capacity may be lower in younger animals as indicated by lower birth weights for calves born from primiparous females than for cows at second or third parity (Kertz et al., 1997). Stress associated with lactation could also compromise fetal survival. Increased feed intake associated with lactation can elevate liver blood flow and metabolism of progesterone during pregnancy (Sangsritavong et al., 2002; Vasconcelos et al., 2003). Luteal progesterone production is needed for the maintenance of pregnancy for the majority of gestation (approximately 200 d; reviewed by Niswender et al., 2000), and the reduced circulating progesterone concentrations associated with increased feed intake during lactation might compromise fetal

development and lead to fetal loss. Recently, López-Gatius et al. (2004) demonstrated that 28-d administration of supplemental progesterone to pregnant, lactating cows beginning at d 36 to 42 reduced pregnancy losses at d 90 of pregnancy. Other hormonal changes associated with lactation (e.g., oxytocin release associated with milk ejection) could also conceivably compromise fetal survival.

Another possible cause of increased fetal loss associated with lactation is mastitis. In the present study, cows with high SCCS near the time of insemination were more likely to experience mid-to-late fetal losses. Similarly, cows diagnosed with mastitis during the first 45 d of gestation were 2.7 times more at risk of abortion within the following 90 d of gestation as compared to cows without mastitis (Risco et al., 1999). A separate study found that mastitis was associated with increased rate of abortions independent of the timing of the first clinical mastitis occurrence during lactation (Santos et al., 2004a). Given that cows that experience mastitis near insemination are also likely to experience mastitis later in lactation (Elvinger et al., 1991b; Sargeant et al., 1998), it is not clear at which stages of pregnancy physiological changes induced by mastitis could interfere with maintenance of pregnancy. Among the potential consequences of mastitis that could affect fetal survival are increased secretion of $PGF_{2\alpha}$ (Hockett et al., 2000) and an increase in circulating concentrations of cytokines, such as TNF-α (Perkins et al., 2002), which has been implicated in pregnancy loss in mice (Gorivodsky et al., 1998). However, changes in blood concentrations of cytokines are not always seen in mastitis (Lehtolainen et al., 2004).

As seen previously (López-Gatius et al., 2002; Silke et al., 2002), differences in milk yield were not associated with rate of fetal loss among lactating females. Such a

result would indicate that the difference in fetal losses between lactating cows and non-lactating heifers is more related to age than to lactation status, or that stresses of lactation causing fetal loss are not the result of the metabolic demands of lactation (which would increase with increasing milk yield), but rather other consequences of lactation independent of milk yield (such as hormonal changes and mastitis).

In the present study, increased days open at the time of pregnancy establishment was associated with increased fetal losses. The reason for this relationship is unclear because there was no relationship between numbers of times inseminated and fetal losses. In other studies, there were no relationships between calving to insemination interval or days open and pregnancy loss by d 84 to 90 of gestation (Silke et al., 2002; López-Gatius et al., 2004). One contribution to days open would be the occurrence of undocumented pregnancy losses, and it is possible that the relationship between days open and fetal loss seen here represents repeatability of fetal loss. The fact that number of times inseminated did not affect pregnancy loss would imply that the ability of a cow to establish pregnancy is not related to ability to maintain that pregnancy in the fetal period. The lack of a strong relationship between pregnancy establishment and fetal survival is not unexpected given the large number of environmental determinants of cow fertility.

It was hypothesized that heat stress during the course of gestation compromises fetal loss and that one result would be an effect of season of breeding on rates of fetal loss. This hypothesis was based on reductions in placental blood flow and size in sheep (Alexander et al., 1987; Bell et al., 1987), as well as reductions in placental hormone secretion and fetal growth caused by heat stress in cattle (Collier et al., 1982). In contrast to our hypothesis, there was no association of season of breeding with rates of fetal loss.

Interpretation of this result is made difficult by the fact that heat stress occurs throughout much of the year in Florida, and gestation in the bovine is 9 months. Thus, all females experienced heat stress at some point of gestation and the lack of a seasonal effect could reflect either a lack of effect of heat stress on fetal survival or effects of heat stress on pregnancy loss occurring at several stages of gestation.

In conclusion, lactating females were more likely to have increased early and mid-to-late fetal loss than non-lactating heifers. Increased fetal losses were associated with increased days open for lactating females, and the occurrence of mastitis was associated with mid-to-late fetal loss. Although season of breeding was not associated with fetal loss, it cannot be determined whether heat stress has no effect on fetal survival or that compromising effects of heat stress on pregnancy loss were obscured by occurrence of heat stress at various points during gestation in most females.

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# BIOGRAPHICAL SKETCH

Frank Dean Jousan was born on September 26, 1977, to Frank and Beverly Jousan Jr. The elder of two children, he graduated from Joaquin High School as valedictorian in 1996. He then enrolled at Texas A&M University where he received his Bachelor of Science degree in animal science with a science option in the spring of 2000. Dean then went to Virginia Polytechnic Institute and State University on a teaching assistantship. He received his Master of Science degree in the summer of 2002 in animal science with the emphasis of his research in Reproductive Physiology under the supervision of Dr. W.E. 'Bill' Beal. He then enrolled at the University of Florida on a teaching assistantship in the fall of 2002 in the laboratory of Dr. Peter J. Hansen. He is currently a candidate for the Doctor of Philosophy degree in the animal molecular and cellular biology program in the Department of Animal Sciences with an emphasis in reproductive physiology. On completion of his degree, Dean will pursue a career in animal agriculture.