INDIVIDUAL BULL VARIATIONS ON SPERM ACROSOME REACTION, SPERM-ZONA BINDING, IN-VITRO EMBRYO PRODUCTION, AND PREIMPLANTATION EMBRYO APOPTOSIS AND GENE EXPRESSION

by

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ABSTRACT

The overall objective of this study was to develop in-vitro tests to predict fertility of bulls in the field. The specific objectives were to investigate 1) the bull effect on sperm acrosome reaction, sperm-zona binding and in vitro embryo production (experiment 1), 2) the effect of sperm pre-incubation time and sperm concentration of bulls on in-vitro fertilization (experiment 2), 3) the bull effect on apoptosis and expression of Bcl-2, Bax, p53, interferon tau and heat shock protein-70 genes in bovine preimplantation embryos produced in-vitro (experiment 3), and 4) the correlation between the above in-vitro tests and the field fertility data. In the first experiment, pre-freeze motility, acrosome reaction at 0 h, increase in acrosome reaction at 4 h and sperm-zona binding were different ($p<0.05$) among bulls. Significant correlations were observed between individual sperm parameters. None of the in-vitro tests was correlated with non-return rates (field fertility data). In the second experiment, significant bull effects were observed on fertilization, when using short and long sperm pre-incubation time with normal and high sperm:oocyte ratio. When using normal sperm:oocyte ratio (25,000:1), the percent difference in normally fertilized zygotes between short and long sperm pre-incubation times showed high degree of correlation with non-return rates ($r = 0.90$; $p<0.05$) of the experimental bulls. In the third experiment, significant bull effects ($P<0.01$) were observed on cleavage and morula to blastocyst development rates; percentage of apoptotic, live and dead cells; and expression levels of heat shock protein 70 and interferon tau genes in morula to blastocyst stage embryos. The expression levels of Bax, Bcl-2 and p53 genes in morula to blastocyst stage embryos were not different among bulls. The field fertility measured by 60-90 day non-return rate was

highly correlated with relative abundance of Bcl-2 mRNA transcripts (r = -0.93) and the ratio of Bax to Bcl-2 gene expression (r = 0.84). The findings of this study conclude that variations exist among individual bulls in sperm acrosome reaction, sperm-zona binding and in-vitro embryo production, apoptosis, and interferon tau and heat shock protein 70 gene expression. Combinations of some of these sperm parameters may be potentially useful for the accurate prediction of bull fertility in the field.

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ABBREVIATIONS

A1	- A Bcl-2 family member, prolongs cell survival
AI	- Artificial insemination
AIF	- Apoptosis inducing factor
ANOVA	- Analysis of variance
Apaf-1	- Apoptosis protease activating factor-1
AR1	- Percentage of acrosome reacted sperm at 0 h incubation
Bak	- Bcl-2 antagonist
Bax	- Bcl-2 associated x protein
Bcl-2	- B-cell lymphoma 2
Bcl-W	- A member of the Bcl-2 family that promotes cell survival
Bcl-XL	- A long isoforms of Bcl-X that inhibits apoptosis
BH	- Bcl-2 homology domain
Bid	- BH-3 Interacting Domain
Bik	- Bcl-2 interacting killer
Bim	- A member of the Bcl-2 family that promotes apoptosis
BIR	- A baculovirus IAP repeat
BO	- Brackett and Oliphant
Bok	- Bcl-2 related ovarian killer
Boo	- Inhibits apoptosis
BSA	- Bovine serum albumin
cAMP	- Cyclic adenosine monophosphate
CARD	- Caspase activation and recruitment domain
Caspase	- Cysteinyl aspartic acid-protease
c-IAP-1	- Cellular inhibitor of apoptosis protein-1
c-IAP-2	- Cellular inhibitor of apoptosis protein-2
DAG	- Diacylglycerol
DED	- Death effector domain
DISC	- Death inducing signaling complex
DNTP	- Deoxyribonucleic acid triphosphate
DPBS	- Dulbecco's phosphate buffered saline
DR5	- Death receptor-5
ERK1	- Extracellular signal regulated kinase-1
ERK2	- Extracellular signal regulated kinase-2
FADD	- Fas associated death domain
FITC	- Fluorescein isothyocyanate
G3PDH	- Glyceraldehyde 3 phosphate dehydrogenase
HSP70	- Heat shock protein 70
IAP	- Inhibitor of apoptosis proteins
ICAD	- Inhibitor of caspase-activated DNAse
ICM	- Inner cell mass
IFNτ	- Interferon tau
InAR	- Increase in percentage of acrosome reacted sperm at 4 h incubation
IP3	- Inositol triphosphate

IVF	- In-vitro fertilization
NAIP	- Neuronal apoptosis inhibitory protein
p53	- A 53 kDa tumor suppressor gene product that promotes apoptosis
PAK2	- P21-activating kinase-2
PARP	- Poly (ADP ribose) polymerase
PIP2	- Phosphatidylinositol 4,5 biphosphate
PKA	- Protein kinase-A
PKC	- Protein kinase-C
PrFM	- Percentage of pre-freeze motile sperm
PSA	- Pisum sativum agglutinin
RT-PCR	- Reverse transcription polymerase chain reaction
SCS	- Superovulated cow serum
STAT	- Signal transducer and activator of transcription
TRAIL	- Tumor necrosis factor (TNF)-related apoptosis-inducing ligand
XIAP	- X-linked inhibitor of apoptosis protein
ZB	- Sperm-zona binding rate
ZP1	- Zona pellucida glycoprotein-1
ZP2	- Zona pellucida glycoprotein-2
ZP3	- Zona pellucida glycoprotein-3

FOREWORD

This thesis is based on the following manuscripts and conference proceedings

1. Giritharan G, Ramakrishnappa N, Balendran A, Cheng KM, Rajamahendran R. 2004. Development of In-Vitro Tests to Predict Fertility of Bulls. Can J Anim Sci. (Accepted; Chapter 3).

2. Giritharan G, Rajamahendran R. 2001. In vitro embryo production using ovaries removed from culled cows. Can J Anim Sci. 81(4), 589-591.

3. Giritharan G, Ramakrishnappa N, Balendran A, Rajamahendran R. 2004. Bull influence on apoptosis in bovine preimplantation embryos. 37th Annual Meeting of the Society for the Study of Reproduction, Aug 1-4, Vancouver, British Columbia, abstr. 528. (Chapter 5).

4. Giritharan G, Aali M, Ramakrishnappa N, Balendran A, Rajamahendran R. 2004. Prediction of fertility of bulls in the field using in vitro fertilization and in vitro embryo production tests. Proc 11th Int Cong Biotech Anim Reprod, September 16-18, Rapotin, Czech Republic. (Chapters 3&5).

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DEDICATION

Dedicated to My Late Father, Mother, Wife and Children
In Deep Appreciation of Their Love and Understanding

CHAPTER 1 – GENERAL INTRODUCTION

Artificial insemination (AI) is one of the most successful reproductive technologies developed to improve reproductive efficiency of farm animals, especially dairy cattle. Accurate evaluation of the fertility of bulls used for AI purposes is of utmost importance since a single ejaculate provides multiple insemination doses and contributes much influence on the reproductive potential of a herd. It is well known that it often takes years and great expense before a young bull is proven for its fertility and genetic merit through progeny testing programs. A major portion of the expenditure goes towards maintenance cost of bulls recruited to the progeny testing program, conducting extensive field insemination trials, non-return rate data collection, and payment of incentives to voluntary farmers. Apart from these, valuable space and materials are necessary for maintaining young bulls and storing frozen semen straws obtained from all the young bulls, which are under the progeny testing program. Therefore, a significant economic advantage for the cattle industry as well as to the AI industry is achievable, if simple laboratory tests are made available to predict fertility of young bulls recruited for progeny testing programs. Numerous laboratory methods have been developed over the years for the laboratory evaluation of semen quality and fertility. Some of these methods measured general characteristics of sperm such as viability, motility patterns, morphology, metabolism, and membrane and acrosome integrity. Despite the progress made in the laboratory evaluation of bull fertility using currently available methods, most of these methods lack the acceptable precision for accurate prediction of fertility in the field. This could possibly be due to the complex

nature of sperm:oocyte interaction and several unknown factors associated with the process of fertilization. The field oriented methods such as recording pregnancy data or measuring 60 day non-return rates are routinely used to evaluate the field fertility of processed semen from bulls. It has been shown that 25–35 % of the low field fertility is due to early embryonic mortality of unknown causes. In addition, it has been suggested that failure of breeding with low fertility bulls is due to fertilization failure, while that of high fertility bulls is due to embryonic death of unknown causes. One of the possible causes for fertilization failure is insemination at an inappropriate time. Although the estrus synchronization and timed insemination technique have been improved very much in the recent past, the ovulation time varies considerably with individual animals. Therefore, the successful completion of fertilization depends on the viability of sperm within the female reproductive tract until they interact with the ovum at an optimum time. Similarly, polyspermy is another contributing factor, which is believed to cause early embryonic mortality ranging from 5–10 %. Sperm concentration, oocyte quality, bull and sperm-oocyte incubation time have been shown to influence the polyspermic fertilization.

Apoptosis and mitosis are the major key events regulating early embryonic development. Proteins of Bcl-2 family, which include both pro- and anti-apoptotic proteins, are involved in the regulation of apoptosis. A balanced expression of pro- and anti-apoptotic proteins is necessary for successful development and growth of the embryo. It has been shown that chromosomes in some of the morphologically normal sperm undergo aberration during the spermatogenesis and these sperm could fertilize normally. In addition, the early embryonic mortality is very high during the first two

weeks of development when activation of embryonic genome and maternal recognition of pregnancy take place. These finding suggest that a defect in the initiation of the expression of developmentally essential genes or the irregular expression of these genes may predispose early embryonic mortality. The question of what extent the bull is responsible for the early embryonic death due to initiation of the expression of developmentally essential genes or the irregular expression of these genes is yet to be answered.

Studies on these bull related parameters might reveal new insights on accurate evaluation of bull fertility in vitro. Therefore, the objectives of the proposed study are to evaluate bull fertility based on a) in vitro sperm function tests such as sperm acrosome reaction, sperm-zona binding and in vitro fertilization; b) in vitro fertilizing ability of spermatozoa after alteration of sperm related conditions; and c) apoptosis and the expression of Bcl2, Bax, p53, interferon tau and heat shock protein-70 in in-vitro produced embryos.

CHAPTER 2 - BACKGROUND

The sperm is a specialized reproductive cell, which has its own unique characteristics, form and function. The sperm should undergo a reversible maturational process, capacitation, during the passage through the female reproductive tract and the acrosome reaction after binding with the zona pellucida of an oocyte to complete the fertilization process. Defects of sperm either in morphology of its head and tail or in functions such as motility, capacitation, sperm-zona binding and acrosome reaction might severely affect its fertilizing potential. In addition, the newly fertilized zygote undergoes a series of developmental changes including activation of the embryonic genome and expression of developmentally essential genes. Hence, this chapter briefly reviews sperm morphology, sperm capacitation, molecules involved in the sperm zona binding, sperm acrosome reaction, fertilization and early embryonic development. Expression of some developmentally essential genes during early embryonic development is briefly reviewed. Different laboratory tests developed over the past, based on the above sperm functions and the relationship of these tests with field fertility measured by 60-90 day non-return rate, will also be reviewed. The rationale, hypothesis and objectives of the study are presented at the end of this chapter.

2.1. MORPHOLOGY OF SPERMATOZOA

The sperm can be structurally divided into head and tail (Saacke and Almquist, 1964a, 1964b). The bovine sperm is about 68-74 microns long in which the head is about 8-10 microns, the neck 0.3-1.5 microns, the mid-piece 2-4 microns, the principal-piece 45-50 microns and the end-piece 2-4 microns (Sullivan, 1978; Cummins and

Woodall, 1985). The head is a flattened oval shaped structure loosely enveloped by a lipid bilayered cell membrane of the entire cell. The widest portion of the head is about 4-5 microns, and at the base, it is about 1.5-1.7 microns. The thickness of the head varies across the length; 0.5 micron at the base, 0.3 micron at the middle and it tapers towards the frontal edge (Sullivan, 1978; Gravance et al., 1996). However, the sperm head dimensions may vary with staining and measuring procedures (Foote, 2003). The head of the sperm contains an acrosome cap and a large nucleus, which occupies most of the available space and is a densely packed crystalline aggregate of deoxyribonucleic acids of the chromosomes and nucleoprotein complexes called chromatin (Wilkins, 1956). The sperm acrosome is a sac-like structure surrounded by the inner and outer acrosomal membranes, which covers 60% of the anterior portion of the nucleus. A homogenous dense ground substance is present in the acrosome of a normal sperm cell. The acrosome folds back at the anterior and the lateral edges to form the apical ridge (Blom and Birch-Andersen, 1961; Sullivan, 1978). A nuclear cap covers the rest of the anterior portion and the posterior portion. The junction of anterior and posterior portions forms the nuclear ring. The mammalian sperm nuclei seem to be composed of parallel stacks of lammellar sheets oriented to the sperm's long axis and the sperm chromatin behaves as a cholesteric liquid in which DNA strands are packaged into parallel planes of linear arrays (Koheler, 1970; Plattner, 1971; Sipski and Wagner, 1977; Koheler et al., 1983; Livolant, 1983). Nodular organization of sperm chromatin has also been shown by recent studies (Allen et al., 1993; Haaf and Ward, 1995; Allen et al., 1996; Fuentes-Mascorro et al., 2000). The sperm nucleus has a uniform thickness of 0.2-0.3 micron, is covered by a porous double membrane, and tapers towards the

frontal edge and is thickened at the base, where the neck is inserted into a concave recess, the implantation fossa. The inner surface of the implantation fossa is lined either partially or completely by a basal plate, which appears to be the continuation of the nuclear membrane and is made up of two membranes. There are two semicircular or triangular basal knobs present on each side of the implantation fossa between the point of attachment of the post nuclear cup and the basal plate (Saacke and Almquist, 1964a; Salisbury and van Dongen, 1964; Blom and Birch-Andersen, 1965; Bahr and Engler, 1970; Sullivan, 1978). The neck connects the head with the mid-piece of the tail. The neck is formed by two implantation plates containing large laminated fibers, which originate by merger of completely independent peripheral coarse fibers. The captulum is a common base formed by merger of two implantation plates and the anterior edge of the captulum fits into the implantation fossa (Blom and Birch-Andersen, 1960). The proximal centriole is situated in the anterior portion of the neck and within the captulum (Blom and Birch-Andersen, 1965).

The tail is divided into the mid-piece, principal-piece and end-piece. The diameter of the mid-piece is about 0.64-0.85 micron, the principal-piece is about 0.5 micron and the principal piece tapers distally to 0.25 micron (Saacke and Almquist, 1964b; Salisbury and van Dongen, 1964; Bahr and Engler, 1970; Sullivan, 1978). The contractile elements for the motility of the tail are present in the centrally located longitudinally coursing axial fiber bundle. The axial fiber bundle is composed of an outer peripheral ring of nine coarse fibers, an inner ring of nine doublets of fine fibers and a pair of centrally located fibers. This arrangement of fibers is commonly described as 9+9+2 fiber pattern (Blom and Birch-Andersen, 1960; Saacke and Almquist, 1964b).

The mid-piece is surrounded by two layers of a single chain right handed mitochondrial helix extending from the neck to Jensen's ring, which separates mid-piece from principle piece and surrounds the axial fiber of the tail (Blom and Birch-Andersen, 1960; Saacke and Almquist, 1964b; Sullivan, 1978). The principal-piece is surrounded by a thin fibrous sheath of closely spaced circular rings. The peripheral coarse fibers in the outer ring of the axial fiber bundle become smaller as they pass posteriorly and eventually disappear at different levels of the tail (Saacke and Almquist, 1964b; Sullivan, 1978). The end-piece of the tail lacks the fibrous sheath and is only surrounded by cell membrane. Only a 9+2 fiber pattern exists at the anterior portion of the end-piece and the doublet character of the peripheral fibers is lost posteriorly (Wu and Newstead, 1966).

2.2. CAPACITATION OF SPERMATOZOA

The mammalian sperm is unable to fertilize an egg immediately after ejaculation and requires a period of incubation either in the female genital tract or in an in-vitro capacitation medium to acquire fertilizing capacity (Yanagimachi, 1994; Breitbart and Naor, 1999; Eisenbach, 1999). Removal of decapacitating factors primarily cholesterol and other components of the seminal plasma from the sperm surface in the female reproductive tract or in the in-vitro capacitation medium triggers many sequential biochemical and physiological changes by which sperm gains the ability to undergo the acrosome reaction and fertilize a mature oocyte. These changes are collectively called as sperm capacitation (Cross, 1996; Cross and Mahasreshti, 1997; Eisenbach, 1999; Abou-Haila and Tulsiani, 2000; Baldi et al., 2002; Visconti et al., 2002; Hunter and

Rodriguez-Martinez, 2004). During capacitation, several intracellular changes are known to occur including increases in cholesterol efflux, membrane fluidity, intracellular pH, Ca^{2+} and cAMP, protein tyrosine phosphorylation and changes in motility pattern (Suarez, 1996; Breitbart and Naor, 1999; Eisenbach, 1999). The lipid redistribution in the plasma membrane or membrane destabilization during the capacitation process results in either exposure or hiding of specific receptors and these changes enhance the binding ability of the sperm to its receptors on the ovum (Eisenbach, 1999). Hence, only completely capacitated sperm can undergo a complete acrosome reaction and the partially capacitated sperm can only undergo a partial acrosome reaction (Jaiswal et al., 1998; 1999).

Although the biological phenomenon of sperm capacitation has been known for several years, the molecular basis of this process still remains obscure. However, the capacitation process seems to be tightly controlled by number of both intrinsic and extrinsic regulators. The observation that capacitation can occur in-vitro spontaneously in a defined medium without the addition of biological fluids suggests that this process is intrinsically modulated by the sperm itself, such that these cells are preprogrammed to undergo capacitation when they are incubated in the appropriate medium. The regulation of capacitation lies more in the de-repression of inhibitory modulators of capacitation through the removal of decapacitating factors than in the stimulation of this process (Yanagimachi, 1994; Visconti and Kopf, 1998). There are several media constituents and pathways involved in the enhancement and regulation of the capacitation process.

The serum albumin acts as a receiving agent for cholesterol and other sterols in the sperm plasma membrane. The removal of cholesterol as well as other sterols is suggested to be responsible for the membrane fluidity changes associated with the capacitation process (Visconti and Kopf, 1998). Cholesterol removal seems to be the primary action of serum albumin in the sperm capacitation process since the capacitation process can be initiated by replacement of albumin with cholesterol-binding proteins, such as high-density lipoproteins or lipid transfer proteins present in follicular or oviductal fluids in in-vitro sperm capacitation medium (Therien and Manjunath, 1996; de Lamirande et al., 1997).

Although, the important roles of Ca^{2+} in sperm signal transduction and capacitation have been documented in several studies, the role of Ca^{2+} in the initiation or regulation of capacitation is currently controversial (DasGupta et al., 1993; Yanagimachi, 1994; Visconti et al., 1995b). The initiation and regulation of capacitation by Ca^{2+} occur via different targets, some of which are involved with cAMP metabolism. Since in sperm Ca^{2+}/calmodulin can activate both the synthesis of cAMP by adenylyl cyclase (Gross et al., 1987), as well as degradation by cAMP cyclic nucleotide phosphodiesterase (Wasco and Orr, 1984), it is not surprising that Ca^{2+} has both positive and negative actions on capacitation and related signaling events. In this respect, Ca^{2+} has a positive effect on mouse sperm by inducing capacitation-associated changes in protein tyrosine phosphorylation (Visconti et al., 1995b). In contrast, Ca^{2+} has been demonstrated to inhibit protein tyrosine phosphorylation in human sperm during the first 2 h of in vitro capacitation (Carrera et al., 1996; Luconi et al., 1998). An increase in intracellular sperm Ca^{2+} during capacitation has been described by some investigators,

whereas others have shown that no changes in Ca^{2+} levels occur during this maturational event (Yanagimachi, 1994). This ambiguity could be due, in part, to the well-demonstrated action of Ca^{2+} on the acrosome reaction and to the inherent difficulties in differentiating between these events. However, the action of Ca^{2+} at the level of effector enzymes involved in sperm signal transduction suggests that this divalent cation is likely to play an important role in capacitation.

The influence of HCO_3^- in the capacitation process has been demonstrated in numerous studies (Lee and Storey, 1986; Neill and Olds-Clarke, 1987; Boatman and Robbins, 1991; Shi and Roldan, 1995; Visconti et al., 1995a). Although very little information is available on the mechanisms of HCO_3^- transport in sperm, the ability of the inhibitors of anion transporters to inhibit the actions of HCO_3^- on various sperm functions suggests that sperm contain functional anion transporters (Okamura et al., 1988; Visconti et al., 1990, 1999; Spira and Breitbart, 1992). The intracellular pH increase during capacitation may be associated with the transmembrane movement of HCO_3^- into sperm (Uguz et al., 1994; Zeng et al., 1996). Because the synthesis of cAMP by mammalian sperm adenylyl cyclase is highly stimulated by HCO_3^-, the action of HCO_3^- could be the regulation of sperm cAMP metabolism (Okamura et al., 1985; Garty and Salomon, 1987; Visconti et al., 1990; Chen et al., 2000). Concurrent increase of intracellular cAMP, HCO_3^- and Ca^{2+} during sperm capacitation implicates an active role of HCO_3^- in the initiation or regulation of adenylyl cyclase activity and the cAMP signaling pathway in sperm capacitation.

Bovine sperm capacitation in vitro can be accomplished in chemically defined medium containing bovine serum albumin, energy substrates, and heparin or oviductal

fluid (Parrish et al., 1988; Miller and Ax, 1990; Miller et al., 1990; Therien et al., 1995). The active capacitating agent in the oviductal fluid is thought to be a heparin-like glycosaminoglycan. Although the mechanism of action of heparin or heparin-like glycosaminoglycans in the process of sperm capacitation is not understood well in the past, it is suggested that the glycosaminoglycans may promote capacitation by binding to and removing sperm plasma membrane adsorbed capacitation inhibiting seminal plasma proteins (Miller et al., 1990; Therien et al., 1995). Interestingly, heparin also increases cAMP synthesis (Parrish et al., 1994), elevates pH, and regulates the capacitation-associated changes in protein tyrosine phosphorylation (Galantino-Homer et al., 1997).

Involvement of several protein kinase mediated signal transduction pathways have been suggested in the sperm capacitation process (Baldi et al., 2002). Activation of protein kinase A (PKA) and protein kinase C (PKC) have been shown during the capacitation process in recent studies (Naor and Breitbart, 1997). However, the PKC activity is lower in sperm during capacitation than its activity in the somatic cells (Bonaccorsi et al., 1998). Extracellular signal regulated kinases (ERK1 and ERK2) and Ras proto-oncoprotein mediated signaling pathways are also active during the process of capacitation (Feng et al., 1998; Luconi et al., 1998; Naz 1998). Reactive oxygen species mediated signaling pathways also suggested to be involved in the capacitation process since direct addition of hydrogen peroxide at low doses promotes capacitation and the addition of catalase prevents the effect of hydrogen peroxide (Griveau et al., 1995; Aitken et al., 1998). The production of superoxides during the capacitation

process and during activation of adenylate cyclase and protein tyrosine phosphorylation have also been shown in the past (Aitken et al., 1998; Leclerc et al., 1996).

2.3. SPERM–ZONA BINDING

It is generally accepted that the interaction between sperm and egg is a carbohydrate-mediated receptor–ligand binding site (Tulsiani et al., 1997; Brewis and Wong, 1999; Wassarman et al., 1999; Topfer-Petersen et al., 2000; Primakoff and Myles, 2002; Wassarman, 1999, 2002; Hoodbhoy and Dean, 2004). The zona pellucida of the oocyte is a cell specific extra-cellular matrix or coat composed of three highly conserved glycoproteins named ZP1, ZP2 and ZP3. The ligand, which binds to sperm receptors, appears to be O-linked carbohydrates of the glycoprotein ZP3 since pre-incubation of sperm with ZP3 in micro molar concentrations strongly inhibits sperm binding to the zona pellucida, whereas pre-incubation of sperm with ZP1 and ZP2 has no effect on sperm binding to the zona pellucida. Chemical or enzymatic removal of all ZP3 oligosaccharides prevents sperm binding to the zona pellucida (Wassarman, 1999). The pre-incubation of sperm with ZP3 oligosaccharides recovered by mild alkaline hydrolysis in reducing conditions or synthesized in the laboratory in micro molar concentrations prevents binding of sperm to the zona pellucida (Litscher et al., 1995; Johnston et al., 1998; Wassarman, 1999). Limited proteolysis (Litscher and Wassarman, 1996), exon swapping (Kinloch et al., 1995) and site directed mutagenesis (Kinloch et al., 1995; Chen et al., 1998) studies have revealed that the oligosaccharides responsible for the sperm binding are located on just two of five serine residues, serine-332 and serine-334, in a region of the polypeptide near the carboxyl terminus encoded by exon-7

of the ZP3 gene. Since these two serine residues are highly conserved, evolutionary changes in the amino acid sequence neighboring these two serine residues may impose changes in the structure of O-linked oligosaccharides added to ZP3 which allows species specificity in sperm-zona binding (Wassarman, 1999).

Several sperm surface egg binding proteins, which include various enzymes to lectin-like protein molecules, from various species have been proposed to function as receptor molecules on the acrosome intact sperm (Wassarman, 1999; Topfer-Petersen et al., 2000). Several studies strongly suggest that sperm-egg binding leading to the acrosomal exocytosis is a complex event that likely reflects interaction between multiple sperm surface receptors and multivalent ZP3 (Brewis and Wong, 1999; Wassarman et al., 1999; Topfer-Petersen et al., 2000; Hoodbhoy and Dean, 2004). Although several putative zona pellucida receptors on the sperm surface have been reported in the last decade, only a few of them are characterized based on their carbohydrate specificity and structure of the carbohydrate-binding domain (Topfer-Petersen et al., 2000). Some of the examples are rabbit sp17, mouse galactosyltransferase, porcine spermadhesins and proacrosin. The rabbit sp17, which shares a consensus sequences with the class-C type lectins, and porcine proacrosin recognize sulfated carbohydrate in the zona pellucida (Topfer-Petersen et al., 2000). However, porcine spermadhesins recognize galactose-containing structures, such as mannose and mannose-6-phosphate, and represent a new class of lectins (Romero et al., 1997). Porcine sperm receptor protein zonadhesin and mouse sp56 have a molecular structure with an unknown ligand-binding specificity (Bookbinder et al., 1995; Hardy and Garbers, 1995; Topfer-Petersen et al., 2000). After binding with the zona pellucida,

mouse p95 tyrosine kinase receptor, human hu9 zona receptor kinase (ZRK) and human fertility antigen FA-1 are autophosphorylated resulting in the induction of intrinsic signaling pathways in the sperm (Burks et al., 1995; Topfer-Petersen et al., 2000). Incubation of sperm with antibodies directed against ZRK, galactosyltransferase and other sperm surface proteins has been shown to initiate the acrosome reaction (McLesky et al., 1998; Shur, 1998). Targeted mutagenesis studies revealed that some of these proteins are not particularly relevant to gamete recognition since male mice, which are homozygous null for β-galactosyltransferase, are fertile (Lu and Shur, 1997). Sperm receptor proteins, proacrosin and PH-20, are located in the wrong compartment of the sperm to participate in gamete recognition or primary binding. Hence, some of these proteins are suggested to be involved in the transient secondary binding of acrosome-reacted sperm during zona penetration (Shur, 1998; Topfer-Petersen, 1999). While porcine p47, spermadhesins and murine proteinase inhibitor-binding protein are peripherally associated to the sperm surface, ZRK, galactosyltransferase, human FA-1 and porcine zonadhesin have been shown to traverse the sperm plasma membrane (McLesky et al., 1998; Shur, 1998; Topfer-Petersen, 1999). Hence, both surface-associated and transmembrane proteins have been suggested to form multimeric receptor binding with the zona pellucida. This multimeric receptor binding induces the aggregation of the signaling molecules of the receptor complex ZRK, galactosyl transferase, zonadhesin or other still unknown components and triggers the different signal transduction pathways of acrosome reaction (Florman et al., 1998).

2.4. ACROSOME REACTION OF SPERMATOZOA

The acrosome reaction is an exocytotic event in which the sperm acrosome undergoes a terminal structural modification leading to fusion, vesiculation and loss of both the outer acrosomal membrane and overlying plasma membrane. The acrosome reaction is a result of signal transduction pathways initiated by carbohydrate mediated sperm binding to the zona pellucida of an egg or suitable ligand (Yanagimachi, 1994; Abou-Haila and Tulsiani, 2000; Baldi et al., 2002; Breitbart, 2002). This exocytotic event results in the release of the trypsin-like acrosin and a variety of enzymes such as acid glycohydrolases, proteinases, phosphatases, esterases and aryl sulfatases, and the exposure of new membrane domains, both of these events are essential for the fertilization process (Allen and Green, 1997; Tulsiani et al., 1998). The sperm creates a groove approximately the width and height of the head to penetrate the zona pellucida with the help of the acrosomal matrix enzymes and the physical pressure of hyperactivated motility obtained by the capacitation process (Allen and Green, 1997).

A number of media constituents, such as progesterone, follicular fluid and cumulus cell secretions containing prostaglandins, sterol sulfate, glycosaminoglycans, and neoglycoproteins, have been shown to induce the acrosome reaction in-vitro (Abou-Haila and Tulsiani, 2000). The zona pellucida glycoprotein ZP3 is the natural agonist which triggers signal transduction pathways resulting in the fenestration and fusion of the sperm plasma membrane and the outer acrosome membrane at multiple sites of the anterior region of the sperm head, sequentially releasing the acrosomal contents at the site of sperm–zona binding and the exposure of the inner acrosomal membrane (Tulsiani et al., 1998). However, there are several reports demonstrating heparin and

heparin-like glycosaminoglycans of the follicular fluid, cumulus cell secretions and female reproductive tract secretions as important factors, which stimulate the acrosome reaction in the sperm (Lee et al., 1985; Miller and Ax 1990; Tulsiani et al., 1998)

The binding of G protein coupled receptor of sperm to ZP3 activates adenylate cyclase and the phospholipase C resulting in an intracellular increase in cAMP and hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2), respectively. The intracellular increase in cAMP concentration triggers phosphorylation of PKA and the hydrolysis of PIP2 resulting the release of diacylglycerol (DAG) and inositol triphosphate (IP3), which leads to PKC translocation and activation. The activation of PKA and release of IP3 stimulate release of Ca^{2+} from the interior of the acrosome to the cytosol of the sperm by a voltage-dependent and IP3-gated Ca^{2+} channel in the outer acrosomal membrane, respectively. This initial increase in the Ca^{2+}concentration may help further activation of phospholipase C and break down of PIP2. The ZP3 also binds to a tyrosine kinase coupled receptor in the sperm plasma membrane and activates the isoforms of phospholipase C and further break down of PIP2 results in release of higher concentrations of DAG and IP3. The breakdown of PIP2 may remove the inhibition of actin-severing proteins to enhance the fusibility of the membranes. The increase DAG concentrations activate specific PKC isoforms to activate a sperm plasma membrane voltage-dependent Ca^{2+} channel and resulting in a second surge of intracellular Ca^{2+} concentration (Spungin and Breitbart, 1996). In addition, the depletion of acrosomal Ca^{2+} by activation of PKA and IP3-dependent Ca^{2+} channels of the outer acrosomal membrane also activates a capacitative Ca^{2+} entry mechanism in the plasma membrane (Breitbart, 2002). The binding G protein or tyrosine kinase coupled receptor of the

sperm can also activate a Na^+/H^+ exchanger in the plasma membrane, resulting in an increase in intracellular pH (Baldi et al., 2002). The increase in intracellular Ca^{2+} activates membrane bound phopholipase-A2 and phospholipase-C which act on membrane phospholipids and release fusogenic lysophospholipids. PKC-mediated protein phosphorylation with the increase in intracellular pH and Ca^{2+} activates actin-severing protein, which results in the dispersion of F-actin. The increase in intracellular pH and Ca^{2+} also promotes the conversion of acrosomal proacrosin into enzymatically active acrosin. The fusogenic lysophospholipids and F-actin dispersion result in the fusion of the plasma and outer acrosomal membranes, leading to vesiculation and acrosomal exocytosis (Breitbart and Naor, 1999; Abou-Haila and Tulsiani, 2000; Baldi et al., 2002; Breitbart, 2002).

2.5. FERTILIZATION

Mammalian fertilization is defined as a complex process, in which the sperm and egg unite thereby restoring the somatic chromosome number and development of a new individual exhibiting the characteristics of the species (Yanagimachi, 1994; Wassarman, 1999). Egg and sperm undergo a series of maturational changes before they fuse successfully and form a viable zygote (Wassarman and Albertini, 1994; Visconti and Kopf, 1998). During maturation, the sperm undergoes capacitation to gain fertilizing capacity and the oocyte undergoes nuclear and cytoplasmic maturation in which the cortical granules move towards the periphery of the oocyte. In the fertilization process, binding of sperm and the zona pellucida of the mature oocyte initiates the sperm acrosome reaction and release of acrosomal contents. With the help

of acrosomal contents, the acrosome reacted spermatozoa penetrate through the zona pellucida and bind with the vitelline membrane of the oocyte. Since previous sections of this chapter reviewed the sperm-zona binding and the sperm acrosome reaction, this section will review the events of fertilization following penetration of the sperm through the zona pellucida.

The oolemma forms microvilli on the surface surrounding the oocyte except the region overlying the mitotic spindle (Longo and Chen, 1984). The observation, that the acrosome reacted sperm binds and fuses only on the region containing microvilli and rarely fuses with the region lacking microvilli, indicates that the oocyte microvilli and equatorial segment of the sperm are rich in molecules involved in sperm egg fusion (Yanagimachi, 1994). Based on antibody and gene targeting studies, several molecules such as a member of the ubiquitous tetraspanin family of proteins CD9, glycosylphosphatidylinositol (GPI)-anchored proteins, epididymal protein DE/cysteine-rich secretory protein-1, fertilin-α, fertilin-β and cyritestin have been suggested to be involved in sperm egg fusion and binding (Cho et al., 1998; Miller et al., 2000; Nishimura et al., 2001; Alfieri et al., 2003; Kaji and Kudo, 2004). The interaction between the sperm and egg initiates a series of biochemical events in the egg called egg activation. The early events of egg activation include an initial transient rise in intracellular Ca^{2+} concentration followed by several hours of Ca^{2+} oscillations resulting in the induction of cortical granule exocytosis and resumption of meiosis (Ben-Yosef and Shalgi, 1998). The initial transient rise in intracellular Ca^{2+} concentration is thought to be due to the release of Ca^{2+} from intracellular stores like the endoplasmic reticulum by increasing concentrations of IP3 after hydrolysis of PIP2 (Swann and Parrington,

1999). The hydrolysis of PIP2 also releases DAG, which activates the PKC (Ben-Yosef and Shalgi, 2001). The exocytosis of cortical granules into the peri-vitelline space leads to alterations in the structure of the zona pellucida glycoproteins, thus establishing a block to polyspermy (Ducibella et al., 1993). Following the early events of egg activation the late events including extrusion of the second polar body, decondensation of the sperm DNA, maternal RNA recruitment, formation of male and female pronuclei, initiation of DNA synthesis, syngamy and cleavage occur (Xu et al., 1994; Schultz and Kopf, 1995). Although it has been suggested that the sperm activates the egg by its binding to a receptor on the egg plasma membrane, evolving recent evidence has strongly suggested that the egg is activated by a sperm protein directly introduced into the egg ooplasm (Parrington et al., 1996; Ben-Yosef and Shalgi, 2001; Runft et al., 2002; Kaji and Kudo, 2004). The mammalian egg can be activated with increases in intracellular Ca^{2+} concentration by activators such as Ca^{2+} ionophores, ethanol or by introduction of IP3 into the ooplasm (Jones and Nixson, 2000). However, these activators give only a single increase in intracellular Ca^{2+} and do not give the transient Ca^{2+} oscillations, which are induced by sperm fusion. It is not yet clear how the sperm activates increases in intracellular Ca^{2+} concentration, transient Ca^{2+} oscillations, cortical reaction and resumption of meiosis. Although other second messengers, PKC and protein tyrosine kinase, were suggested as possible inducers for early events of egg activation, their role in the egg activation is yet to be proved (Sun et al., 1997; Raz et al., 1998; Eliyahu et al., 2002; Kaji and Kudo, 2004).

2.5.1. Polyspermy

In the fertilization process, the sperm fusion with vitelline membrane of the oocyte initiates the release of cortical granules into the peri-vitelline space, which causes chemical changes in the zona pellucida to prevent the entry of other sperm, the zona block (Cherr and Ducibella, 1990; Wassarman, 1999). Defects in the process of zona block resulting in the penetration of more than one sperm and can lead to polyspermy (Hyttel et al., 1988; Cherr and Ducibella, 1990; Yanagimachi, 1994; Ducibella, 1996). Compared to in-vivo embryos, in-vitro produced embryos show a high incidence of polyspermy (Xu and Greeve, 1988). In the in-vitro fertilized embryos, polyspermic penetration is associated with the observation of undispersed cortical granule contents in the invaginations of oolemma resulting in an incomplete block to polyspermy (Hyttel et al., 1988). It has been suggested that polyspermy appears to be a result of a delay in the migration the cortical granules during maturation of oocytes in-vitro (Hyttel et al., 1986, 1989). Polyspermy has been shown as a most prevalent abnormal fertilization procedure resulting in the formation of polyploidic zygotes (Xu and Greve, 1988; Saeki et al., 1991). The frequency of polyspermic fertilization ranges from 6-37% in in-vitro produced embryos and 5-10% in in-vivo produced embryos (Iwasaki et al., 1989; King, 1991; Lechniak, 1996). It has been shown that polyspermic zygotes rarely develop beyond the morula and blastocyst stages or develop as androgenotes (Iwasaki et al., 1989; Pinto-Correia et al., 1992; Long et al., 1993). Improper maturation of oocytes (Niwa et al., 1991; Chian et al., 1992; Long et al., 1994; Agca et al., 2000), concentration of sperm (Long et al., 1994), source of sperm (Kreysing et al., 1997), fertilization medium (Tajik et al., 1993; Pavlok, 2000) and

sperm oocyte co-incubation time (Long et al., 1994) have been shown to influence fertilization and polyspermy.

2.6. EARLY EMBRYONIC DEVELOPMENT

The oviduct provides an optimum environment for sperm-egg transport, fusion at the ampulla region in the fertilization process and early embrynic development. Paracrine and/or autocrine systems involving various growth factors and glycoproteins secreted by oviductal epithelial cells mediate embryo growth promoting action of the oviductal microenvironment (Martus et al., 1997; Pushpakumara et al., 2002). Granulosa cell co-culture or synthetic oviductal fluid provides a similar environment for embryo development in-vitro. The developing bovine embryo enters the uterus 72-84 h after fertilization and starts attaching to the uterine wall from day 22 of gestation after hatching on day 9 of gestation. During migration from the oviduct to the uterus and before attachment with the uterine wall, the developing embryo undergoes a series of biochemical and morphological changes. The zygote starts with the mitotic divisions and the duration of first, second, third and fourth cell cycles of in-vitro produced embryos have been estimated as 32-34, 9-14, 10-14, and 48-54 h, respectively (Grisart et al., 1994; Holm et al. 1998). However, in-vivo studies showed that the duration of cell cycles 1-4 are about 32, 13, 14 and 24 h, respectively. This indicates there is a relatively long fourth cell cycle and a delay in the development of the in-vitro embryos after the 8-16 cell stage (Holm et al. 2002). The two major events in early embryonic development are compaction and cavitation, which follow the fifth cell cycle. As embryonic cell cleavage proceeds past the early 8 cell stage, the developmental potency

of the dividing cells is gradually restricted with a combination of several events that include the relative in/out positions of the cells within the embryonic structure and the gradual polarization of the outer cell layer resulting in the segregation into two mutually exclusive cell lineages at the time of compaction (Johnson and Ziomek, 1981). Compaction occurs around the 32-cell stage in both in-vivo and in-vitro derived embryos and appears to be a pre-requisite for the formation of the trophectoderm (Van Soom et al., 1997). A cluster of small and apolar cells devoid of microvilli located on the inside of the compacted embryo and the appearance of these cells differs considerably with the development of apical and baso-lateral domains in the outer cells. The differentiation of outer cells into the trophectoderm of the embryo is associated with the peripheral re-organization, profound changes in the cytoskeleton of the outer cells and the establishment of apical junctional complexes between them (Wiley et al., 1990). Formation of a tight permeability seal around the embryo, coupled with the vectorial transporting activity of the trophectoderm, results in the accumulation of fluid between cells and the progressive expansion of a central cavity known as the blastocoele which displaces the clump of apolar inner cells towards an eccentric position (Biggers et al., 1988). Thus, outer and inner cells acquire distinct morphological and functional characteristics, and are exposed to different microenvironments, the external uterine milieu and the blastocoele fluid, respectively. In response to these combined events, inside cells evolve into the inner cell mass (ICM), which contains the founder cells of the fetus. Experimental evidence showing that the descendants of the first-dividing blastomere of the two-cell stage embryo locate preferentially in the ICM (Graham and Deussen, 1978; Kelly et al., 1978) further

suggests that the rapid accentuation of ICM-trophectoderm cell divergence during blastocyst expansion depends on the preliminary unequal distribution of cell determinants before the time of compaction (Edwards and Beard, 1997). Progressive segregation of key cytoplasmic constituents, such as transcription factors, during successive rounds of cleavage between fertilization and compaction has been postulated as a mechanism for the generation of embryonic cells with unique complements of developmental information, and thereby with different ICM trophectoderm commitments (Antczak and Van Blerkom, 1997). As development proceeds throughout the blastocyst stage and the early phase of implantation, the ICM cells sub-divide into all three germ layers (Gardner, 1983). The interval between insemination and the formation of a tight compact morula and early blastocyst in-vitro is 110-140 h and 135-155 h post insemination, respectively (Holm et al. 1998). It is well known that the stage of development in which embryonic genome activation occurs is species specific. The bovine embryonic genome activation generally occurs during the fourth cell cycle and this is paralleled by marked ultrastructural changes in the blastomere nucleoli (Laurincik et al., 2000). Although activation of major portions of the bovine embryonic genome takes place during the fourth cell cycle, the embryonic genome is not completely inactive in the preceding cycles and some embryonic transcriptions are detected as early as the first cell cycle (Hay-Schmidt et al., 2001). During the proliferation of blastomeres by mitosis and the development of the embryo, the unwanted cells, which includes chromosomally aberrated blastomeres, are eliminated by a programmed cell death process.

2.7. APOPTOSIS IN THE PREIMPLANTATION EMBRYO

Development of multicellular organisms is controlled not only by cell proliferation and differentiation, but also by the elimination of unwanted cells with minimal disturbance to the organism. The elimination of unwanted cells is carried out by a programmed cell death or cell suicidal procedure called apoptosis. Apoptosis is an energy requiring genetically regulated multistep physiological procedure maintaining balanced tissue homeostasis during cell proliferation (Antonsson, 2001). Apoptosis is a tightly regulated process that initiates cleavage of many proteins to mediate final cell death alteration in specific cells and leads them to cell suicidal program (Bloom and Muscarella, 1998). Apoptosis is characterized morphologically by cell shrinkage, membrane blebbing, chromatin aggregation, nuclear and cytoplasmic condensation, internucleosomal DNA fragmentation, and partition of cytoplasm and nucleus into membrane-bound vesicles called apoptotic bodies which are subsequently phagocytosed by resident macrophages or adjacent cells (Kerr et al., 1972). Chromatin condensation during the apoptosis process is associated with a specific type of DNA fragmentation in which DNA is first cleaved into large 300- and 50-kb (Ploski and Aplan, 2001) fragments and then subsequently cleaved between nucleosomes to generate fragments of DNA that are multiples of 185 bp resulting in a specific DNA ladder pattern on agarose gel electrophoresis (Wyllie, 1980). The stimulus for apoptosis can be intracellular such as DNA damage, excess production of reactive oxygen species, or extracellular such as heat stress or ionizing radiation. The mitochondrial pathway and the death receptor pathway have been identified as two major pathways for programmed cell death (Ingo et al., 2000). Both pathways are regulated by cysteine-dependent

aspartate-specific proteases called caspases, which require a cysteine in their active site for activity and proteolytically cleave target proteins at an aspartate residue within a 4-amino acid cleavage site (Vanags et al., 1996). Fourteen caspases have been identified and are classified into three functional groups. Group 1 caspases, which include caspase-1, -4, -5, -11, -12, -13, and -14, are involved in the processing of proinflammatory cytokines and do not appear to play a role in apoptosis. Group 2 caspases, which include caspase-2, -8, -9, and -10, are regulatory caspases and play a role in initiating the apoptotic pathway. Group 3 caspases, which include caspase-3, -6, and -7, are called effector caspases and play a key role in enzymatically cleaving a variety of cellular proteins that lead to an orderly demise of the cell (Mirkes, 2002). The Bcl-2 family of proteins are involved in the regulation of the mitochondrial apoptotic pathway. To date, at least 17 members of the Bcl-2 family have been identified and classified into three functional groups. Group 1 members, which include Bcl-2, Bcl-XL, A1, Boo, Bcl-w and Mcl-1, have anti-apoptotic activity and contain four conserved Bcl-2 homology (BH1-4) domains that localize the protein to the outer membrane of the mitochondria and the membranes of the endoplasmic reticulum. Group 2 members, which include Bax, Bak and Bok, have pro-apoptotic activity and their structure is similar to group 1 members, but the BH4 domain is absent in this group. Group 3 members, which include Bim, Bik, Bid, Bad, Bmf, Hrk, Noxa and PUMA, have pro-apoptotic activity, and contain a diverse collection of proteins that share only the BH3 domain with group 1 and 2 (Antonsson, 2001; Mirkes, 2002). Another family of anti-apoptotic proteins known as inhibitors of apoptosis (IAPs) have been identified recently based on their highly conserved approximately 70 amino acid baculoviral IAP repeat

(BIR) domain that functions to suppress apoptosis triggered by various apoptotic stimuli known to activate both mitochondrial and receptor-mediated apoptotic pathways (Deveraux and Reed, 1999). Eight members of IAP family; XIAP, NAIP, C-IAP-1, C-IAP-2, p-IAP, Survivin, LIVIN, and BRUCE or APOLLON, have been identified and some of these act on various caspases to inactivate the apoptotic pathways (Bennett et al., 1998; Deveraux et al., 1997, 1998; Roy et al., 1997; Kasof and Gomes, 2001). In the death receptor-mediated apoptotic pathway, the ligand binding of the death receptors, which include Fas/CD95, TNFR1, DR3/APO-3, DR4 and DR5, activate the receptor by inducing receptor clustering (Mirkes, 2002). The activated receptor recruits a cytosolic adapter protein known as Fas associated death domain (FADD). FADD recruits and binds procaspase-8 via common death effector domains (DED) and forms a complex called death-inducing signaling complex (DISC). Within the DISC, the procaspase-8 is autocatalytically processed resulting in the active caspase-8 containing large and small subunits. The activated caspase-8 enzymatically cleaves and activates the downstream effector caspases such as caspase-3 (Chen and Wang, 2002; Mirkes, 2002). In the mitochondrial apoptotic pathway, instead of caspase-8/-10, procaspase-9 is activated through unclear upstream pathways. A variety of apoptotic signals act on the mitochondria to release cytochrome-c into the cytoplasm where cytochrome c activates a protein called apoptosis protease activating factor-1 (Apaf-1) to form an oligomeric complex known as an apoptosome. Subsequently, procaspase-9 is recruited to the apoptosome through protein-protein interactions mediated by caspase recruitment domains (CARD) in Apaf-1 and caspase-9 (Tsujimoto and Shimizu, 2000; Mirkes, 2002). Although the activation of procaspase-9 is not completely understood,

autocatalytic processing of the proenzyme within the apoptosome yields the activated, caspase-9, which activates downstream effector caspases, such as procaspase-3. Both the death receptor and the mitochondrial apoptotic pathways involve caspase cascades that activate one or more effector caspases. The activated effector caspases act on various cellular proteins containing a caspase cleavage motif to either activate, e.g., P21- Activating Kinase 2 (PAK2) or inactivate, e.g., Poly (ADP-ribose) Polymerase (PARP), inhibitors of caspase activated DNAse (ICAD). Inactivation of PARP and ICAD, and cleavage of lamin -A, actin, Gas-2, gelsolin and fodrin by effector caspases results in DNA fragmentation, and cell shrinkage and membrane blebbing, respectively (Kothakota et al., 1997). Effector caspase mediated cleavage of lamins, which are intermediate filament scaffold proteins of the nuclear envelope, also appears to be involved in nuclear fragmentation because over expression of lamins with mutated caspase cleavage sites delays the onset of chromatin condensation (Rao et al., 1996). Although the relationship between the cleavage of some of the specific substrates and subsequent functional and morphological events of apoptosis has been established, the relationship between cleavage of most effector caspase target proteins, and the functional and morphological events of apoptosis is yet to be established.

p53, a tumor suppresser protein, has been shown to regulate both the death receptor and the mitochondrial pathways and apoptosis by transcription dependent and independent ways. p53 regulates apoptosis following a variety of stimuli such as DNA damage, cytotoxic drugs, free radicals, irradiation, growth factor withdrawal, hypoxia, metabolic change, virus infection, cytokines, or deregulated expression of cell cycle genes (Canman and Kastan, 1998). p53 initiates the mitochondrial apoptotic pathway by

up-regulation of pro-apoptotic genes such as Bax and down-regulation of anti-apoptotic genes such as bcl-2 by transcriptional activation or repression, resulting in alteration of the relative quantities of Bax to Bcl-2 and shifting the balance towards apoptosis (Miyashita et al., 1994). Activation of p53 induces the death receptor mediated apoptotic pathway by translocation of an intracellular pool of Fas located in the Golgi complex to the cell surface, which induces FADD recruitment to Fas, and caspase activation. Recent studies revealed that various stages of p53 dependent and independent apoptotic pathways are negatively regulated by heat shock protein-70 (Beere et al., 2000; Gabai et al., 2000).

An irregular expression pattern of pro and anti apoptotic proteins regulating both the death receptor and the mitochondrial pathways causes cell death and embryo mortality during early stages of development since apoptosis and mitosis are essential developmentally regulated functions for development and differentiation of the early embryo (Hardy, 1997; Matwee et al., 2000; Kolle et al., 2002). Compared to in-vivo produced embryos, in-vitro produced embryos show higher numbers of apoptotic cells (Jurisicova et al., 1998) and this might be one of the reasons for the lower survival rates of in-vitro produced embryos (Xu et al., 1992; Keskintepe and Brackett, 1996). External stress factors change the expression patterns of pro and anti apoptotic proteins to predispose blastomeres to signal induced apoptosis (Jurisicova et al., 1998). Apoptosis has been observed in 8-16 cell, morula and blastocyst stage embryos, but has not been observed in zygotes (with 2 pronuclei), 2-cell, or 3 to 7-cell stage embryos (Matwee et al., 2000). Furthermore, the percentage of apoptotic nuclei observed decreases at the morula stage before increasing again at the blastocyst stage (Byrne et al., 1999).

Although expression of several IAPs, which protect cells from apoptosis, have been shown, their roles in development and the embryo response to apoptotic stimuli are unknown.

2.8. Bax, Bcl-2 and p53

Bcl-2 associated protein (Bax) is one of the major pro-apoptotic proteins first isolated with the key regulatory anti-apoptotic protein B-cell lymphoma-2 (Bcl-2) by co-immunoprecipitation (Oltvai et al., 1993). In normal cells, Bax is primarily localized in the cytosol as a monomer and apoptotic stimulation specifically translocates Bax to the mitochondria (Hsu et al. 1997; Hsu and Youle 1998; Gross et al. 1998; Zhang et al. 1998b; Antonsson et al. 2000). Activation and translocation of Bax have been accompanied by conformational changes in its quaternary structure in which the C-terminal α-helix is removed from the BH3 cleft making the BH3 domain accessible to interactions leading to complex formation (Suzuki et al. 2000). After activation, Bax is found inserted into the outer mitochondrial membrane as large oligomers (Antonsson et al. 2001).). It has been shown that Bax forms large clusters containing oligomers of thousands of Bax molecules in the outer mitochondrial membrane in cells undergoing apoptosis (Nechushtan et al. 2001). Bax levels have been reported to change during apoptosis in several cell types (Krajewski et al. 1995; Ekegren et al. 1999). p53 has been shown to mediate transcriptional activation of the Bax promoter resulting in up-regulation of Bax protein expression (Miyashita and Reed 1995). Bax appears to induce apoptosis with the release of cytochrome c and other proteins, including AIF, adenylate kinase and endonuclease G from the mitochondrial intermembrane space by forming

specific channels or pores in the outer membrane and/or by opening the permeability transition pore resulting in mitochondrial matrix swelling and outer membrane rupture (Daugas et al. 2000; Verhagen et al. 2000; Li et al. 2001).

Bcl-2, the founder of the Bcl-2 protein family was first identified as a gene translocated in B-cell lymphoma, thus linking its activity to tumor growth (Tsujimoto et al. 1985). Anti-apoptotic proteins like Bcl-2 are localized in several intracellular membranes, including the mitochondria, endoplasmic reticulum, and nuclear envelope (Krajewski et al. 1993). Anti-apoptotic members such as Bcl-2 tend to form heterodimers with BH3 domains of the pro-apoptotic members, blocking their function, such that cell fate may be determined by the ratio of pro- to anti-apoptotic members (Oltvai et al., 1993). Since Bcl-2 inhibits Bax activation and oligomerization by either direct or indirect interactions, the molecular mechanism for Bcl-2 prevention of Bax activation remains unclear and appears to be dependent on cell type or the apoptotic stimulation (Mahajan et al., 1998; Antonsson et al., 2001; Mikhailova et al. 2001). Pro-apoptotic and cell cycle inhibitory functions of Bcl-2 have also been reported (O'Reilly et al., 1996; Vairo et al., 1996; Cheng et al., 1997; Shinoura et al. 1999). Bcl-2 can also modulate the cell cycle in a way that is different from the inhibitory effect on apoptosis (Linette et al. 1996). Bcl-2 promotes exit into quiescence and retards re-entry into the cell cycle (Mazel et al., 1996; Adams and Cory, 1998). It has been shown that over expression of the Bcl-2 protein increases the half-life of the Bax protein (Miyashita et al., 1995) indicating that the Bcl-2 gene functions like a pro-apoptotic gene in some circumstances. It has been reported that caspase-3 cleaves Bcl-2 at Asp34 and transforms the Bcl-2 protein into an inducer of cell death (Cheng et al., 1997). The anti-

apoptotic function of the Bcl-2 can be lost by multi-site phosphorylation (Haldar et al. 1995). The regulation of the function of Bcl-2 mainly involves interactions with other proteins of the Bcl-2 protein family, but phosphorylation may also be a crucial event in the regulation of its function (Haldar et al. 1995; Yamamoto et al. 1999). Jnk was repeatedly indicated as a potential Bcl-2 kinase, which phosphorylates Bcl-2 at four serine/threonine sites (Maundrell et al. 1997; Blagosklonny, 2001). However, it has been suggested that several kinases may be involved in the phosphorylation of Bcl-2 (Blagosklonny, 2001). Although Bcl-2 at a low level of expression was anti-apoptotic, Bcl-2 at a high level of expression was pro-apoptotic to Fas-mediated apoptosis (Shinoura et al., 1999).

Expression of Bcl-2 and Bax was demonstrated in the bovine (Kolle et al., 2002; Matwee et al., 2000; Yang and Rajamahendran, 2002), human (Liu et al., 2000; Spanos et al., 2002; Jurisicova et al., 2003), and mouse (Exley et al., 1999) pre-implantation embryos. Changes in expression of Bcl-2 and other members of Bcl-2 family are associated with fragmentation of embryos at the pronucleate and blastocyst stages (Jurisicova et al., 1998; Exley et al., 1999). Bcl-2 family members are involved in the regulation of apoptosis during mouse preimplantation development. The anti-apoptotic members including Bcl-2, Bcl-W, and Bcl-XL are expressed at high mRNA levels at the pronucleate and blastocyst stages and lowest at the 2-8 cell stages, suggesting that the message is both inherited from the oocyte and transcribed from the embryonic genome (Jurisicova et al., 1998; Warner et al., 1998; Exley et al., 1999). Of these, Bcl-XL is expressed at the highest copy number, with Bcl-2 lowest (Jurisicova et al., 1998). Bax is constitutively expressed, increasing from low copy number to higher levels after

embryonic genome activation, with pro-apoptotic members Bak and Bad also expressed (Jurisicova et al., 1998). Jurisicova et al. (2003) studied seven Bcl-2 genes in normal and fragmenting embryos, and found that levels of the pro-apoptotic genes of the Bcl-2 family were up regulated in fragmented embryos.

p53 is a 53 kDa sequence-specific protein transcription factor that is widely recognized to function during the cell cycle as an inducer of cell cycle arrest and as a mediator of apoptosis (Yonish-Rouach et al., 1991; Levine, 1997; Sionov & Haupt, 1999). The elimination of excess, damaged or infected cells by p53 mediated apoptosis is essential for the proper regulation of cell proliferation and for the control of propagation of damaged DNA in multicellular organisms (Huang and Strasser, 2000). p53 exerts its tumor suppressor effect through regulation of cell proliferation by regulating cell cycle checkpoints and mediating growth arrest, and also by mediating apoptosis by both transactivation of genes involved in different cellular functions and activation of transcription-independent mechanisms of apoptosis (Lane, 1992; Haupt et al., 1995; Agarwal et al., 1998). The external and internal stress signals, such as DNA damage, free radicals, irradiation, virus infection and nucleotide depletion, activate p53 and promote its nuclear accumulation in an active deregulated expression of cell cycle genes resulting in either viable cell growth arrest or apoptosis (reviewed by Bennett, 1999). Under normal conditions, p53 has a short half-life regulated by p53 inhibitors (Giaccia and Kastan, 1998). p53 activation involves stabilization of the protein, and enhancement of its DNA binding and transcriptional activity. These changes in p53 are mediated by extensive post-translational modifications of p53 and protein-protein interactions with cooperating factors. The activation of p53 leads to either cell growth

arrest or apoptosis depending on the summation of incoming signals and the cellular context (Jin and Levine, 2001). A multitude of mechanisms, which include stimulation of a wide network of signals that act through two major apoptotic pathways by induction of specific apoptotic target genes and promotion of apoptosis by a transcription-independent mechanism under certain conditions, are employed by p53 to ensure efficient induction of apoptosis in a stage, tissue and stress signal specific manner (Giaccia and Kastan, 1998; Lohrum and Vousden, 1999). p53 can activate the extrinsic apoptotic pathway through the induction of genes encoding three transmembrane proteins Fas, DR5 and PERP (Muller et al., 1998). p53 may also rapidly sensitize cells to Fas-induced apoptosis by increasing Fas receptors at the cell surface by promoting Fas receptor trafficking from the Golgi before the transcription-dependent apoptotic effect (Bennett et al., 1998). p53 also induces DR5/KILLER, the death-domain-containing receptor for the TNF-related apoptosis-inducing ligand (TRAIL), in response to DNA damage (Wu et al., 1997). p53 regulates the key Bcl-2 family pro-apoptotic proteins of the intrinsic apoptotic pathway such as Bax, Noxa, PUMA and Bid (Cory and Adams, 2002; Kuwana et al., 2002; Thornborrow et al., 2002). It appears that in response to DNA damage p53 activates the intrinsic mitochondrial apoptotic pathway by inducing the expression of at least three Bcl-2 pro-apoptotic family members, shifting the balance towards pro-apoptotic effects (Adams and Cory, 2002). p53 also induces Apaf-1 expression through a response element within the Apaf-1 promoter (Rozenfeld- Granot et al., 2002). The p53 also induces apoptosis by direct action in the mitochondria through signal-induced localization resulting in cytochrome c release and procaspase-3 activation to induce apoptosis (Marchenko et al., 2000). p53 also forms

complexes with the protective Bcl-XL and Bcl-2 proteins and promotes permeabilization of the outer mitochondrial membrane (Mihara et al., 2003). p53 regulates transcription of pro-apoptotic Bid which is distinguished by its unique ability to connect activation of the extrinsic death receptor pathway to activation of Bax and Bak associated with the intrinsic pathway (Sax et al., 2002). Hence, p53 appears to promote the convergence of the intrinsic and extrinsic pathways of apoptosis through Bid regulation.

2.9. INTERFERON TAU (IFNτ)

Bovine trophoblast IFNτ is a 195 amino acid pre-protein. It is coded by a 595 bp open reading frame, containing a 23 amino acid signal sequence that is cleaved to yield the mature protein. It has molecular masses of 22,000 to 24,000 kDa, each with multiple isoforms, and is glycosylated with N-linked oligosaccharides (Demmers et al., 2001). IFNτ is a well characterized embryonic signal for the maternal recognition of pregnancy. It exerts its function through a reduction in the expression of estrogen and oxytocin receptors in the endometrium, which in turn reduces or prevents the oxytocin mediated pulsatile secretion of prostaglandin $F_{2\alpha}$ and luteolysis (Godkin et al., 1997; Wolf et al., 2003). In addition to maintenance of the corpus luteum of pregnancy, IFNτ induces expression of a number of genes such as STAT (signal transducer and activator of transcription) 1 and 2 (Stewart et al., 2001), β2 microglobulin (Vallet et al., 1991), IFN-regulatory factor 1 (Spencer et al., 1998), ubiquitin conservative protein (Johnson et al., 1999), Mx protein (Ott et al., 1998), granulocyte chemotactic protein 2 (Teixeira et al., 1997) and 2'5'-oligoadenylate synthase (Johnson et al., 2001). Further, IFNτ

stimulates the expression of granulocyte-macrophage colony-stimulating factor, a cytokine with putative positive effects on the conceptus, in stroma cells of the endometrium (Emond et al., 2000). Other effects of IFNτ in endometrial cells include a reduction of oxytocin-induced cyclooxygenase-2 and prostaglandin F-synthetase expression (Xiao et al. 1999). Thus, IFNτ supports the maintenance of pregnancy via multiple mechanisms. Inadequate reaction of the endometrium to IFNτ or insufficient secretion of IFNτ by the conceptus is assumed to be the major reason for early embryonic loss and pregnancy failure. Therefore, the level of IFNτ secretion has been discussed as a parameter for the assessment of embryo quality (Hernandez-Ledezma et al. 1993). IFNτ is expressed transiently during embryo development with the peak production on day 16 in sheep (Godkin et al., 1982) and day 17 in cattle (Bartol et al., 1985). It is detectable until day 20 in sheep and day 25 in cattle when the embryo starts the implantation process. The rapid onset and cessation of IFNτ expression is regulated by number of transcription factors such as Ets-2 (Ezashi et al., 1998) and granulocyte–macrophage colony-stimulating factor acting via the proto-oncogene c-*jun* and an AP-1 site (Imakawa et al., 1993; Yamaguchi et al., 1999). In addition, negative regulatory domains have been shown in the bovine IFNτ promoter that may be involved in the precisely timed cessation of gene expression (Guesdon et al., 1996; Yamaguchi et al., 1999).

2.10. HEAT SHOCK PROTEIN-70

Stress or heat shock proteins (HSPs) were first discovered as a set of highly conserved proteins whose expression was induced by different kinds of stressors

(Ritossa, 1962). Mammalian HSPs have been classified into four major families according to their molecular size: HSP90, HSP70, HSP60 and the small HSPs. HSPs act as molecular chaperones which regulate some of the important house keeping functions such as import of proteins into cellular compartments; folding of proteins in the cytosol, endoplasmic reticulum and mitochondria; degradation of unstable proteins; dissolution of protein complexes; prevention of protein aggregation; control of regulatory proteins; and refolding of misfolded proteins (Bakau and Horwich, 1998). The specificity of chaperone activity is determined by the structure of the chaperone, and the size and localization of the protein to be chaperoned

HSP70 functions as ATP-dependent molecular chaperones under normal conditions by assisting the folding of newly synthesized polypeptides, the assembly of multi-protein complexes and the transport of proteins across cellular membranes (Murakami et al., 1988; Beckmann et al., 1990; Shi and Thomas, 1992). When HSP70 is expressed under various stress conditions their synthesis enhances the ability of stressed cells to cope with increased concentrations of unfolded or denatured proteins (Nollen et al., 1999). HSP70 has been shown to inhibit apoptosis and thereby increase the survival of cells exposed to a wide range of lethal stimuli and overexpression of HSP70 protects cells from stress-induced apoptosis, both upstream and downstream of the caspase cascade activation (Mosser et al., 1997). HSP70 helps ensure the survival of cells by directly interacting with various components of tightly regulated programmed cell death machinery (Buzzard et al., 1998; Parcellier el al., 2003). HSP70 also seems to protect cells from energy deprivation associated with cell death (Wong et al., 1998). HSP70 has been shown to inhibit apoptosis downstream of the release of cytochrome c

and upstream of the activation of caspase-3 (Li et al., 2000). The direct interaction of HSP70 with APAF-1 to prevent the recruitment of procaspase-9 to the apoptosome has also been reported (Beere et al., 2000; Saleh et al., 2000). HSP70 may prevent cell apoptosis by interacting with proteins mediating caspase independent cell death pathways (Creagh et al., 2000). HSP70 directly interacts with apoptosis inducing factor (AIF) and neutralizes the apoptogenic effects of AIF such as AIF-induced chromatin condensation. (Ravagnan et al., 2001). HSP70 does not preclude the activation of caspase-3 but prevents downstream morphological changes that are characteristic of dying cells in TNF-induced apoptosis (Jaattela et al., 1998). HSP70 has also been shown to associate with the pro-apoptotic proteins p53 and c-myc (Beere et al., 2000; Gabai et al., 2000). Expression of HSP70 in the developing embryo, which undergoes various stress conditions, is important for the survival of the embryo and HSP70 has been shown to play a key role in protection of developing preimplantation embryos from various stress factors. The expression of HSP70 is developmentally regulated (Edwards et al., 1997; Paula-Lopes and Hansen, 2002). Heat stress induced expression of HSP70 was reported in very early 2 cell stage embryos (Chandolia et al., 1999)

2.11. METHODS DEVELOPED TO PREDICT BULL FERTILITY

2.11.1. Motility, Morphology and Viability

Among the parameters necessary for a basic analysis of semen, sperm concentration, motility, viability and morphology estimates would be considered the most important. Semen quality traits that are viability-related or of a morphological nature (Saacke, 1982) could be assessed by microscopic examination of either unstained

semen samples or stained semen samples by simple staining procedures. The relationship of sperm morphology to field fertility has been shown recently (Sailer et al., 1996; Aziz et al., 1998; Ostermeier et al., 2001). Acceptable standards for a "probable fertile" specimens of bull semen are the presence of over 500 x 10^6 spermatozoa per ml and more than 50% of motile sperm making forward progression (Hafez, 1987).

Several studies have compared the basic traits of semen quality with fertility estimates and correlations have ranged from zero to very high (Saacke, 1982). At present, advanced technologies such as computer-assisted semen analysis systems have become commercially available. Such systems allow an analysis of sperm translational movement, thus providing an alternative to subjective sperm motion analysis (Macnutt, 1990). Budworth et al. (1988) used this technique to examine the relationship between sperm motility and fertility, and found a correlation between the two, and suggested that computerized motility analysis may be useful in the prediction of fertility of bull spermatozoa. However, Bailey et al. (1994) found no correlation between any of the seven computer determined motility parameters and in-vivo fertility of cryopreserved bovine spermatozoa.

Other than motility, parameters like the live:dead ratio, acrosomal status and morphological abnormalities are considered important for predicting the fertilizing ability of bovine spermatozoa. Assessment of sperm viability has conventionally depended on supravital staining techniques. These are based on the principle that live cells possess intact plasma-acrosomal membranes and thus do not readily allow the passage of the macromolecular stain. Eosin-nigrosin, trypan blue or Congo red have

been conventionally used for assessing membrane integrity of bull spermatozoa. Since early reports of the use of nucleic acid specific stains (bisbenzamide) for the sorting of live cells according to their DNA content (Arndt-Jovin and Jovin, 1977; Visser, 1980), commercially available stains such as Hoechst 33258 and Hoechst 33345 (Sigma, St. Louis, MO, USA) have been found useful for assessing membrane integrity of spermatozoa in a wide variety of species including the human (Cross et al., 1989). In spite of their importance, these tests fail to predict fertility of bulls in the field.

The maximum permissible limits for the abnormal spermatozoa in bull semen were set over 70 years ago. Williams and Savage (1925) found that if abnormal spermatozoa exceeded 18%, fertility declined. Even though a wide variety of morphological abnormalities of spermatozoa have been reported, there is no clear experimental evidence of a relationship between specific morphological characteristics and fertility; however a high frequency of abnormal spermatozoa has been associated with reduced fertility (Sullivan, 1978). The maximum permissible limit for head abnormalities is set at 5%, and 20 % for total abnormalities (all categories). Any sample exceeding this limit is considered unfit for AI. Based on the above findings, it has been suggested that tests based on sperm qualitative parameters (concentration, morphology and viability) might be useful for the first step elimination of bad semen producers and development of tests based on sperm function might be necessary for selection of highly fertile semen producers.

2.11.2. Biochemical Parameters

Measurements of metabolic activity of spermatozoa have also been considered as possible predictors of fertility. The use of metabolic tests such as oxygen uptake

(Bishop and Salisbury, 1955), pyruvate oxidation (Melrose and Terner, 1953), fructolysis index (Secrist and Schultze, 1952), methylene blue reduction (Branton et al., 1951), and resazurin reduction (Erb and Ehlers, 1950) have been suggested, but were not found useful for routine evaluation of semen. Pace and Graham (1970) attempted to use enzyme-loss as fertility-index. They measured the release of glutamic-oxaloacetic-transaminase (GOT) from spermatozoa and found significant correlations between such measurements and fertility. A kit for rapidly and conveniently assessing sperm viability by measuring ATP loss is now commercially available (Sperm Viability Test, FireZyme Diagnostic Technologies Limited, Halifax, NS, Canada). This test uses an enzyme, Luciferase (derived from Fire-Fly), which oxidizes luciferin (substrate) in proportion to the concentration of ATP present, resulting in the emission of light. Since ATP disappears within seconds following cell death, only the viable spermatozoa will contain ATP to contribute to the light producing reaction. The light produced is measured in a bioluminometer. Major AI companies are currently using the kit priced at around $3,500.00 on a trail basis. Published information on the usefulness of this test for making fertility estimates is still not available. Although this method gives considerable accuracy in predicting fertility, the cost of this kit renders it less useful for routine semen analysis.

2.11.3. Swim-up Tests and Sperm Binding to Genital Epithelium

Spermatozoa have an innate ability to traverse fluids of a certain viscosity, which led to the so-called "swim-up tests" where spermatozoa are assessed for their capacity to pass through fluid barriers as happens in-vivo (Rodriguez – Martinez et al., 1997). Studies with frozen–thawed bull semen, using "swim-up tests" across a column

of culture medium have indicated the number of viable spermatozoa with linear motility and this test reflected the innate fertilizing ability of a semen sample (Zhang et al., 1998a). Binding of spermatozoa to oviductal epithelial cells prolongs their life in vitro, presumably because the binding occurs only with non-capacitated spermatozoa (Lefebvre and Suarez, 1996). Sperm co-culture with oviductal explants is being used to determine the capacity of a semen sample to colonize the reservoir with a marginal relation to fertility.

2.11.4. Sperm Capacitation and Acrosome Reaction

Once the acrosome reaction has occurred, spermatozoa tend to die rapidly. In order to prolong the life span of spermatozoa, which may be essential for successful fertilization, acrosomal integrity should be maintained. Saacke (1970) found a significant correlation between post thaw motile life and maintenance of the acrosome in bull spermatozoa. Saacke and White (1972) found a positive correlation between the percentage of intact acrosomes and non-returns of first insemination, whereas only a weak correlation was obtained when motility estimates were compared with non-returns. Following this report, many workers have examined the relationship between fertility and either acrosomal integrity or the ability of spermatozoa to undergo the acrosome reaction under in-vitro conditions (Ambrose et al., 1995; Whitfield and Parkinson, 1995; Januskauskas et al., 2000). There is strong evidence to show that the ability of spermatozoa to acrosome react under the influence of heparin or other agents like calcium ionophore A23187 or lysophosphatidylcholine has a definite relationship to fertility (Ax et al., 1985; Parrish et al., 1985; Ax and Lenz, 1986; Graham and Foote 1987a; 1987b; Whitfield and Parkinson, 1992). A relationship between the binding

affinity of heparin to spermatozoa and fertility has also been demonstrated (Marks and Ax, 1985; Lalich et al., 1989; Bellin et al., 1993). A test based on calcium ionophore-induced acrosome reaction in human spermatozoa has been found useful in identifying semen samples of sub-fertile/infertile men, indicating acrosomal dysfunction as a likely cause of fertilization failure. Cummins et al. (1991) have shown this test to have a predictive value for fertility. Results of these studies strongly suggest that the ability of spermatozoa to undergo the acrosome reaction in-vitro may be useful in predicting the fertility of bulls. Rajamahendran et al. (1994) demonstrated the use of anti-human sperm monoclonal antibody HS-11 as a marker to assess bovine sperm capacitation and acrosome reaction in-vitro. Ambrose et al. (1995) using the HS-11 antibody further concluded that a) between bull differences exist in HS-11 binding to spermatozoa and cleavage rate of embryos in vitro, and b) HS-11 binding to spermatozoa is correlated with fertility, as determined by the cleavage rate of bovine oocytes matured and fertilized in vitro.

2.11.5. Sperm Zona Binding/Accessory Sperm Counts

The effective binding of spermatozoa to the zona pellucida is a critical step in the process of fertilization that has sperm capacitation as a pre-requisite and relates to the acrosome reaction induced by the zona pellucida (Topper et al., 1999) being the rationale for in vitro sperm-zona binding tests. Using zona-binding tests significant correlations have been obtained with fertility in bulls (Zhang et al., 1998a). Spermatozoa that did not penetrate the zona pellucida entirely during fertilization (due to effective block to polyspermy by the oocyte) are trapped in the zona pellucida of the oocytes or early embryos and named "accessory spermatozoa". These spermatozoa have

demonstrated all the attributes needed to penetrate the zona pellucida and therefore been considered as potentially fertile (Saacke et al., 1998). The number of accessory spermatozoa bound to zona pellucida in-vivo significantly correlated with in-vivo fertilization rates of bulls (Nadir et al., 1993; Saacke et al., 2000), and therefore was used to measure the fertilizing capacity of a given semen sample. Although the sperm-zona binding assay showed a significant correlation with field fertility of bulls, the repeatability of this test was very low. This could be attributed to the testing procedure since frozen immature oocytes are used for this test procedure and the sperm receptor proteins on the zona pellucida undergo structural alterations by freezing procedure. Therefore, modification of the test procedure by using fresh matured oocytes might give higher repeatability in zona binding assays.

2.11.6. Oocyte Penetration Assays

Oocyte penetration assays (Bousquet et al., 1983; Boatman et al., 1988; Wheeler and Seidal, 1987; Graham and Foote, 1987a; 1987b; Fazeli et al., 1993; Fazeli et al., 1995) have been developed and examined as predictors of fertility. Normally, sperm of a species can penetrate ova only of the same species due to the species-specific barrier provided by the zona pellucida. However, the pioneering work of Yanagimachi (1972) demonstrated that capacitated and acrosome reacted spermatozoa of most species could penetrate hamster eggs whose zona has been removed in-vitro. For some unexplained reason, zona free hamster oocytes seem most receptive to spermatozoa from heterologous species. Zona free hamster eggs have been used to assess the fertilizing capacity of human spermatozoa, and have also been shown to allow penetration by the capacitated spermatozoa of various species including cattle (Bousquet and Brackett,

1982; Brackett et al., 1982a), horse (Brackett et al., 1982b), pig (Imai et al., 1980), sheep (Flechon and Pavlok, 1986), goat (Bou and Hanada, 1985) and buffalo (Takahashi et al., 1989). Attempts to determine the fertilizing capacity of cattle, using this system, and to rank breeding bulls in order of their fertility status were not successful since the results did not fit with the rank of bulls based on their non-return rates (Kruip et al., 1992).

2.11.7. Correlation Between In-vitro and In-vivo Fertility

Brackett et al. (1982a) was the first to report the birth of the first live calf produced by an in-vitro fertilization (IVF) techniques. Since then, the potential applications of bovine IVF have generated tremendous interest in this technology. As a result, though with limited success, bovine IVF has now become a feasible technology for the production of embryos for both research and commercial applications (Gordon and Lu, 1990; Trounson, 1992). Attempts have been made to correlate the results of bovine IVF with in-vivo fertility based on 60-90 day non-return-rates, but conflicting results have been obtained (Oghoda et al., 1988; Hillery et al., 1990; Marquant-Le Guienne et al., 1990). The non-return rate for a given bull is defined as that percentage of females not returning to estrus within a given period (usually 60-90 day) after being bred with semen from that bull. Thus, the higher the non-return rate, the better the fertility of the bull under question. Shamsuddin and Larsson (1993) found that 56-60 day non-return rates were significantly correlated with the first cleavage in-vitro, but further embryonic development in-vitro was not correlated with non-return rate.

2.12. RATIONALE FOR THE STUDY

Reproductive efficiency has important economical values in a dairy farm operation. The fertility of bulls used for AI has an important role in determining the reproductive efficiency. Huge expenditures are incurred by AI organizations in terms of time, labour and management costs to prove young bulls for their fertility and genetic merit. This indicates that significant advantages to the cattle industry as well as to the AI industry could be achievable, if simple laboratory tests were made available to predict fertility of young bulls recruited for progeny testing program. Although several laboratory semen evaluation methods have been developed over the past, most of them lack repeatability or precise prediction of bull fertility in the field. The success of the reproductive process is measured by the birth of viable calf. In this process sperm and egg fuse and the zygote develops in a highly controlled ordered stepwise process beginning with sperm capacitation, sperm-zona binding, sperm acrosome reaction, penetration, fusion, activation of embryonic genome and development of an embryo. Hence, the irregular occurrence of any of these processes is detrimental and reflected by low fertility. Since several new molecular techniques have been developed in the past to evaluate cell functions at gene expression levels, the embryo developmental capacity can be evaluated more accurately at molecular level than just evaluating morphology and sperm functions. Hence, it was decided to evaluate the fertility of bulls by determining sperm functions before fertilization (sperm-zona binding and acrosome reaction), at fertilization (sperm pre-incubation time and concentration on normal and polyspermic fertilization), and after fertilization (embryo development, apoptosis and gene expression).

2.13. HYPOTHESES

Although there have been several studies conducted to evaluate fertility of bulls accurately, few single sperm parameters have been found to significantly correlated with field fertility. The more the sperm parameters are tested, the more accurate will the prediction of bull fertility be. In this respect, the following hypotheses are proposed for this study.

1. Bovine in-vitro sperm acrosome reaction, sperm-zona binding and fertilization are partially male factor dependant and therefore will have a direct influence on fertility of bulls.
2. Viability of bovine spermatozoa within the female reproductive tract is partially male factor dependant and has a direct influence on fertility of bulls.
3. Apoptosis and gene expression of bovine pre-implantation embryos are partially male factor dependant and have a direct influence on fertility of bulls.

2.14. OBJECTIVES

The overall objective of the proposed study is to evaluate the fertility of bull using in vitro sperm function tests. Based on this general objective, the following specific objectives are proposed for this study.

1. To investigate the bull effect on sperm acrosome reaction, sperm-zona binding and in-vitro embryo production (experiment 1)
2. To study the effect of sperm pre-incubation time and sperm concentration of bulls on in-vitro fertilization (experiment 2).

3. To study the bull effect on apoptosis and expression of Bcl-2, Bax, p53, interferon tau and heat shock protein-70 genes in bovine in-vitro produced embryos (experiment 3).
4. To study the correlation between the above in-vitro tests and field fertility data.

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CHAPTER 3 – BULL EFFECTS ON SPERM ACROSOME REACTION, SPERM-ZONA BINDING AND IN-VITRO EMBRYO PRODUCTION*

3.1. PREFACE

Bull effect on sperm acrosome reaction, sperm-zona binding and in-vitro embryo production, and the correlation of this effect to field fertility measured by 60-90 day non-return rate were investigated in this study. Frozen semen from three separate ejaculates of eight genetically unrelated young bulls was used. Upon thawing, ejaculates from each bull were pooled, motile sperm separated by swim-up and a) subjected to an immunofluorescent assay at 0 and 4 h of incubation in capacitation media, to assess the acrosome status; b) used in an in-vitro fertilization assay system, to assess cleavage and blastocyst production rates; and c) a sperm-zona binding assay was carried out to determine the number of sperm bound to the zona pellucida of mature oocytes. Percentage of pre-freeze motile sperm (PrFM) and non-return rate data were obtained from an artificial insemination center. PrFM, percentage of acrosome reacted sperm at 0 h (AR1), increase in percentage of acrosome reacted sperm after 4 h (InAR), and sperm-zona binding rates (ZB) differed ($p<0.05$) among sperm samples obtained from different young bulls. Significant correlations ($p<0.05$) were observed between PrFM and AR1 ($r = -0.31$), InAR ($r = 0.36$), and ZB ($r = 0.32$). AR1 was negatively correlated to ZB ($r = -0.27$) and cleavage rate ($r = -0.20$), while InAR was positively correlated with ZB ($r =$

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0.31) and cleavage rate (r=0.26). None of the in-vitro tests was correlated with non-return rate. Our findings indicate that along with pre-freeze motility, a combination of in-vitro tests including the percentage of spontaneously acrosome reacted sperm at thawing, might be useful in predicting bull field fertility. Such a combination of assays, however, yet to be determined.

3.2. INTRODUCTION

Artificial insemination (AI) is one of the most successful reproductive technologies developed to improve reproductive efficiency of farm animals, especially dairy cattle. Accurate evaluation of the fertility of bulls used for AI purposes is of utmost importance since a single ejaculate provides hundreds of insemination doses and may have considerable influence on the reproductive potential of a herd (Rodriguez-Martinez and Larsson, 1998). Therefore, the basic purpose of semen evaluation procedures is to ensure that only good quality and highly fertile semen is used for AI purposes. Recording pregnancy data or measuring 60 day non-return rates provides the field fertility of processed semen from bulls (Zhang et al., 1999). A relatively high number of variables affect the success of these methods. From the paternal side, ejaculate, semen handling, storage, season and technician, and from the maternal side, estrus detection, farm management, parity and season influence the outcome of AI (reviewed by Foote, 2003). To increase the precision of this method one has to increase the number of animals inseminated, which is both costly and time consuming (reviewed by Foote 2003). Numerous methods have been developed for the laboratory evaluation of semen quality and fertility (Branton et al., 1951; Saacke, 1982; Budworth et al.,

1988; Critser and Noiles, 1993; Bailey et al., 1994; Collin et al., 2000). Some of these methods measure general characteristics of spermatozoa (i.e., viability, motility patterns, morphology, sperm metabolism, membrane and acrosome integrity) and among these, semen fertility appears to be more closely related to membrane integrity than to the other general characteristics (reviewed by Larsson and Rodriguez-Martinez, 2000).

Considering that fertilization is a complex process, semen samples can be subjected to various in-vitro tests that are related to the process of fertilization. Among these, selecting sperm by swim-up protocols, the ability of the sperm to bind genital epithelia, the ability to undergo sperm capacitation and acrosome reaction in-vitro, the ability to bind to the zona pellucida, accessory sperm counts on the zona pellucida, and in-vitro fertilization are potential predictors of fertility of bulls in the field (reviewed by Larsson and Rodriguez-Martinez, 2000). These tests are very important for young bulls recruited to a "progeny testing" program. It is well known that it often takes years before a young bull is proven for his genetic merit through progeny testing programs (Leitch, 1989; Norman et al., 2001, 2003). In the process, huge expenditures are incurred by the AI organizations in terms of time, labour and management costs. A major portion of the expenditure goes towards maintenance cost of bulls recruited to progeny testing programs, conducting extensive field insemination trials, non-return rate data collection, and payment of incentives to voluntary farmers (Leitch, 1989). Apart from these expenses, valuable space and materials are tied up to maintain young bulls and to store frozen semen straws obtained from young bulls that subsequently score poorly in progeny tests or show low field fertility and are consequently removed

from the young sire testing program. It is therefore of significant advantage to the cattle industry as well as to the AI industry if simple laboratory tests become available to predict fertility of young bulls recruited for progeny testing programs.

Thus, this study was designed to determine, whether the in-vitro sperm acrosome reaction, in-vitro fertilization and sperm-zona binding assay could be used as simple laboratory tests to predict fertility of young bulls recruited to a commercial progeny-testing program in Canada.

3.3. MATERIALS AND METHODS

Eight genetically unrelated young bulls (YS_1-YS_8) were randomly selected for this study based on their pedigree information over the last 4-5 generations. The age of the bulls, during collection, ranged from 13-17 months. Frozen semen from three separate ejaculates per bull collected at the beginning, middle and at the end of the test collection period was obtained from the British Columbia Artificial Insemination Center (Westgen). Sperm pre-freeze motility and 60-90 day non-return rate data for each bull recorded by the Westgen staff was also obtained. Ejaculates from each bull collected at the three different collection periods were thawed and pooled before being subjected to in-vitro tests described below to minimize variability among ejaculates. In all, nine replicate tests per bull were carried out.

3.3.1. In-vitro Fertilization (IVF) and Culture Assay

Bovine ovaries were collected at a local slaughterhouse and transported to the laboratory in normal saline (0.9% NaCl; Sigma-Aldrich Canada Ltd, Oakville, ON) supplemented with penicillin-G (100 IU/mL; Sigma-Aldrich Canada Ltd) and

streptomycin sulphate (0.2 μg/mL; Sigma-Aldrich Canada Ltd) at 30-32 °C in a thermos flask. Cumulus oocyte complexes from small follicles (< 7 mm) were aspirated using an 18-G needle and 10 mL syringe into a mixture of Dulbecco's phosphate buffered saline (DPBS; GIBCO BRL, Canadian Life Technologies, Burlington, ON), 0.3% bovine serum albumin (BSA; Sigma-Aldrich Canada Ltd) and 50 μg/mL gentamicin (Sigma-Aldrich Canada Ltd). Oocytes with an evenly granulated cytoplasm and surrounded by more than three layers of cumulus cells were selected for maturation. These oocytes were cultured in maturation medium for 24 h at 38.5 °C in humidified air containing 5% CO_2. The maturation medium consisted of tissue culture medium 199 (TCM199; Sigma-Aldrich Canada Ltd), 0.01 mg/mL follicle stimulating hormone (Folltropin; Vetrepharm Canada Inc., Belleville, ON), 5% superovulated cow serum (SCS; Boediono et al., 1994) and 50 μg/mL gentamicin. Straws containing frozen semen from a test bull, collected at three different times, were thawed at 37 °C, pooled and washed twice by centrifugation at 500 *g* for 5 min in Brackett and Oliphant medium (BO medium; Brackett and Oliphant 1975). The viable spermatozoa were swim-up separated, diluted to 5 x 10^6 sperm/mL in BO medium, and supplemented with 2.5 mM caffeine sodium benzoate (Sigma-Aldrich Canada Ltd) and 20 μg/mL heparin (Sigma-Aldrich Canada Ltd; Giritharan and Rajamahendran, 2001). Sperm droplets (100 μL) were prepared under mineral oil and pre-incubated at 38.5 °C in humidified air containing 5% CO_2 for 1 h. Twenty matured oocytes were placed in each of these semen droplets and incubated at 38.5 °C in humidified air containing 5% CO_2 for 16-18 h (Giritharan and Rajamahendran, 2001). The presumptive zygotes were then cultured in media prepared by mixing TCM-199, 5% SCS, 5 μg/mL insulin (Sigma-Aldrich

Canada Ltd) and 50 μg/mL gentamicin (Boediono et al., 1994) in four-well culture dishes at 38.5 °C in humidified air containing 5% CO_2. The culture media was changed every 72 h. Cleavage and blastocyst formation were assessed 72 h after insemination and 9 d of embryo culture, respectively (Plate 3.1.).

3.3.2. Sperm Acrosome Reaction (AR) Assay

Acrosome status of sperm from a test bull was assessed immediately after thawing (0 h) and after 4 h of incubation of the sperm sample (prepared for IVF assay) in capacitation medium, using an indirect immunofluorescent assay with fluorescein isothiocyanate (FITC) labeled Pisum sativum agglutinin (PSA; Sigma-Aldrich Canada Ltd; Cross and Meizel, 1989; Cross and Watson, 1994). Three separate smears per bull were prepared from the test sample (concentration 5 x 10^6 sperm per mL) after incubation at 0 and 4 h in capacitation medium. Five micro liter of semen preparation was placed on the wells of teflon coated multi-well slides, spread well to make thin smears and air-dried. The slides were then fixed in methanol for 10 min. Each smear was washed three times with BO medium containing 0.6% BSA and 50 μL of FITC labeled PSA (10 μg/mL) was placed on each smear. The slides were incubated in a dark humidifying chamber. After 45 min of incubation in the dark chamber, excess FITC labeled PSA solution was removed by pipetting and the smears were washed three times with BO medium containing 0.6% BSA. A drop of 80% glycerol diluted in DPBS was placed on the smear and covered with an appropriate cover slip. Slides were examined under a fluorescence microscope at x100 to x400 magnification using a B-2A filter (excitation filter of 450 – 490 nm and barrier filter of 520 nm) and the status of sperm acrosomes was assessed. The acrosomal region of acrosome-intact spermatozoa would

normally show a bright green fluorescent staining with FITC labeled PSA, whereas acrosome reacted spermatozoa would show no staining or a band of green fluorescence (Plate 3.2.; Cross and Meizel, 1989; Cross and Watson, 1994). Acrosome status was assessed on randomly selected fields until 100 spermatozoa were counted from each well of the slides. In each field, the number of total spermatozoa was counted under dark field illumination, immediately followed by the counting of FITC-labeled sperm under fluorescent light in a dark field to obtain the percent acrosome intact sperm.

3.3.3. Sperm Zona Binding (ZB) Assay

Thirty good quality mature oocytes were denuded with 0.1% hyaluronidase (Sigma-Aldrich Canada Ltd) by vortexing for 3 min. Denuded mature oocytes were placed (10 oocytes/bull) into 50 μL semen droplets (sperm concentration - 1 x 10^6 per ml) prepared for IVF assay, covered with mineral oil and incubated at 38.5 °C in humidified air containing 5% CO_2 for 4 h. After incubation, sperm oocyte complexes were removed and washed 10 times in DPBS containing 0.5% BSA to remove loosely attached sperm. Sperm oocyte complexes were then fixed in 2.5 % Glutaraldehyde (Sigma-Aldrich Canada Ltd) for 10 min and washed three times in DPBS containing 0.5 % BSA. Sperm oocyte complexes were thereafter incubated in 50 μL drops of HOECHST 33342 (bis-benzamide) stain solution (0.1 mg/mL; Sigma-Aldrich Canada Ltd) in a dark chamber for 10 min. Sperm-oocyte complexes were then transferred to a glass slide and were slightly compressed under a cover slip supported on the corners by Vaseline. Prepared slides were then stored in a dark humidified chamber until counting the sperm attached to zona pellucida. Sperm attached to zonae were counted under a

fluorescence microscope using UV-2A filter combination (excitation filter of 330 – 380 nm and barrier filter of 420 nm) at x 100 to x 400 magnification (Plate 3.2.).

3.3.4. Field Fertility Data

The field fertility data of all experimental bulls were obtained from Westgen to estimate the correlation between the field fertility and in-vitro tests performed. Field fertility data for each bull were based on 60-90 day non-return rate after the first insemination with semen of that particular bull. The number of inseminations ranged from 335 to 637.

3.3.5. Statistical Analyses

Data analysis was done by analysis of variance (ANOVA) after arcsine transformation of data using JMP statistical software (SAS Institute Inc., Sas Campus Drive, Cary, North Carolina 27513, USA). Mean separation procedure was performed using Fisher's LSD multiple comparison test when ANOVA showed significant F-Values. Non-return rate data were analyzed by Chi-square test using JMP statistical software. For all experiments, results are reported as the mean values for each set of data ± standard error of the means and the level of statistical significance was defined at a p value of less than 0.05. The Pearson pair-wise correlation was used to establish correlations between in-vitro tests and field fertility.

3.4. RESULTS

Results of this study are shown in Table 3.1. and 3.2. Pre-freeze motility of the sperm samples from different young bulls was significant ($p<0.05$) different. Sperm

from bull YS_1 (74.2±2.0 %) and YS_8 (69.8±0.8 %) showed highest and lowest pre-freeze motility, respectively.

Mean percent spontaneous acrosome reacted sperm at 0 h ranged from 28.9±2.1 to 44.5±1.7%. Sperm samples from bulls YS_4 and YS_1 showed highest and lowest acrosome reacted sperm, respectively. Sperm samples showed significant ($p<0.05$) differences in percent spontaneous acrosome reacted sperm at 0 h incubation due to bull. Sperm samples also showed significant ($p<0.05$) difference in the increase in acrosome reacted sperm at 4 h incubation due to bull. Sperm sample from bull YS_1 showed highest percentage increase in acrosome reacted sperm at 4 h.

The mean number of sperm from the test bulls bound to the zona pellucida ranged from 84.7±4.8 to 145.9±8.4. Sperm samples significantly ($p<0.05$) differed due to bull in their binding ability to the zona pellucida of in-vitro matured oocytes. Sperm from bulls YS_2 and YS_7 had highest binding, whereas sperm samples from bull YS_8 showed lowest binding. However, there was no significant difference in the percentage of cleaved embryos or blastocysts produced by fertilization of oocytes with sperm from different young bulls. In addition, chi-square analysis revealed that there was no significant difference among bulls in non-return rates.

Pre-freeze motility was positively correlated ($p<0.05$) with an increase in percentage of acrosome reacted sperm at 4 h ($r = 0.36$) and the number of sperm that bound to zonae ($r = 0.32$), and negatively correlated with percentage of acrosome reacted sperm at 0 h ($r = -0.31$). The percentage of acrosome reacted sperm at 0 h was negatively correlated with the number of sperm that bound to zonae ($r = -0.27$) and cleavage rate ($r = -0.20$). The increase in percentage of acrosome reacted sperm at 4 h

was positively correlated with the number of sperm that bound to zonae (r=0.31) and cleavage rate (r=0.26). None of the in-vitro tests was correlated with 60-90 day non-return rate.

3.5. DISCUSSION

Since sperm motility is necessary for successful fertilization, motility parameters are routinely used as a first step selection procedure to select semen samples for AI. However, the correlation of this parameter with fertility is questionable. Some studies have shown a positive correlation with field fertility (Januskauskas et. al., 2001), whereas others failed to show any correlation (Bailey et al., 1994; Zhang et al., 1999). In our study, although the percentage of motile sperm pre-freeze was not correlated with non-return rate, it was correlated positively with increase in the percentage of acrosome reacted sperm at 4 h post-thaw and the rate of sperm-zona binding. Conversely, the percentage of motile sperm before freezing was negatively correlated with the percentage of acrosome reacted sperm at 0 h post-thaw. This observation is in agreement with observations in a number of studies where sperm motility has been linked with in-vitro tests such as sperm morphology, sperm concentration, acrosome integrity, and sperm migration capacity (Correa et. al., 1997).

Several early reports have suggested that the ability of spermatozoa to undergo the acrosome reaction in-vitro may be useful in predicting the fertility of bulls (Ambrose et al., 1995; Whitfield and Parkinson, 1995; Januskauskas et al., 2000). On average, 30-40% sperm were spontaneously acrosome reacted at 0 h in our study, showing that membrane damage due to freezing procedures is very high in these semen

samples. Significant differences due to bull in percentage of acrosome reacted sperm at 0 h indicates that either membrane integrity of sperm varies among bulls or the incidence of membrane damage due to cryopreservation varies among bulls. When correlating the percentage of acrosome reacted sperm at 0 h to pre-freeze motility, sperm-zona binding and the increase in the percentage of acrosome reacted sperm at 4 h, negative correlations were observed. This shows that sperm having superior membrane integrity and motility are less susceptible to membrane cryo-damage. This finding is in agreement with studies reported earlier (Hammerstedt et al., 1990; Parks and Graham, 1992; Ambrose et al., 1995; Watson, 2000).

In our study, the pre-freeze sperm motility was very high in the sample from bull YS_1 and it also showed the lowest rate of spontaneous acrosome reactions at 0 h, highest net increase in the rate of acrosome reactions at 4 h, as well as highest sperm-zona binding rate. Moreover, cleavage and blastocyst development rates and the 60-90 day non-return rate also tended to be higher in this bull. This finding suggests that sperm with high pre-freeze motility can withstand cryopreservation, thus resulting higher in-vitro and in-vivo fertility.

The binding of sperm to the zona pellucida is an important step in the process of fertilization and appears correlated with in-vivo fertility in bulls (Zhang et al., 1998). However, in the present study, even though sperm from different bulls differed in binding to the zona pellucida, there was no correlation with 60-90 day non-return rate. Interestingly, poor correlations were also obtained between zona-binding test and in-vitro fertility as measured by cleavage and blastocyst production rates. However, significant correlations were obtained between the percentage of motile sperm pre-

freeze and sperm-zona binding as well as the rates of acrosome reacted sperm at 0 and 4 h pre-incubation.

Attempts have been made to correlate the results of in-vitro fertilization rates with in-vivo fertility based on 60-90 day non-return rates, but conflicting results have been reported (Ohgoda et al., 1988; Hillery et al., 1990; Shamsuddin and Larson, 1993). In the present study, there was no difference in cleavage rate or blastocyst development rate among embryos produced by in-vitro fertilization using sperm from the different bulls. Also, there was no correlation either between cleavage rate and non-return rate or between blastocyst development rate and non-return rate. This might indicate that the large number of sperm in the fertilization droplet masks the effect of bull on embryo development in vitro, because a very low number of sperm reaches the site of fertilization in in-vivo. Eyestone and First (1989), and Kjaestad and Stubbings (1992) reported similar findings after using larger, as well as smaller, numbers of sperm for in-vitro fertilization. Although there was no effect of bull on cleavage rate or blastocyst development rate among embryos produced by experimental bulls, cleavage rate was correlated with the increase in acrosome reacted sperm at 4 h incubation. The increase in acrosome reacted sperm at 4 h of incubation was also correlated to the sperm-zona binding rate. Neither the non-return rate nor the cleavage and blastocyst development rates were affected by bull.

Although several studies have tried to establish an in-vitro test to measure field fertility, few have showed high correlation with field fertility measured by non-return rate (Ambrose et. al., 1995; Zhang et. al., 1998). In our study, none of the in-vitro tests was correlated with non-return rate and this is in agreement with most of the other

studies (Linford et. al., 1976; Bailey et. al., 1994). Although the non-return rate gives the actual field fertility of a bull, several environmental and maternal factors influence the accuracy of this measurement. Using non-return rates corrected for ejaculate, season, inseminator and parity to minimize the influence of these factors Zhang et. al. (1999) showed highest correlation with in-vitro tests. The chance of obtaining a higher correlation between an in-vitro test and field fertility increases when there is a high variation in non-return rate between bulls (Linford et. al., 1976; Zhang et. al., 1999). This might be one of the reasons for the very poor correlation in our study, because the non-return rate of test bulls ranged only from 64.9 to 71.9 percent.

3.6. CONCLUSION

In conclusion, assays of sperm function such as acrosome reaction, zona binding ability and in-vitro fertilization showed between bull variations and inter function correlations. Hence, these sperm parameters may be potentially useful with routine semen analysis tests in predicting of field fertility. However, it remains to be established that these tests can be used to predict in-vivo fertility accurately. This justifies further research into the exact relationship between these parameters and field fertility. Calculation of a fertility index from the outcome of the combination of these tests might result in much accurate prediction of field fertility.

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Table 3.1. Percentage of motile sperm before freezing (PrFM), percentage of acrosome reacted sperm at 0 h (AR1) after thawing, increase in the percentage of acrosome reacted sperm at 4 h incubation in capacitation media (InAR), average number of sperm bound to zona pellucida of mature oocytes after 4 h co-incubation in in-vitro fertilization medium (ZB), percentage of inseminated oocytes that cleaved (CL), percentage of inseminated eggs that developed to the blastocyst stage after 9 d culture (BL) and 60-90 day non-return rates (NRR). Frozen semen from three separate ejaculates were thawed and pooled for each of eight unrelated young bulls (YS_1-YS_8) were pooled then used.

Bull ID	PrFM(%)[$] (Mean±SE)[w]	Acrosome Reaction (%)[$]		ZB[$] (Mean±SE)[z]	CL(%)[$]	BL(%)[$]	NRR(%)[*]
		AR1 (Mean±SE)[x]	InAR (Mean±SE)[y]				
YS_1	74.2±2.0*a*	28.9±2.1d	23.4±3.6*a*	123.4±8.4*bc*	67.2	19.4	71.3
YS_2	70.0±2.2*bc*	33.9±2.5c	22.9±3.1*ab*	145.9±8.4*a*	59.4	16.1	70.5
YS_3	71.7±1.1*abc*	36.1±3.6*bc*	16.8±3.0*bc*	103.5±9.3*cde*	59.4	14.4	68.4
YS_4	71.7±1.1*abc*	44.5±1.7*a*	18.1±1.1*abc*	110.4±9.5*bcd*	57.2	18.9	71.8
YS_5	71.0±1.0*abc*	37.8±3.0*bc*	20.1±4.0*ab*	105.1±8.6*cde*	56.1	15.0	71.2
YS_6	73.0±1.2*ab*	37.7±2.3*bc*	19.9±3.6*ab*	89.6±6.5*de*	60.6	20.6	64.9
YS_7	73.3±1.1*ab*	40.4±3.4a*b*	14.9±2.4*c*	130.1±7.8*ab*	61.1	17.8	67.5
YS_8	69.2±0.8*c*	39.9±3.2a*b*	17.9±3.5*abc*	84.7±4.8*e*	61.1	22.2	71.9

a,b,c,d,e - Means with different letters within a column differ ($P<0.05$).

w,x,y,z – Arithmetic mean and standard error of mean for percentage of motile sperm before freezing, percentage of acrosome reacted sperm 0 h after thawing, percentage of acrosome reacted sperm 4 h after incubation in capacitation medium, and number of sperm bound per matured oocyte, respectively.

[$] - Data within these columns are based nine replicates (n = 9)

* - The non-return rates are based on the number of inseminations ranged from 335 to 637.

Table 3.2. Pair-wise comparison of sperm pre-freeze motility (PrFM), sperm acrosome reaction at 0 h (AR1), increase in sperm acrosome reaction from 0 to 4 h (InAR), sperm-zona binding (ZB), embryo cleavage (CL). blastocyst production (BL) and 60-90 day non-return (NRR) rates.

	PrFM	AR1	InAR	ZB	CL	BL
AR1	-0.31*					
InAR	0.36*	-0.47*				
ZB	0.32*	-0.27*	0.31*			
CL	0.12	-0.20*	0.26*	0.15		
BL	0.03	0.10	0.01	0.19	0.41*	
NRR	-0.46	-0.02	0.29	0.14	-0.04	0.01

* comparisons showing significant correlation at $P<0.05$.

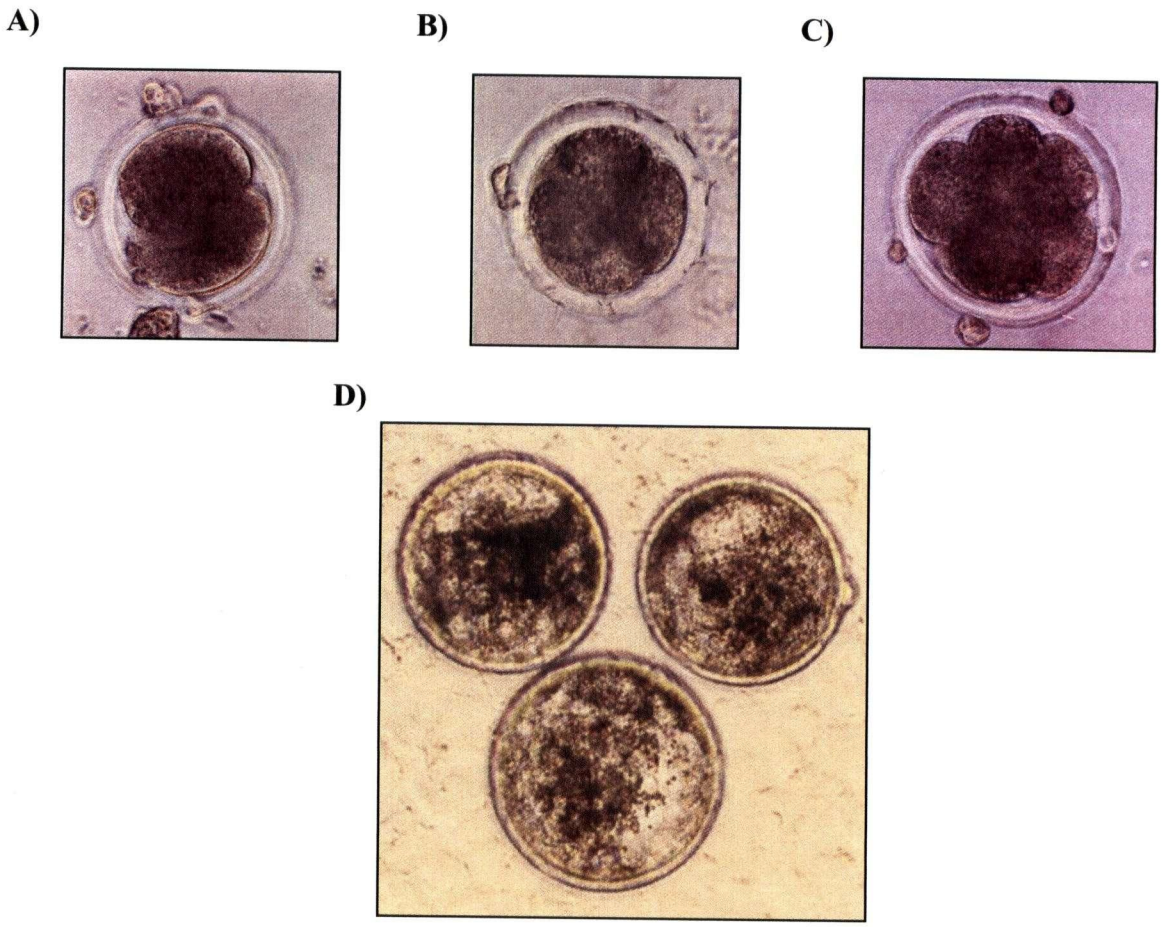
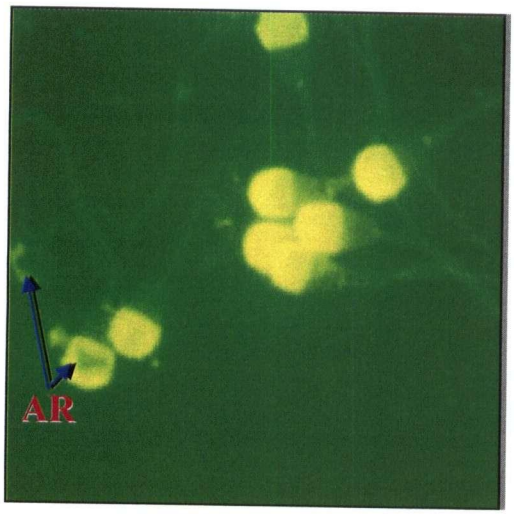
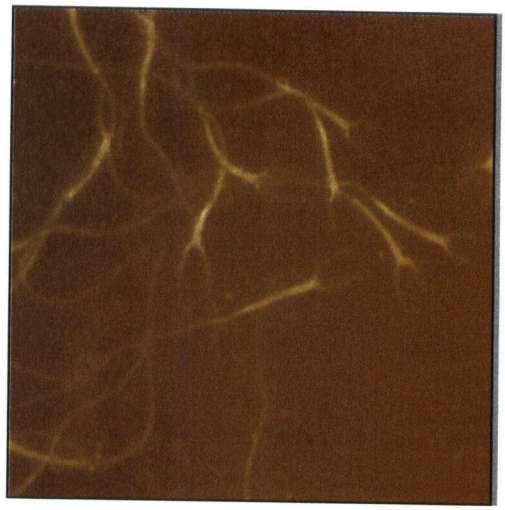


Plate 3.1. Light microscopic images of bovine 2-cell **A)**, 4-cell **B)**, 8-cell **C)**, and blastocyst **D)** stage embryos. The photographs were taken at x400 magnification.

A)



B)



C)

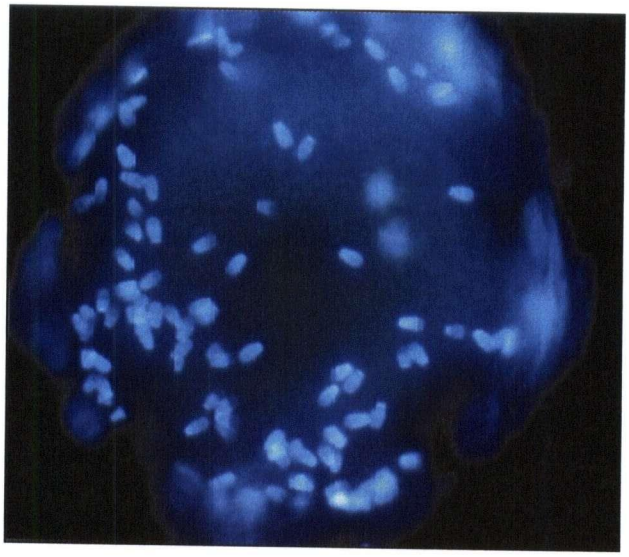


Plate 3.2. Fluorescence **A)**, and light microscopic **B)**, images of bovine spermatozoa, and spermatozoa bound to zona pellucida of mature oocytes **C)** stained by fluorescein isothiocyanate coated pisum sativam agglutinins and bis-benzamide, respectively. The photographs were taken at 400x magnification using B-2A (excitation filter of 450 – 490 nm and barrier filter of 520 nm) and UV-2A (excitation filter of 330 – 380 nm and barrier filter of 420 nm) filter combination and dark field illumination **B)**. The sperm acrosome shows green fluorescence and bis-benzamide stained sperm heads shows blue fluorescence. The arrow indicates partial and complete acrosome reaction.

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CHAPTER 4 – THE EFFECT OF SPERM PRE-INCUBATION TIME AND SPERM CONCENTRATION OF BULLS ON IN-VITRO FERTILIZATION

4.1. PREFACE

In this study, the bull effect on in-vitro fertilization (including normal and polyspermy) was evaluated using normal (25,000:1) and high (50,000:1) sperm:oocyte ratio with short (0 h) and long (6 h) sperm pre-incubation in capacitation medium. The degree of correlation of this effect with the non-return rates, sperm pre-freeze motility and other in-vitro sperm parameters obtained in the first experiment (Chapter 3) was also investigated. In addition to this, data were pooled to determine the effect of sperm concentration and pre-incubation time on in-vitro fertilization. Frozen semen from two separate ejaculates of six unrelated bulls was used. Ejaculates from each bull were alternately used for in-vitro fertilization in six replicates (3 from one set of ejaculates and other 3 from second set of ejaculates) to minimize variability among ejaculates. Oocytes obtained from slaughterhouse ovaries were matured and co-incubated with sperm from experimental bulls using a normal (25,000:1) and high (50,000:1) sperm:oocyte ratio and short (0 h) and long (6 h) sperm pre-incubation in capacitation medium at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. After 14-16 h exposure to sperm, presumptive zygotes were stained with bisbenzamide and the percentage of fertilized oocytes containing 2 pronuclei and more than 2 pronuclei was determined. The non-return rates and sperm pre-freeze motility data of the experimental

bulls were obtained from the records of the artificial insemination center. The sperm acrosome reaction, sperm-zona binding and in-vitro embryo development data were obtained from the first experiment (Chapter 3). Percentage of zygotes (including both normal and polyspermic) and normally fertilized zygotes containing 2 pronuclei was different among bulls with short sperm pre-incubation in normal ($p<0.01$) and high ($p<0.06$) sperm:oocyte ratios. Percentage of zygotes was different among bulls with long sperm pre-incubation in high sperm:oocyte ratio ($p<0.05$), but not in the normal sperm:oocyte ratio. However, percentage of normally fertilized zygotes was different among bulls with long sperm pre-incubation in normal sperm:oocyte ratio ($p<0.05$), but not in the high sperm:oocyte ratio. However, the percentage of polyspermic zygotes was different among bulls with long sperm pre-incubation in the high sperm:oocyte ratio ($p<0.05$). The percentage of difference in normally fertilized zygotes between short and long sperm pre-incubation in the normal sperm:oocyte ratio showed a high degree of correlation with non-return rates ($r = 0.90$; $p<0.05$) of the experimental bulls. A high degree of correlation was also observed between sperm pre-freeze motility, and percentage of zygotes ($r = 0.88$; $p<0.05$) and normally fertilized zygotes ($r = 0.85$; $p<0.05$) with the long sperm pre-incubation in the normal sperm:oocyte ratio. Increase in acrosome reaction at 4 h was highly correlated with percentage of polyspermic zygotes when using long sperm pre-incubation in normal sperm:oocyte ratio ($r = 0.93$; $p<0.05$). The cleavage rate was correlated with the percentage of zygotes when using long sperm pre-incubation in the normal sperm:oocyte ratio. The percentage of difference in normally fertilized zygotes between short and long sperm pre-incubation in the high sperm:oocyte ratio had a correlation with blastocyst production rate of the

experimental bulls ($r = 85$; $p<0.05$). Increase in sperm concentration and pre-incubation time resulted significant ($p<0.01$) increases and decreases in percentage of zygotes and normally fertilized zygotes, respectively. The present study concludes that the sperm of bulls showing high non-return rates exhibits a significant reduction in in-vitro fertilizing ability within 6 h of pre-incubation in capacitation medium.

4.2. INTRODUCTION

Every year the dairy industry loses a greater amount of revenue primarily due to reproductive inefficiency either by fertilization failure due to misdiagnosis of estrus, inappropriate timing of AI, or early embryonic mortality of unknown causes (Senger, 1994). It has also been suggested that failure of breeding with low fertility bulls is due to fertilization failure and that of high fertility bulls is due to embryonic death of unknown causes (Saacke et al., 2000). Bulls differ in the ability of their sperm to survive in the female reproductive tract during the interval between insemination and ovulation and very big differences in fertility are observed when cows are inseminated early in the estrus than when they are inseminated at a later stage of estrus (Shannon, 1978; Maatje et al., 1997). Both of these differences are found when frozen and fresh semen from bulls with low fertility are compared to those with high fertility (Shannon 1978). Hence, the fertility of bulls in the field is mainly dependent on the viability of sperm in the female reproductive tract and the evaluation of the fertile lifespan of sperm might be helpful in accurate laboratory prediction of field fertility of bulls.

Mammalian fertilization is a complex process, in which the sperm and egg unite, thereby, restoring the somatic chromosome number and developing a new individual

exhibiting the characteristics of the species (reviewed by Yanagimachi, 1994; Wassarman, 1999). Egg and sperm undergo a series of maturational changes before they fuse successfully and form a viable zygote during the fertilization process (Wassarman and Albertini, 1994; Visconti and Kopf, 1998). During maturation, sperm undergoes capacitation to gain fertilizing capacity, and the oocyte undergoes nuclear and cytoplasmic maturation in which the cortical granules move towards periphery of the oocyte. Hence, success of the fertilization process depends on the correct timing of insemination to allow sufficient time for the sperm and egg to undergo maturational changes before fertilization. Generally, there are two technical difficulties associated with studies designed to evaluate the optimal time of artificial insemination. These are inadequate numbers of cows for valid statistical comparisons (Trimberger and Davis, 1943; Trimberger 1948; Maatje et al., 1997) and knowledge of the onset of estrus due to the low frequency and efficiency of the methods used for estrus detection (Foote, 1978; Nebel et al., 1994). The fertile lifespan of sperm and egg, the transport time of viable sperm from the site of artificial insemination to fertilization, and the ovulation time in association with artificial insemination are the critical biological events that influence the timing of artificial insemination and fertilization. Studies by Rajamahendran et al. (1989) and Walker et al. (1996) showed that the interval from the onset of estrus to ovulation was 28-36 h. The transport of viable sperm to the oviduct requires a minimum of 6 h to obtain a sperm population capable of fertilization; sperm numbers in the oviduct progressively increase over 8 to 18 h (Thibault, 1973; Wilmut and Hunter, 1984; Hawk, 1987). The chance of pregnancy was highest when artificial insemination was performed 11.8 h after the onset of estrus signs (Maatje et al., 1997). Therefore,

successful fertilization appears to depend on the functional lifespan of the sperm during transport in the female reproductive tract. The fertility due to number of functional viable sperm at the site of fertilization varies with bull and sperm concentration used for insemination (den Daas et al., 1998). Non-return rates are low when too many as well as too few sperm are used for artificial insemination (Foote, 1970; Saäcke, 1982) Hence, studies based on sperm parameters such as sperm concentration and in-vitro fertilizing capacity after pre-incubation for 6 h may reveal the fertilizing potential of sperm from different bulls in-vivo.

In the fertilization process, the binding of sperm to the zona pellucida of the mature oocyte initiates the sperm acrosome reaction and the release of acrosomal contents. With the help of acrosomal contents, the capacitated sperm penetrate through the zona pellucida and bind with the vitelline membrane of the oocyte. This initiates the release of cortical granules into peri-vitelline space, which causes chemical changes in the zona pellucida, the zona block, to prevent the entry of other sperm (Cherr and Ducibella, 1990; Wassarman, 1999). Defects in the process of the zona block resulting in the penetration of more than one sperm can lead to polyspermy (Hyttel et al., 1986; Cherr and Ducibella, 1990; Ducibella, 1996). Polyspermy is the most prevalent abnormal fertilization procedure in which more than one sperm enters into a mature female egg and forms polyploidic zygotes (Xu and Greve, 1988; Saeki et al., 1991). The polyspermic zygotes rarely develop beyond morula and blastocyst stages or develop as androgenotes (Iwasaki et al., 1989; Pinto-Correia et al., 1992; Long et al., 1993). Improper maturation of oocytes (Niwa et al., 1991; Chian et al., 1992; Long et al., 1994; Agca et al., 2000), concentration of sperm (Long et al., 1994), source of sperm

(Kreysing et al., 1997), in-vitro fertilization medium (Tajik et al., 1993; Pavlok, 2000) and sperm oocyte co-incubation time (Long et al., 1994) have been shown to influence fertilization and polyspermy. Some of the above factors, which are determined by time of insemination and the quality of semen sample, influence the in-vivo fertilizing capacity of sperm (Dransfield et al., 1998).

In this study, it is hypothesized that the fertile lifespan of bovine sperm within the female reproductive tract is male factor dependant and has a direct influence on bull fertility. The objective of this study is to determine the effect of bull on in-vitro fertilization with sperm pre-incubation time and concentration, the relationship of these sperm fertilization parameters to in-vivo fertility and other laboratory parameters of sperm, and the effect of sperm pre-incubation and concentration on in-vitro fertilization.

4.3. MATERIALS AND METHODS

4.3.1. Experimental Design

Six genetically unrelated young bulls (S_1-S_6) were randomly selected for this study based on their pedigree information over the last 4-5 generations. Frozen semen from two separate ejaculates per young bull collected at two different collection periods was obtained from a local AI center. Sperm from one set of ejaculates were used at a time with 0 and 6 h pre-incubation time before being subjected to in-vitro fertilization in a sperm:oocyte ratio of 25,000 to 1 and 50,000 to 1 as described below. Both ejaculates were used alternately for in-vitro fertilization to minimize variability between ejaculates in six replicates (3 from one set of ejaculates and other 3 from the second set of ejaculates) using 240 oocytes per bull.

4.3.2. In-vitro Fertilization

Bovine ovaries were collected at a local slaughterhouse and transported to the laboratory in normal saline (0.9% NaCl; Sigma-Aldrich Canada Ltd, Oakville, ON) supplemented with penicillin-G (100 IU/mL; Sigma-Aldrich Canada Ltd) and streptomycin sulphate (0.2 μg/mL; Sigma-Aldrich Canada Ltd) at 30-32 °C in a thermos flask. Cumulus oocyte complexes from small 2-8 mm follicles were collected into an aspiration medium using an 18-G needle and a 10-mL syringe. The medium contained Dulbecco's phosphate buffered saline (DPBS; GIBCO BRL, Canadian Life Technologies, Burlington, ON), 0.3% bovine serum albumin (BSA; Sigma-Aldrich Canada Ltd) and 50 μg/mL gentamicin (Sigma-Aldrich Canada Ltd). Oocytes with an evenly granulated cytoplasm and surrounded by more than three layers of cumulus cells were selected for maturation. These oocytes were cultured in maturation medium for 24 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. The maturation medium consisted of tissue culture medium 199 (TCM199; Sigma-Aldrich Canada Ltd), 0.01 mg/mL follicle stimulating hormone, 5% superovulated cow serum (SCS; Boediono et al., 1994) and 50 μg/mL gentamicin. Frozen semen from different experimental bulls was thawed at 37 °C, washed twice by centrifugation at 500g for 5 min, diluted to 5 x 10^6 sperm/mL and 10 x 10^6 sperm/mL in Brackett and Oliphant medium (Brackett and Oliphant, 1975), and supplemented with 2.5 mM caffeine sodium benzoate (Sigma-Aldrich Canada Ltd), 0.3% BSA and 20 μg/mL heparin (Sigma-Aldrich Canada Ltd). Sperm droplets (50 μL) were prepared under mineral oil and pre-incubated at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 0 and 6 h. Ten matured oocytes were placed in each of these semen droplets and incubated at 38.5 °C in a humidified

atmosphere of 5% CO_2 in air. After 14–16 h exposure to sperm, the cumulus cells attached to presumptive zygotes were removed by vortexing for 2-3 min and the denuded presumptive zygotes were washed three times in DPBS supplemented with 0.3 % BSA and stained by nuclear staining procedure described below

4.3.3. Nuclear Staining

The denuded presumptive zygotes were washed three times in DPBS solution containing 0.25% pronase (Sigma-Aldrich Canada Ltd) to remove accessory sperm and then fixed in a 2.5% glutaraldehyde solution (Sigma-Aldrich Canada Ltd) for 10 min. The presumptive zygotes were then washed three times in DPBS solution supplemented with 0.3% BSA to remove the glutaraldehyde and incubated in 50 μL drops of 10 μg/mL bisbenzamide stain (Sigma-Aldrich Canada Ltd) at 38.5 °C in a dark humidified atmosphere of 5% CO_2 in air. After 10 min incubation, presumptive zygotes were washed three times in DPBS solution supplemented with 0.3% BSA before being transferred to a clean glass slide, and slightly compressed under cover slip supported on the corners by a mixture vaseline and mineral oil (Sigma-Aldrich Canada Ltd). The slides were stored in dark humidified chamber until counting the number of pronuclei under the fluorescent microscope using UV-2A filter combination (excitation filter of 330 – 380 nm and barrier filter of 420 nm) at 100 to 400 times magnification. The oocytes containing either a bright blue germinal vesicle, or one polar body and metaphase spindle were considered as unfertilized (Plate 4.1.). The oocytes containing 2 bright blue polar bodies in the peri-vitelline space and 2 or more than 2 bright blue pronuclei were considered fertilized and thus called zygotes (Plate 4.1.). The zygotes

containing 2 polar bodies and 2 pronuclei were considered normally fertilized and those containing 2 polar bodies and more than 2 pronuclei were considered polyspermic.

4.3.4. Field Fertility Data and Other Sperm Parameters

The field fertility and pre-freeze motility data of all the experimental bulls in question were obtained from the records of the AI center in order to estimate the degree of correlation between the field fertility and in-vitro fertilization parameters. The field fertility data for each bull were based on 60-90 day non-return rate to first insemination with semen of that particular bull. The number of inseminations ranged from 335 to 637. The sperm acrosome reaction, sperm-zona binding and the in-vitro embryo production data used in this study were obtained from the previous experiment (Chapter 3).

4.3.5. Statistical Analyses

Analysis of variance was used to compare the sperm concentration and pre-incubation time related effects of bull on in-vitro fertilization. An arcsine transformation was conducted on all percentage data before analysis and a Fisher's Least Significant Difference test was used to locate differences among experimental bulls of all treatment groups. Pearson's correlation coefficient was used to determine the degree of correlation between in-vivo fertilization and in-vitro fertilization parameters.

4.4. RESULTS

4.4.1. In-vitro Fertilization Rates

The in-vitro fertilization results of this study are summarized in table 4.1. Percentage of zygotes was different among bulls in the treatment groups where 0 h pre-

incubated sperm was used in a sperm:oocyte ratio of 25,000:1 ($p<0.01$) and 50,000:1 ($p<0.05$). Percentage of normally fertilized zygotes containing 2 pronuclei was also different among bulls in the treatment groups where 0 h pre-incubated sperm were used in a sperm:oocyte ratio of 25,000:1 ($p<0.01$) and 50,000:1 ($p=0.062$). Percentage of zygotes was different among bulls in the treatment groups where 6 h pre-incubated sperm was used in a sperm:oocyte ratio of 50,000:1 ($p<0.05$). Percentage of normally fertilized zygotes was different among bulls in the treatment groups where 6 h pre-incubated sperm was used in a sperm:oocyte ratio of 25,000:1 ($p<0.05$). However, percentage of zygotes in the treatment groups where 6 h pre-incubated sperm was used in a sperm:oocyte ratio of 25000:1 and percentage of normally fertilized zygotes in the treatment groups where 6 h pre-incubated sperm was used in a sperm:oocyte ratio of 50,000:1 were not different among bulls. Whereas, percentage of polyspermic zygotes was different among bulls in the treatment groups where 6 h pre-incubated sperm was used in a sperm:oocyte ratio of 50,000:1 ($p<0.05$). Percentage of polyspermic zygotes in the treatment groups where 0 h and 6 h pre-incubated sperm was used in a sperm:oocyte ratio of 25,000:1 was not different among bulls.

The effect of sperm concentration and sperm pre-incubation time on percentage of zygotes, and normally fertilized and polyspermic zygotes was determined by pooled data and showed in Fig. 4.1. and 4.2. When the sperm:oocyte ratio is increased from 25,000:1 to 50,000:1 the percentage of zygotes as well as normally fertilized zygotes increased in all experimental bulls (56.4±2.1, 68.5±2.0; 51.8±1.9, 61.9±1.7, respectively; $p<0.01$). When sperm pre-incubation time increased from 0 to 6 h the

percentage of zygotes and normally fertilized zygotes was reduced in all experimental bulls (68.3±2.1, 56.5±2.1; 61.8±1.9, 51.9±1.8, respectively; $p<0.01$).

4.4.2. Correlation Between Sperm Parameters

The sperm parameters showing significant correlations are shown in Table 4.2. The percent differences in normally fertilized zygotes between treatment groups where 0 h and 6 h pre-incubated sperm were used in a sperm:oocyte ratio of 25,000:1 showed a high degree of correlation with non-return rates ($r = 0.90$; $p<0.05$) of the experimental bulls. High degrees of correlation were also observed between sperm pre-freeze motility, and percentage of zygotes ($r = 0.88$; $p<0.05$) and normally fertilized ($r = 0.85$; $p<0.05$) zygotes in the treatment group where 6 h pre-incubated sperm were used in a sperm:oocyte ratio of 25,000:1. Increase in acrosome reaction in 4 h pre-incubated sperm was highly correlated with percentage of polyspermic zygotes in the treatment group in which 6 h pre-incubated sperm was used in a sperm:oocyte ratio of 25,000:1 ($r = 0.93$; $p<0.05$). The cleavage rate showed a high degree of correlation with percentage of zygotes in the treatment group in which 6 h pre-incubated sperm was used in a sperm:oocyte ratio of 25000:1 ($r = 0.90$; $p<0.05$). The percent differences in normally fertilized zygotes between treatment groups where 0 h and 6 h pre-incubated sperm were used in a sperm:oocyte ratio of 50,000:1 showed a high degree of correlation with blastocyst production rate of the experimental bulls ($r = 0.85$; $p<0.05$). The sperm-zona binding was not correlated with any of the sperm in-vitro fertilization parameters.

4.5. DISCUSSION

In the present study, a very efficient and less time consuming nuclear staining technique was used to determine the effect of bull on in-vitro fertilization using short (0 h) and long (6 h) sperm pre-incubation time and normal (25,000:1) and high (50,000:1) sperm:oocyte ratios. The degrees of correlation between these effects on in-vitro fertilization, and in-vivo fertility of bulls measured by 60-90 day non-return rates and other sperm parameters measured in the laboratory were also assessed. In addition, using pooled data of all the bulls, the effect of sperm pre-incubation and sperm concentration on in-vitro fertilization was determined. This is the first study, which showed very high positive correlation between non-return rate and percent difference in normally fertilized zygotes with the increase in sperm pre-incubation time from 0 to 6 h using normal sperm:oocyte ratio. This indicates that when non-return rates go up the fertile life span of sperm in in-vitro capacitation medium goes down with the increase in sperm pre-incubation time.

Male dependent variability in in-vitro as well as in-vivo fertilization rates has been observed despite intensive testing and selection of bulls for high fertilization rates (Hillery et al., 1990; Zhang et al. 1995; Kurtu et al., 1996; Larocca et al., 1996; Kreysing et al., 1997). Similarly, a marked variability among individual bulls and different ejaculates from the same bull in their suitability for in-vitro embryo production has been reported (Brackett et al.1982; Ohgoda et al., 1988; Kurtu et al. 1996). In the present study, when using normal and high sperm:oocyte ratio, sperm from different experimental bulls showed significant differences in fertilizing capacity with short (0 h) pre-incubation. This supports the findings in the recent studies where sperm

concentrations ranging from 0.065 to 0.5 x 10^6 sperm/ml were used, and significant differences in fertilization rates of the sperm from bulls having non-return rates range from 57 to 78.5 was shown (Ward et al., 2002; 2003). A high degree of correlation between non-return rates and in-vitro fertilization rates at a concentration of 0.5 x 10^6 sperm/ml was also demonstrated. However, in the present study bull effect on the fertilization at short sperm pre-incubation was not correlated with non-return rates with normal and high sperm:oocyte ratio. This can be attributed to the high concentration (5 x 10^6 sperm/ml) used in the in-vitro fertilization procedure of present study. In the current study, when using normal sperm:oocyte ratio the polyspermic fertilization was not significantly different among sperm from different experimental bulls in both short and long (6 h) pre-incubation groups. This indicates that polyspermic fertilization is not affected by sperm pre-incubation time at normal sperm:oocyte ratio and this is in agreement with the research findings by Saeki et al. (1995).

The exposure of oocytes to high numbers of capacitated sperm has been shown to increase the incidence of polyspermy (Hunter 1991; Kim et al., 1997). In the present study, in high sperm:oocyte ratio, different experimental bulls showed significant differences in their fertilizing capacity in the short and long pre-incubation groups. However, normal fertilization was not significantly different among bulls in the long pre-incubation group. When using the high sperm:oocyte ratio with short sperm pre-incubation, bulls marginally affected the polyspermic fertilization. However, bulls significantly affected polyspermic fertilization with long sperm pre-incubation and high sperm-oocyte ratio. Based on in-vivo and in-vitro studies it has been documented that a high number of sperm in the inseminate increases availability of viable and fertile

sperm for fertilization, and thereby increases fertilization rates (Koops et al., 1995; Shannon and Vishwanath, 1995; Heeres et al., 1996; Kommisrud et al., 1996; Fearon and Wegener, 2000; Ward et al., 2003). This is the case in the present study in which the increase from normal to high sperm:oocyte ratio significantly increased the percentage of zygotes and normally fertilized zygotes in both short and long sperm pre-incubation groups.

The increase in sperm pre-incubation time from 0 to 6 h significantly reduced the percentage of fertilized as well as normally fertilized zygotes in both low and high sperm:oocyte ratios. This indicates that either in-vitro fertilizing capacity or viability of the sperm is significantly affected by longer pre-incubation in sperm capacitation medium. This supports the findings of the recent studies in which the fertile lifespan of sperm was assessed in-vitro with various media supplements after 8 h pre-incubation (Fukui et al., 1990; Gliedt et al., 1996; Pavlok 2000; Lechniak et al., 2003). The bovine serum albumin and heparin supplements in the sperm pre-incubation and the in-vitro fertilization media significantly reduced the percentage of penetrated oocytes, after 8 h pre-incubation only 3.5 % of the oocytes showed penetration. However, in the present experiment, the sperm pre-incubation and in-vitro fertilization in the same medium also showed a reduction in fertilization rate after a longer sperm pre-incubation, but with 56.5% zygotes showing two or more than two pronuclei. This indicates that the reduction in the in-vitro fertilizing capacity with sperm pre-incubation varies with experimental conditions in the different laboratories and is very high after 6 h pre-incubation in capacitation medium.

In the current study, the bulls showing higher non-return rates showed significant reductions in in-vitro fertilizing capacity with increased sperm pre-incubation time with the normal sperm:oocyte ratio. A very high positive correlation was observed between non-return rate and percentage difference in normally fertilized zygotes with the increase in sperm pre-incubation time from 0 to 6 h using normal sperm:oocyte ratio. This indicates that sperm from these bulls lose their fertilizing capacity rapidly and if they are present in large numbers, it will result in abnormal fertilization with more than two pronuclei. This is documented in the present study in which sperm from bulls showing higher non-return rates tended to show higher percentages of polyspermic fertilization in normal as well as high sperm:oocytes ratio. In the in-vitro fertilization system, an increase in the time of sperm:oocyte co-culture increases the incidence of oocyte penetration and polyspermy (Chian et al., 1992; Sumantri et al., 1997; Kreysing et al., 1997). Hence, exposure of oocytes to high numbers of fertile sperm for a long period increases the chances of polyspermic fertilization. The polyspermic embryos undergo degeneration within two weeks after fertilization.

In the present study, very high positive correlation was observed between non-return rate and percentage difference in normally fertilized zygotes with the increase in sperm pre-incubation time from 0 to 6 h using normal sperm:oocyte ratio. This might be an indication that only very good quality sperm fertilize the oocytes and most of the defective sperm lose their viability after 6 h in-vitro pre-incubation. This notion supports the previous finding that the optimum time for artificial insemination is 4-14 h after the onset of estrus. Because if the animal is inseminated 4-14 h after onset of

estrus, only the good quality sperm, which show long fertile lifespan, fertilize the oocytes and most of the defective sperm are neither fertile nor viable at the time of ovulation (Dransfield et al., 1998; Nebel et al., 2000). In addition, the fertilized zygote is surrounded by a very small number of viable as well as highly fertile sperm if the animal is inseminated early in estrus. This concept is also supported by previous findings in which animals inseminated 24 h after the onset of estrus, although showing high fertilization rates and high numbers of accessory sperms, yielded poor quality blastocysts compared to 0 and 12 h insemination groups (Foote, 1978; Dalton et al., 2001). This is attributed to the availability of high numbers of viable and fertile sperm at the time of fertilization in the animals inseminated 24 h after the onset of estrus.

Motility is a very important characteristic of sperm to help reaching the oocyte after transport through the female reproductive tract and the cumulus investment of the oocyte to bind and penetrate through the zona in a successful fertilization process. Hence, sperm motility is routinely used as evaluation criteria for the selection of bulls with varying degrees of correlation to field fertility (Bailey et al., 1994; Correa et al., 1997). In the current study, although the pre-freeze motility is not correlated with field fertility, it is correlated with percentage of zygotes and normally fertilized zygotes in the treatment group where longer pre-incubated sperm were used in normal sperm:oocyte ratio. A high degree of correlation was observed between percentage of polyspermic zygotes produced by using longer sperm pre-incubation with normal sperm:oocyte ratio, and increase in acrosome reaction in 4 h pre-incubated sperm. This supports the previous findings in which the exposure of oocytes to a high number of capacitated sperm has been shown to increase the incidence of polyspermy (Hunter

1991; Kim et al., 1997). The cleavage rate of the experimental bulls showed a high degree of correlation with the percentage of zygotes produced by using longer sperm pre-incubation with normal sperm:oocyte ratio. The percentage of difference in normally fertilized zygotes between treatment groups where short and long pre-incubated sperm were used in a high sperm:oocyte ratio showed a high degree of correlation with the blastocyst production rates of the experimental bulls. This supports the research findings of Zhang et al. (1999) and Ward et al. (2003) in which the cleavage and blastocyst production rates are correlated with field fertility measured by 60-90 day non-return rates. This correlation changes with number of sperm used for in-vitro fertilization.

4.6. CONCLUSION

In the present study, bull influence on fertile lifespan of sperm was assessed by in-vitro fertilization technique using 0 and 6 h pre-incubated sperm in sperm:oocyte ratios of 25,000:1 and 50,000:1. Based on this study, it is concluded that the fertile lifespan of sperm from bulls showing higher field fertility measured by 60-90 day non-return rates reduces with in-vitro pre-incubation in capacitation medium. This measure may be potentially used for the prediction of bull fertility in the field. The increase in pre-incubation time reduces in-vitro fertilizing capacity of sperm, whereas increase in the concentration increases the in-vitro fertilizing capacity of sperm.

Table 4.1. Effect of sperm pre-incubation time and sperm concentration of bulls on the percentage of fertilized, normally fertilized and polyspermic zygotes obtained 14-16 h post insemination. The semen from six experimental bulls (S_1-S_6) was used with oocytes collected from slaughterhouse ovaries to produce presumptive zygotes. The presumptive zygotes were stained by bisbenzamide and evaluated under the fluorescent microscope. The zygotes showing two polar bodies and two pronuclei were considered as normally fertilized. The zygotes showing two polar bodies and more than two pronuclei were considered as polyspermic. The fertilized zygotes included both normally fertilized and polyspermic zygotes.

	Sperm pre-incubation time	Bull Numbers					
		S_1	S_2	S_3	S_4	S_5	S_6
Percentage of zygotes (M±SEM)[z] (sperm to oocyte ratio of 25000:1)	0 h	*ab* 71.7±4.8	*c* 45.0±4.3	*a* 83.3±6.2	*bc* 65.0±5.0	*c* 51.7±4.8	*c* 50.0±4.5
	6 h	50.0±3.7	51.7±6.0	63.3±9.9	58.3±9.8	51.7±3.1	50.0±5.8
Percentage of normally fertilized zygotes (M±SEM)[z] (sperm to oocyte ratio of 25000:1)	0 h	*a* 66.7±4.9	*b* 45.0±4.3	*a* 71.7±7.0	*ab* 60.0±3.7	*b* 43.3±3.3	*b* 46.7±4.2
	6 h	*ab* 48.3±4.0	*b* 45.0±4.3	*a* 58.3±7.9	*a* 58.3±9.8	*b* 46.7±3.3	*ab* 46.7±6.2
Percentage of polyspermic zygotes (M±SEM)[z] (sperm to oocyte ratio of 25000:1)	0 h	5.0±2.2	0	11.7±6.0	5.0±2.2	8.3±3.1	3.3±3.3
	6 h	1.7±1.7	6.7±4.9	5.0±3.4	0	5.0±3.4	3.3±2.1
Percentage of zygotes (M±SEM)[z] (sperm to oocyte ratio of 50000:1)	0 h	*abc* 78.3±4.0	*c* 56.7±5.6	*a* 90.0±3.7	*ab* 78.3±4.8	*abc* 73.3±8.0	*bc* 71.7±6.0
	6 h	*c* 51.7±4.8	*c* 53.3±4.2	*a* 75.0±8.5	*abc* 63.3±4.9	*ab* 71.7±3.1	*bc* 58.3±6.5
Percentage of normally fertilized zygotes (M±SEM)[z] (sperm to oocyte ratio of 50000:1)	0 h	*a* 71.7±3.1	*b* 51.7±4.8	*a* 78.3±6.5	*ab* 70.0±5.2	*ab* 65.0±8.5	*ab* 66.7±3.3
	6 h	51.7±4.8	51.7±4.8	63.3±6.1	60.0±5.2	61.7±4.8	51.7±4.8
Percentage of polyspermic zygotes (M±SEM)[z] (sperm to oocyte ratio of 50000:1)	0 h	6.7±2.1	5.0±2.2	11.7±6.5	8.3±5.4	8.3±4.8	5.0±3.4
	6 h	*c* 0	*c* 1.7±1.7	*a* 11.7±3.1	*bc* 3.3±2.1	*ab* 10.0±4.5	*abc* 6.7±3.3

a,b,c – Means with different superscripts within rows differ ($P<0.05$).

(For percentage of normally fertilized zygotes using 50,000:1 sperm-oocyte ratio and 0 h pre-incubation time group $p=0.062$).

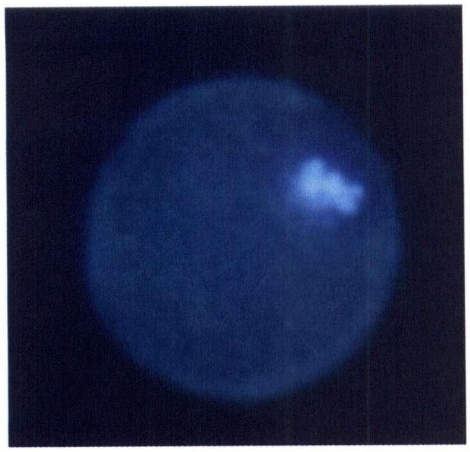
z – Arithmetic mean and standard error of the percentage of zygotes, and normally fertilized and polyspermic zygotes obtained by fertilization of oocytes with sperm from different experimental bulls.

Table 4.2. Pair-wise comparison of percentage of zygotes when 6 h pre-incubated sperm was used in sperm to oocyte ratio of 25,000:1 (T2C1-Zy), percentage of normally fertilized zygotes when 6 h pre-incubated sperm was used in sperm to oocyte ratio of 25,000:1 (T2C1-2PN), percentage of polyspermic zygotes when 6 h pre-incubated sperm was used in sperm to oocyte ratio of 25,000:1 (T2C1->2PN), percentage of difference in normally fertilized zygote between 0 and 6 h pre-incubated sperm used in sperm to oocyte ratio of 25,000:1 (C1Dif-2PN), percentage of difference in normally fertilized zygotes between 0 and 6 h pre-incubated sperm used in sperm to oocyte ratio of 50,000:1 (C2Dif-2PN), sperm pre-freeze motility (PrFM), increase in acrosome reaction from 0 to 4 h pre-incubation of sperm (InAR), cleavage rate(CL), blastocyst production rate (BL) and 60-90 day non-return rates (NRR).

	T2C1-Zy	T2C1-2PN	T2C1->2PN	C1Dif-2PN	C2Dif-2PN	PrFM	InAR	CL	BL
T2C1-2PN	0.97*								
T2C1->2PN	-0.36	-0.57							
C1Dif-2PN	-0.12	-0.15	0.20						
C2Dif-2PN	0.50	0.59	-0.57	0.24					
PrFM	0.88*	0.85*	-0.46	-0.27	0.65				
InAR	-0.07	-0.29	0.93*	0.30	-0.26	-0.18			
CL	0.90*	0.77	0.08	-0.12	0.28	0.76	0.36		
BL	0.61	0.63	-0.38	-0.14	0.85*	0.72	-0.06	0.53	
NRR	-0.16	-0.23	0.36	0.90*	-0.12	0.36	0.36	-0.11	-0.48

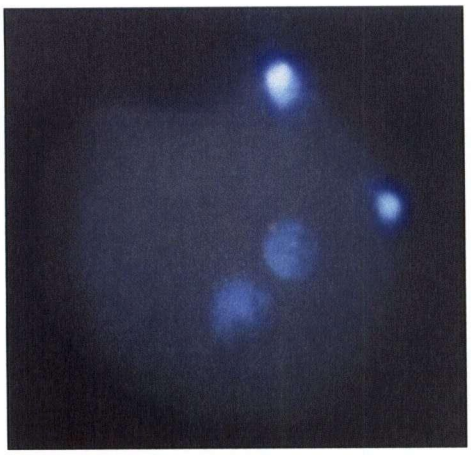
* Comparisons showing significant correlations at $P<0.05$.

A)



B)

C)



D)

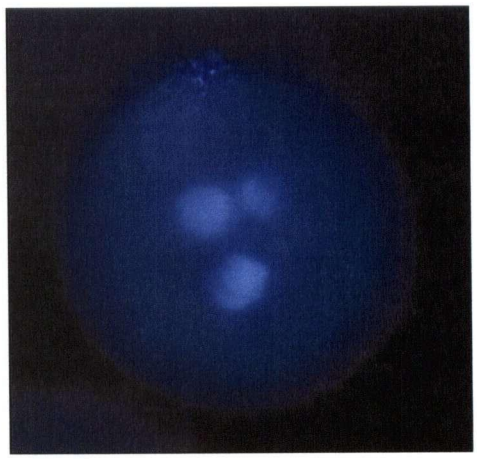


Plate 4.1. Fluorescence microscopic images of bovine unfertilized oocytes, and zygotes showing normal and abnormal fertilization after staining with bisbenzamide. The photographs were taken at 400x magnification using UV-2A filter combination (excitation filter of 330 – 380 nm and barrier filter of 420 nm). The oocytes containing either bright blue germinal vesicle **(A)** or one polar body and metaphase spindle **(B)** were considered as unfertilized. The zygotes containing 2 polar bodies and 2 pronuclei were considered as normally fertilized **(C)** and the zygotes containing 2 polar bodies and more than 2 pronuclei were considered as polyspermic **(D)**.

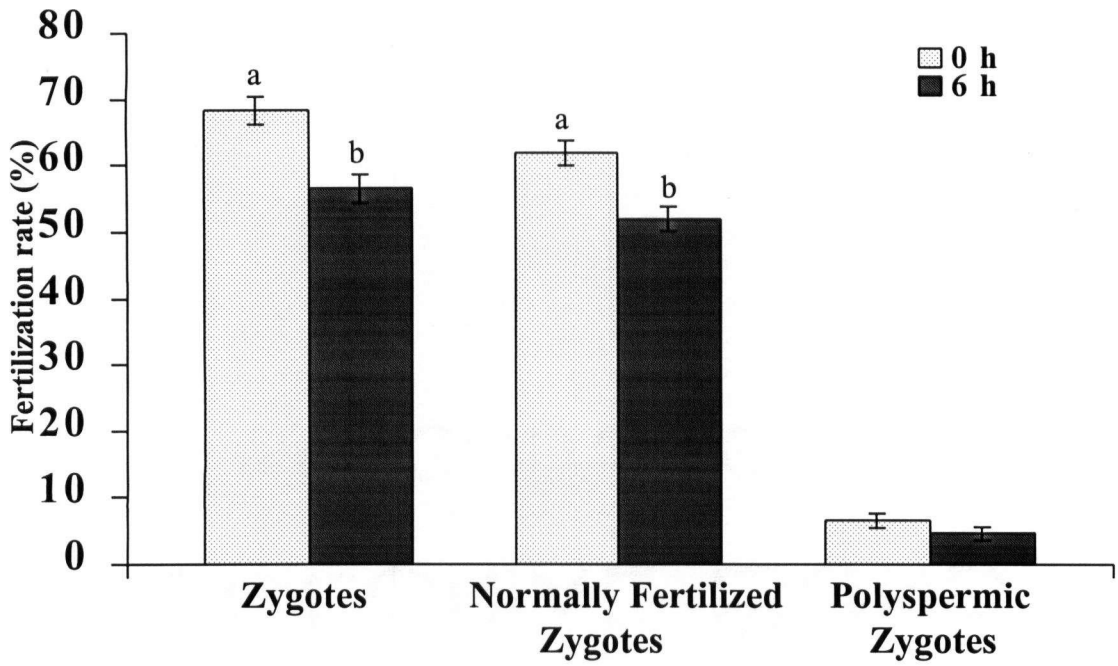


Figure 4.1. Percentage of zygotes, and normally fertilized and polyspermic zygotes obtained 14–16 h post insemination using 0 and 6 h pre-incubated sperm. a,b – Bars with different superscripts within each group of bars differ significantly ($P<0.01$).

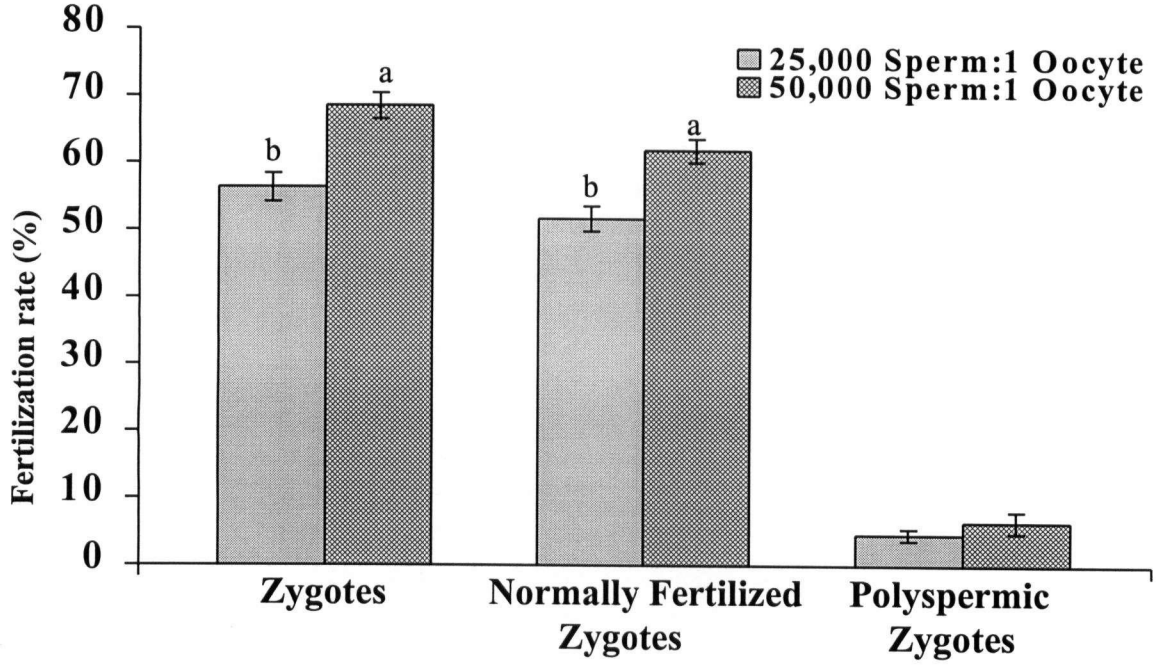


Figure 4.2. Percentage of zygotes, normally fertilized and polyspermic zygotes obtained 14–16 h post insemination using sperm:oocyte ratio of 25,000:1 and 50,000:1. a,b – Bars with different superscripts within each group of bars differ significantly ($P<0.01$).

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CHAPTER 5 – BULL INFLUENCE ON APOPTOSIS, AND EXPRESSION OF Bcl2, Bax, P53, HEAT SHOCK PROTEIN 70 AND INTERFERON TAU GENES IN PREIMPLANTATION EMBRYOS

5.1. PREFACE

The bull effects on development, apoptosis, and expression of Bax, Bcl-2, p53, heat shock protein 70 (HSP70) and interferon tau (IFNτ) genes in in-vitro produced embryos were investigated in this study. The degree of correlation of this effect with the 60-90 day non-return rates was also investigated. Frozen semen from three separate ejaculates of six genetically unrelated bulls (B_1–B_6) was used. Ejaculates from each bull were pooled to minimize variability among ejaculates. Oocytes obtained from slaughterhouse ovaries were matured and incubated with sperm from experimental bulls in a standard in-vitro fertilization and embryo culture procedure to obtain morula to blastocyst stage embryos. Cleavage and morula to blastocyst development rates were determined at 72 h and 168 h post insemination, respectively. The number of live, apoptotic, and dead cells of morula to blastocyst stage embryos was counted after staining with annexin V, propidium iodide, and bisbenzamide. Bax, Bcl-2, p53, HSP70 and IFNτ gene expression levels in morula to blastocyst stage embryos were determined by reverse transcription polymerase chain reaction. The non-return rate data for all experimental bulls were obtained from a local artificial insemination center. Cleavage and morula to blastocyst development rates were different ($P<0.01$) among bulls. Percent apoptotic, live and dead cells in morula to blastocyst stage embryos were

different ($P<0.01$) among bulls. The expression levels of HSP70 and IFNτ genes in morula to blastocyst stage embryos were different ($P<0.01$) among bulls. The expression levels of Bax, Bcl-2 and p53 genes in morula to blastocyst stage embryos were not different among bulls. Percentage of live cells was positively correlated with cleavage rate ($r = 0.61$), morula to blastocyst development rate ($r = 0.73$), and expression levels of interferon tau ($r = 0.58$) and heat shock protein 70 ($r = 0.57$) genes. Percentage of apoptotic cells was negatively correlated ($p<0.05$) with cleavage rate ($r = -0.57$), morula to blastocyst development rate ($r = -0.67$), and IFNτ ($r = -0.56$) and HSP70 ($r = -0.51$) gene expression levels. The field fertility measured by 60-90 day non-return rate is highly correlated ($p<0.05$) with relative abundance of Bcl-2 mRNA transcripts ($r = -0.93$) and the ratio of Bax to Bcl-2 gene expression ($r = 0.84$;). None of the other in-vitro embryo parameters was correlated with 60-90 day non-return rate.

5.2. INTRODUCTION

There are approximately 30% and 40% embryonic losses observed in cattle by day 7 and by day 8-17 after fertilization, respectively. This indicates that early embryonic mortality is the main cause of reproductive wastage (Humblot 2001; Thatcher et al., 1994, 2001; Bilodeau-Goeseels and Kastelic, 2003). The main reasons for embryonic losses could be due to intrinsic defects within embryo (Gustafsson and Larsson 1983), suboptimal oviductal and uterine environment (Lafrance et al., 1989; Gray et al., 2001) or insufficient interaction of the embryo with the oviduct and the endometrium (Goff, 2002; Hansen, 2002). There is very little information available on intrinsic defects within the embryo. Apoptosis is an energy-requiring, genetically

regulated multi-step process that initiates the cleavage of many proteins. The cleavage of these proteins mediates final cell death alteration in some specific cells in a cell suicidal program (Bloom and Muscarella, 1998). Apoptosis and mitosis are the key events regulating early embryonic differentiation and development (Hardy, 1997; Kolle et al., 2002). Hence, some of the intrinsic defects in the embryo may be reflected by an increase in number of blastomeres undergoing apoptosis. The stimulus for apoptosis can be intracellular such as DNA damage, excess production of reactive oxygen species, or extra cellular such as heat stress. The DNA damage in the embryo can be reflected by an increase in the expression of p53, a 53 kDa tumor suppressor protein, which acts as a transcription factor for several pro-apoptotic genes (Betts and King, 2001). The mitochondrial pathway and the death receptor pathway have been identified as two major pathways for programmed cell death (Ingo et al., 2000). Members of Bcl-2 protein family are involved in the regulation of the mitochondrial pathway. These proteins are subdivided into two major groups: pro- and anti-apoptotic proteins (Antonsson, 2001). Expression of pro- and anti-apoptotic proteins was demonstrated in bovine pre-implantation embryos (Kolle et al., 2002; Matwee et al., 2000; Yang and Rajamahendran, 2002). A balanced expression of pro- and anti-apoptotic proteins is necessary for successful development and growth of the embryo (Warner et al., 1998; Kolle et al., 2002). External stress factors originating from oviductal and uterine environment change the expression pattern of pro- and anti-apoptotic proteins to predispose blastomeres to signal induced apoptosis (Jurisicova et al., 1998). One of the main factors in this category is heat stress. The survival of the embryo depends on the degree to which it can either adjust its own physiology to withstand external stress

factors or act on the mother to restore its favorable microenvironment (Hansen, 2002). The embryo can adjust its own physiology by producing increased amounts of heat shock protein 70 (HSP70), a 70 kDa protein. Expression of this protein in bovine pre-implantation embryos subjected to various stress factors has been shown in the past (Edwards and Hansen, 1997; Edwards et al., 1997; Al-Katanani and Hansen, 2002). The embryo produces various signals to manipulate its oviductal and uterine microenvironment. The high failure rate in maintaining the pregnancy is assumed to be due to insufficient communication between embryo and mother. One of the main signals produced by the embryo is the expression of IFNτ, which acts on the endometrium to prevent the production of PGF2 alpha and thereby maintain the corpus luteum of pregnancy (Wolf et al., 2003). The quality of the embryo is determined at the time of oocyte maturation, in vitro fertilization, initial embryonic cell divisions and embryonic genome activation, and by sufficient expression of developmentally essential genes at correct time points (Hansen, 2002). Therefore, the successful execution of embryo developmental program is partially determined at the time of fertilization by genetic and non-genetic inheritance that an embryo receives from the oocyte or sperm. It has been shown that chromosomes in some of the morphologically normal spermatozoa undergo aberration during spermatogenesis and these spermatozoa could fertilize normally (Bochenek et al., 2001). Sperm chromatin damage due to stress varies with bull and significantly reduces the fertility due to embryonic mortality at the time of embryonic genome activation and the expression of developmentally essential genes (Evenson, 1999). Paternal influence on first cleavage division of the embryo and its relationship to embryo development was also reported recently (Warner et al., 1998;

Comizzoli et al., 2000;Ward et al., 2001). Hence studies based on these parameters in the embryo can reveal the fertility of the male.

Accurate prediction of bull fertility in the field by laboratory evaluation of semen samples is a long time goal for the AI industry. Although bulls are selected for high production traits and various sperm parameters, their fertilizing ability shows considerable variation in the field. Since embryonic mortality is very high during the first week of development when activation of the embryonic genome takes place, it was hypothesized that the bull has influence on embryo apoptosis and expression of developmentally essential genes, and this influence can be reflected by field fertility.

The objective of this experiment was to investigate the effect of the bull on viability of in vitro produced embryos by measuring cleavage and blastocyst development rates, and percentage of live, apoptotic and dead cells, and expression levels of Bcl-2, Bax, p53, HSP70 and IFNτ genes in morula to blastocyst stage embryos and to determine the degree of correlation of this bull effect with 60-90 day non-return rates.

5.3. MATERIALS AND METHODS

5.3.1. Experimental Design

Frozen semen from three separate ejaculates collected from six genetically unrelated bulls (of which three exhibited high field fertility and the other three low field fertility) were selected based on their pedigree information over the 4-5 generations. Ejaculates from each bull collected at three different collection periods were pooled before being subjected to in-vitro fertilization to minimize variability among ejaculates.

Oocytes collected from slaughterhouse ovaries were randomly assigned for in-vitro fertilization with spermatozoa from each bull to produce embryos for staining (n = 10) and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR; n = 15). The in-vitro fertilization was repeated four times per bull and 480 oocytes were allocated per bull. Both staining and semi-quantitative RT-PCR were also repeated four times with a total of 100 morula to blastocyst embryos per bull.

5.3.2. In-vitro Embryo Production

Bovine ovaries were collected at a local slaughterhouse and transported to the laboratory in normal saline (0.9% NaCl; Sigma-Aldrich Canada Ltd, Oakville, ON) supplemented with penicillin-G (100 IU/mL; Sigma-Aldrich Canada Ltd) and streptomycin sulphate (0.2 μg/mL; Sigma-Aldrich Canada Ltd) at 30-32 °C in a thermos flask. Cumulus oocyte complexes from small 2-8 mm follicles were collected into an aspiration medium using an 18-G needle and a 10-mL syringe. The medium contained Dulbecco's phosphate buffered saline (DPBS; GIBCO BRL, Canadian Life Technologies, Burlington, ON), 0.3% bovine serum albumin (BSA; Sigma-Aldrich Canada Ltd) and 50 μg/mL gentamicin (Sigma-Aldrich Canada Ltd). Oocytes with an evenly granulated cytoplasm and surrounded by more than three layers of cumulus cells were selected for maturation. These oocytes were cultured in maturation medium for 24 h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. The maturation medium consisted of tissue culture medium 199 (TCM199; Sigma-Aldrich Canada Ltd), 0.01 mg/mL follicle stimulating hormone, 5% superovulated cow serum (SCS; Boediono et al., 1994) and 50 μg/mL gentamicin. Three straws containing frozen semen from a test bull, collected at three different times, were thawed at 37 °C, pooled and washed twice

by centrifugation at 500g for 5 min in Brackett and Oliphant medium (BO medium; Brackett and Oliphant, 1975). The viable spermatozoa were swim-up separated, diluted to 5 x 10^6 sperm/mL in BO medium, and containing 2.5 mM caffeine sodium benzoate (Sigma-Aldrich Canada Ltd) and 20 μg/mL heparin (Sigma-Aldrich Canada Ltd). Six sperm droplets (100 μL each) were prepared under mineral oil and pre-incubated at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 1 h. Twenty matured oocytes were placed in each of these sperm droplets and incubated at 38.5 °C in a humidified atmosphere of 5% CO_2 in air for 16-18 h. The presumptive zygotes were then cultured in media prepared by mixing TCM-199, 5% SCS, 5 μg/mL insulin (Sigma-Aldrich Canada Ltd) and 50 μg/mL gentamicin (Boediono et al., 1994), in four-well culture dishes at 38.5 °C in a humidified atmosphere of 5% CO_2 in air. The culture media was changed every 72 h. The uncleaved presumptive zygotes were removed and the cleavage rate was assessed at 72 h post insemination. During in vitro culture, morula to early blastocyst stage embryos (n = 25) were randomly collected at 168 h post insemination. Their cumulus cells were removed by gentle pipetting in 0.3% hyaluronidase solution before ten were stained, and fifteen were subjected to the RT-PCR procedure.

5.3.3. Differential Embryo Staining

Annexin V-FITC Apoptosis Detection kit (Sigma-Aldrich Canada Ltd) was used with bisbenzamide (Sigma-Aldrich Canada Ltd) to differentially stain embryos to detect live, apoptotic and dead blastomeres. The kit manufacturer's protocol was followed with slight modification to fit the experimental conditions. Briefly, the embryos (n = 10) were washed twice in DPBS supplemented with 0.3% BSA, incubated in 0.25%

Pronase (Sigma-Aldrich Canada Ltd) solution for 1 min and observed under a dissecting microscope. When the zona pellucida appeared thin and flexible the embryos were transferred to DPBS containing 0.3% BSA and the zona pellucida was removed by gentle pipetting. The embryos were then washed twice in DPBS containing 0.3% BSA and transferred to a mixture containing 20 μg/mL bisbenzamide, 10 μg/mL propidium iodide, 2 μg/mL FITC conjugated annexin V and the binding buffer provided with the kit for 30 min. The stained embryos were washed in DPBS containing 0.3% BSA, transferred onto a clean glass slide and carefully pressed under a cover slip mounted on a wax and mineral oil mixture at the four corners. The slides were kept in a dark humidified chamber until counting. The embryos were observed under fluorescence microscope and the numbers of live, apoptotic and dead cells were counted at x100 to x400 magnification using UV-2A (excitation filter of 330 – 380 nm and barrier filter of 420 nm), B-2A (excitation filter of 450 – 490 nm and barrier filter of 520 nm) and G-2A (excitation filter of 510 – 560 nm and barrier filter of 590 nm) filter combinations. The live, apoptotic and dead cells appeared bright blue, green and red color, respectively (Plates 5.1.). The staining procedure was repeated four times per bull with different sets of morula to blastocyst embryos. During preliminary work, serial concentrations of the above stains were tested with a series of different incubation times to find an optimum stain concentration and incubation time for true positive staining without background or false positive staining.

5.3.4. Semi-quantitative RT-PCR Procedure

5.3.4.1. Reverse Transcription

Reverse transcription used in this study was accomplished by utilizing the commercially available first strand cDNA synthesis kits (Cells to cDNA II kit, Ambion

Inc. The RNA Company, Austin, Texas, USA). The reverse transcription reactions were performed by following the kit manufacturer's protocol with slight modification to match the experimental conditions. During initial attempts, different numbers of embryos and various concentrations of oligo-dT and random decamers were tested to determine optimum conditions for reverse transcription, which produce a sufficient amount of first strand cDNA for PCR amplification of the genes of interest. The embryos (n = 15) were washed three times in nuclease free phosphate buffered saline provided with the kit, pooled and lysed in 15 μL of cell lysis buffer by incubation at 75 °C for 10 min. The genomic DNA was removed by addition of DNase I (0.04 U/μL) and incubation at 37 °C. The DNase I in the solution was inactivated by incubation at 75 °C for 6 min. The reverse transcription reaction was performed by using 15 μL of cell lysate, 5 μM of random decamers, 2.5 μM of oligo dT primers, deoxyribonucleoside triphosphate mixture (0.5 mM each), 4 μL of 10XRT buffer pH 7.4, RNase inhibitor (0.5 U/μL), M-MLV reverse transcriptase (0.5 U/μL) and nuclease free water in a 40 μL reaction mixture and by incubation of the reaction mixture at 42 °C for 1 h. The reverse transcriptase was inactivated by incubating the reaction mixture at 94 °C for 10 min and the product was stored at –20 °C for future use in PCR amplification. For each bull the reverse transcription was repeated four times with different sets of in-vitro produced morula to blastocyst stage embryos.

5.3.4.2. Gene Specific PCR Amplification

The polymerase chain reaction (PCR) was performed using Jumpstart REDTaq ReadyMix PCR reaction mix (Jumpstart; Sigma-Aldrich Canada Ltd) and gene specific primers for Bcl-2, Bax, HSP70, p53, IFNτ and the house keeping gene G3PDH. The

gene specific primers Bax, HSP70, p53 and IFNτ were designed by using Primer3 software (Whitehead Institute for Biomedical Research; http://frodo.wi.mit.edu/primer3/primer3_code.html; Rozen and Skaletsky, 2000.) and the messenger RNA sequence obtained from the National Center for Biotechnology Information (NCBI) web site (http://www.ncbi.nlm.nih.gov/). The gene specific primer sequences for Bcl-2 and G3PDH were obtained from previously published data (Reyes and Cockerell, 1998). The primer sequence, fragment size, annealing temperature, number of PCR cycles and gene reference identification number are provided in Table 5.1. The PCR reaction was performed by following manufacturer's protocol with a slight modification to fit the experimental conditions. Briefly, gene specific primers, $MgCl_2$, nuclease free water and 2 μL of cDNA template were added to 12.5 μL of Jumpstart to make 25 μL reaction mixture. The reaction mixture was composed of 10 mM Tris-HCl, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM of each dNTP (dATP, dCTP, dGTP, dTTP), inert dye, stabilizers, 0.03 U/μL Taq DNA polymerase, Jumpstart Taq antibody, 0.8 – 1.6 μM gene specific primers, cDNA template and nuclease free water. For the amplification of the HSP70 gene sequence instead of using 2.5 mM $MgCl_2$, 4 mM $MgCl_2$ was used in the reaction mixture. The typical reaction cycles consisted of an initial denaturation step at 94 °C for 2 min followed by 34 – 38 cycles of denaturation at 94 °C for 30 sec, annealing at 56 – 62 °C for 30 sec and elongation at 72 °C for 45 sec with a final elongation step at 72 °C for 5 min. The PCR products were analyzed by gel electrophoresis using ethidium bromide (0.4 μg/mL) stained 2-3% agarose gels. The gels were photographed under ultraviolet illumination and the optical density of individual bands was analyzed using Scion Image Beta 4.02

for Windows computerized image analyzing software (Scion Corporation, Frederick, Maryland, USA; http://www.scioncorp.com/). The PCR procedure was repeated four times with different sets of cDNA synthesized by reverse transcription. The PCR products were nucleotide sequenced and the sequence identity was confirmed by standard nucleotide-nucleotide blasting at the NCBI web site.

5.3.5. Field Fertility Data

Based on 513,469 Holstein first inseminations, the Holstein breed average field fertility was determined as 67% and this was considered as fertility solution equal to 0. Fertility of different bulls is expressed in relation to this fertility solution value, and the bulls showing 60-90 day non-return rates above this value were considered as high fertility and the bulls showing non-return rates below this value were considered as low fertility. Based on this scaling and the number of inseminations which ranged from 335 to 467, three of the experimental bulls showed fertility values above 67%, high fertility and the other three showed fertility values below 67%, low fertility.

5.3.6. Statistical Analyses

Data analysis was done by one-way analysis of variance (ANOVA) after arcsine transformation of percentage data. Mean separation procedure was performed when analysis of variance showed significant F-Values using Fisher's Least Significant Difference. Non-return rate data were analyzed by Chi-square test. The results were reported as the mean values for each set of data ± standard error of the means and the level of statistical significance was defined at a probability level of less than 0.05. Pearson's pair-wise correlation coefficient was used to determine the degree of

correlation between in-vitro, in-vivo fertilization, and apoptosis and gene expression in morula to blastocyst stage embryos.

5.4. RESULTS

5.4.1. Validation of Semi-quantitative RT-PCR

The semi-quantitative RT-PCR method was used to determine the relative abundance of mRNA transcripts for Bcl-2, Bax, p53, HSP70 and IFNτ in bovine preimplantation embryos produced in-vitro. A linear relationship (log phase) was found between 34 and 38 cycles for PCR amplification of different gene transcripts and the internal control G3PDH, (Fig. 5.1., 5.2., 5.3., 5.4., 5.5. and 5.6.). The expected PCR amplicon size for Bcl-2, Bax, P53, HSP70, IFNτ and G3PDH gene transcripts were 156 bp, 223 bp, 363 bp, 376 bp, 386 bp and 318 bp, respectively. This method of semi-quantitative RT-PCR measurement of mRNA transcripts using G3DPH as an internal control was previously described in the literature (Mamluk et al., 1998; 1999). The possibility of PCR cross contamination and genomic DNA amplification was ruled out because no PCR products were observed in negative controls (without template and without reverse transcriptase).

5.4.2. Bull Effects on Embryo Apoptosis and Development

The in-vitro embryo development results measured by cleavage and morula to blastocyst development rates are shown in Fig. 5.7.and 5.8., respectively. The results of embryo viability measured by percentage of live, apoptotic and dead blastomeres in morula to blastocyst stage embryos produced by fertilization of oocytes with spermatozoa from various experimental bulls are shown in Fig. 5.9., 5.10. and 5.11., respectively. In order to investigate the in-vitro fertility of experimental bulls the

cleavage rate and morula to blastocyst development rates were measured and the data revealed that the cleavage and morula to blastocyst development rate were different ($P<0.01$) among bulls. Percent apoptotic, live and dead cells in morula to blastocyst stage embryos were also different ($P<0.01$) among bulls.

5.4.3. Bull Effects on Embryonic Gene Expression

The semi-quantitative RT-PCR results for relative abundance of mRNA transcripts for Bcl-2, Bax, p53, HSP70 and IFNτ are shown in Fig. 5.12., 5.13., 5.14., 5.15 and 5.16. Although the number of live, apoptotic and dead blastomeres were significantly different, the relative abundance of transcripts for pro-apoptotic genes Bax and p53, and the anti-apoptotic gene Bcl-2 were not significantly different in morula to blastocyst stage embryos produced by fertilization of oocytes with spermatozoa from various experimental bulls. However, the HSP70 and IFNτ gene transcripts were significantly different in morula to blastocyst embryos produced by fertilization of oocytes with spermatozoa from various experimental bulls.

5.4.4. Bull Effects on Non-return Rates

The field fertility measured by 60-90 day non-return rates to first service is shown in Fig. 5.17. The Chi square test revealed that the non-return rates were significantly different among bulls ($p<0.01$).

5.4.5. Correlation Between Bull Fertility Parameters

The significant correlations between different in-vitro sperm fertility parameters and the field fertility measured by 60-90 day non-return rates are summarized in Table 5.2. The cleavage rate was positively correlated with percentage of live blastomeres ($r = 0.61$; $p<0.05$) and IFNτ gene expression levels ($r = 0.42$; $p<0.05$), and negatively

correlated with percentage of apoptotic blastomeres ($r = -0.57$; $p<0.05$). The morula to blastocyst development rate was also positively correlated with percentage of live blastomeres ($r = 0.73$; $p<0.05$) and IFNτ gene expression levels ($r = 0.42$; $p<0.05$), and negatively correlated with percentage of apoptotic blastomeres ($r = -0.67$; $p<0.05$). Cleavage or morula to blastocyst development rates were not correlated with Bcl-2, Bax, p53 and HSP70 gene expression levels. The percentage of live blastomeres was positively correlated with IFNτ ($r = 0.58$; $p<0.05$) and HSP70 ($r = 0.57$; $p<0.05$) gene expression levels. Percentage of apoptotic blastomeres was negatively correlated with IFNτ ($r = -0.56$; $p<0.05$) and HSP70 ($r = -0.51$; $p<0.05$) gene expression levels. Percentage of live and apoptotic blastomeres in morula to blastocyst stage embryos was not correlated with Bcl-2, Bax and p53 gene expression levels. The percentage of dead blastomeres was positively correlated with bcl-2 gene expression ($r = 0.87$; $p<0.05$) and not correlated with expression levels of other genes. The field fertility measured by 60-90 day non-return rate is correlated with relative abundance of Bcl-2 mRNA transcripts ($r = -0.93$; $p<0.05$) and the ratio of Bax to Bcl-2 gene expression ($r = 0.84$; $p<0.05$). None of the other in-vitro embryo parameters was correlated with 60-90 day non-return rates.

5.5. DISCUSSION

The present study investigated bull effects on embryo viability at various levels such as in-vitro embryo development, apoptosis and gene expression. This study is the first one showing the bull influence on percentage of apoptotic, live and dead

blastomeres, and expression of interferon tau and HSP70 genes in in-vitro produced morula to blastocyst stage embryos.

In the present study the bull dependent variation in the percentage of live, apoptotic and dead blastomeres in in-vitro produced embryos indicated that the in-vitro fertility of bulls is determined by viability of embryos based on the percentage of live, apoptotic and dead blastomeres. This is supported by correlation data in which the in-vitro fertility of bulls assessed by cleavage and blastocyst production rates is positively correlated with percentage of live blastomeres and negatively correlated with percentage of apoptotic blastomeres. All the experimental embryo samples showed apoptosis and some of them showed an excessive percentage of apoptotic blastomeres. This finding supports the notion that occurrence of apoptosis is most common in the developing embryo, which undergoes rapid cell division resulting in a chance for a high degree of chromosomal abnormalities and that apoptosis is a process by which faulty as well as unwanted cells are removed from the system (Mirkes, 2002). Although apoptosis is the most commonly occurring process to remove faulty and unwanted cells during cell proliferation, excessive cell apoptosis is detrimental and leads to eventual death of the whole organism (Antonsson, 2001). The embryos, which showed a high percentage of apoptotic blastomeres in the current experiment, were either highly susceptible to stress factor induced apoptosis or had imbalances in the tightly regulated cascade of apoptosis resulting in excessive cell apoptosis and death as indicated by Zakeri and Lockshin (2002). The viability of embryos showing high percentages of apoptotic blastomeres is low as there is a negative correlation observed in the present study with percentage of live blastomeres, and in-vitro embryo development assessed

by cleavage and blastocyst production rates. This supports the finding by Yang and Rajamahendran (2002) in which they showed that embryos, which show high levels of apoptosis measured by TUNEL, and the expression levels of Bcl2 and Bax proteins, have high levels of fragmentation. When cells are exposed to apoptotic stimulation, pro-apoptotic proteins are activated through post-translational modifications or changes occur in their conformation and mitochondria to release cytochrome c by increasing membrane permeability. In the cytosol, cytochrome c forms a complex with apoptosis protease activating factor 1 which activates the caspase cascade ultimately leading to cell death (Antonsson, 2001; Mirkes, 2002). The death receptor pathway is stimulated by the binding of tumor necrosis factor α, FAS ligand and other death initiating factors to their receptors, and direct activation of downstream caspases, which lead to cell death (Mirkes, 2002). The balance between pro- and anti-apoptotic protein expression regulates the fate of the cell since pro-apoptotic activity of Bax can be inhibited by over expression of Bcl-2 (Korsmeyer et al., 1993). Irregular expression pattern of these proteins causes cell death and embryo mortality during early stages of development (Hardy, 1997). In the present experiment, expression levels of HSP70 mRNA transcripts were influenced by the bull and negatively correlated with the percentage of apoptotic cells in the experimental embryo samples. HSP70 has been shown to modulate the apoptosis process at different levels of the downstream caspase cascade (Parcellier el al., 2003). Hence, HSP70 may have played a major role in the difference in the percentage of apoptotic blastomeres in the experimental embryo samples.

In the present study, expression of IFNτ genes varied with embryos produced from fertilization of oocytes with spermatozoa from various experimental bulls. This

indicates that the expression of IFNτ can be influenced by heritable and non-heritable factors, which affect the expression of some of the transcription factors. Because, the rapid onset and cessation of IFNτ expression is regulated by number of transcription factors such as Ets-2 (Ezashi et al., 1998) and granulocyte–macrophage colony-stimulating factor acting via the proto-oncogene c-*jun* and an AP-1 site (Imakawa et al., 1993; Yamaguchi et al., 1999). Also, negative regulatory domains have been shown in the bovine IFNτ promoter that may be involved in the precisely timed cessation of gene expression (Guesdon et al., 1996; Yamaguchi et al., 1999). This also supports the recent finding in which bull dependent variability in IFNτ production in in-vitro produced embryos has been reported (Kubisch et al., 2001). IFNτ expression was highly correlated with in-vitro embryo development measured by cleavage and blastocyst production rates indicating that the viability of embryos is represented by IFNτ expression. The observation, that the viability of the embryos is represented by IFNτ expression in the present study, supports the previous finding by Hernandez-Ledezma et al. (1993). Inadequate reaction of the endometrium to IFNτ or insufficient secretion of IFNτ by the embryo is assumed to be the major reason for early embryonic losses and pregnancy failure. Therefore, the level of IFNτ secretion has been discussed as a parameter for the assessment of embryo quality (Hernandez-Ledezma et al. 1993). Hence, in the current study the expression of IFNτ gene should have been correlated with in-vivo fertility measured by 60-90 day non-return rates, but it was not correlated because of unknown reasons. This may be attributed to various paternal, maternal and management factors which influence the success of AI and in-vivo embryo development.

In the present study, it was shown that there is a bull influence on the expression levels of HSP70 mRNA transcripts in in-vitro produced embryos. The bull influence is also correlated with the developmental competence of in-vitro produced embryos assessed by cleavage and morula to blastocyst production rates. This indicates that the viability of in-vitro produced embryos has been also determined by expression of HSP70 and bull related variability in the viability of in-vitro produced embryos is associated with expression of HSP70 gene. HSP70 ensures the survival of cells by acting as cellular chaperones to correct the folding and prevent the aggregation of stress-accumulated misfolded proteins by directly interacting with various components of tightly regulated programmed cell death machinery, and playing a role in proteosome mediated degradation of selected proteins under stress conditions (Parcellier el al., 2003). Our findings support the notion that the HSP70 plays a key role in the protection of developing preimplantation embryos from various stress factors and the expression of this protein is developmentally regulated (Edwards et al., 1997; Paula-Lopes and Hansen, 2002).

Several researchers have compared in-vitro fertility of bulls with in-vivo fertility to establish suitable method to predict fertility in the laboratory with conflicting results. Some of the investigators established significant correlation between in-vivo and in-vitro fertility (Hillery et al. 1990; Kjaestadt et al. 1992; Eid et al. 1994; Zhang et al. 1995; Bredbacka et al. 1997; Lansbergen et al. 1997) whereas, others has shown no correlation or poor correlation (Ohgoda et al. 1988; Palma et al. 1996; Schneider et al. 1996). In the current study, the field fertility measured by 60-90 day non-return rate was not correlated with in-vitro fertility measured either by cleavage and embryo production

rates. Also, this is in agreement with the findings of the first study of this thesis (Chapter III). However, the non-return rates are negatively correlated with Bcl-2 expression and positively correlated with the ratio of Bax to Bcl-2 expression and not correlated with expression levels of p53, HSP70 and IFNτ genes. Also, the Bcl-2 expression is correlated with the percentage of dead blastemeres. This indicates that the cells, which are not undergoing apoptosis, may start producing higher amounts of Bcl-2 as a compensatory preventive measure for the survival of embryos. Since a pro-apoptotic function of Bcl-2 has been demonstrated (Chen et al., 1996; Shinoura et al., 1999), the higher expression of Bcl-2 in the embryos produced by low fertility bulls is an indication that they are undergoing apoptosis. Also, when Bcl-2 is expressed at higher levels caspase 3 acts on Bcl-2 and cleaves it to a pro-apoptotic protein (Cheng et al., 1997). This is a good indication that the bulls showing low fertility in the field may produce very susceptible embryos in which the blastomeres undergo irregular apoptosis by unknown pathways.

5.6. CONCLUSION

In the present study, bull influence on embryo viability was assessed by in-vitro embryo development, embryo apoptosis, and the expression levels of Bcl-2, Bax, p53, HSP70 and IFNτ genes. Based on this study, it is concluded that the bull affects the preimplantation embryo development, apoptosis, and expression levels of HSP70 and IFNτ genes. Measurement of either the Bcl-2 gene or a ratio of Bax to Bcl-2 gene expression levels in morula to blastocyst stage embryos produced in-vito may be useful in predicting bull field fertility.

Table 5.1. Primers used in the RT-PCR amplification of specific mRNA transcripts in morula to blastocyst stage embryos.

Gene	Primer Sequence (5'-3')	Anneal. T °C	PCR cycles	Fragment Length	Accession Number
G3PDH	5' TGTTCCAGTATGATTCCACCC 3' AGGAGGCATTGCTGACAATC	58	34-36	318 bp	U85042
Bax	5' TGCTTCAGGGTTTCATCCAG 3' AACATTTCAGCCGCCACTC	58	34	223 bp	U92569
Bcl-2	5' TTCGCCGAGATGTCCAGTCAGC 3' GTTGACGCTCTCCACACACA	62	37	156 bp	U92434
p53	5' GCACCACCATCCACTACAA 3' GCTCCAAGGCATCATTCAG	56	36	363 bp	X81704
HSP70	5' CACTTCGTGGAGGAGTTCA 3' GGTTGATGCTCTTGTTGAGG	58	38	376 bp	AY149619
IFNτ	5' GACTCTCTCCTCATCCCTGTCT 3' GGCTCTCATCATCTCCACTCT	57	35	386 bp	AF196325

Table 5.2. Pair-wise comparison of cleavage rate (CL), blastocyst production rate (BL), percentage of live blastomeres (PL), percentage of apoptotic blastomeres (PA), percentage of dead blastomeres (PD), expression of Bcl-2 gene (Bcl-2), ratio of expression of Bax and Bcl-2 genes (Bax/Bcl-2), expression of interferon tau gene (IFNτ), expression of heat shock protein 70 gene (HSP70), 60-90 day non-return rates (NRR). The percentage of live, apoptotic and dead blastomeres, and all the gene expression levels are measured in morula to blastocyst stage embryos.

	CL	BL	PL	PA	PD	Bcl-2	Bax/ Bcl-2	p53	IFNτ	HSP70
BL	0.89*									
PL	0.61*	0.73*								
PA	-0.57*	-0.67*	-0.91*							
PD	-0.25	-0.31	-0.46*	0.04						
Bcl-2	-0.28	-0.15	-0.08	0.04	0.87*					
Bax/Bcl-2	0.33	0.29	0.26	-0.31	0.05	-0.58*				
p53	-0.13	-0.14	0.06	0.001	-0.15	-0.04	0.08			
IFNτ	0.42*	0.42*	0.58*	-0.56*	-0.19	-0.19	0.20	0.17		
HSP70	0.36	0.29	0.57*	-0.51*	-0.26	-0.22	0.11	0.44*	0.58*	
NRR	0.45	0.26	0.28	-0.06	-0.65	-0.93*	0.84*	-0.03	0.42	0.47

* Comparisons showing significant correlations at $p<0.05$.

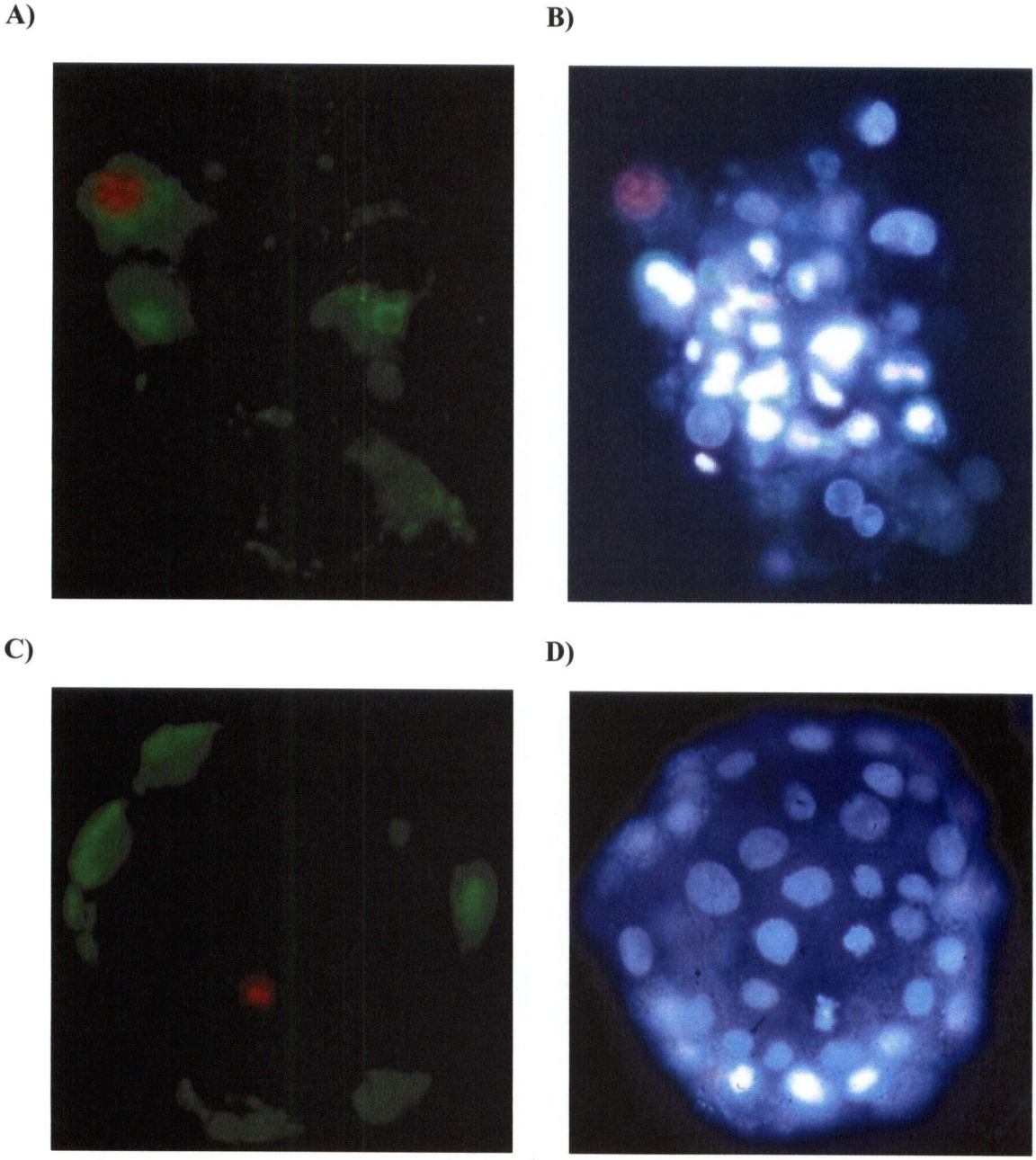
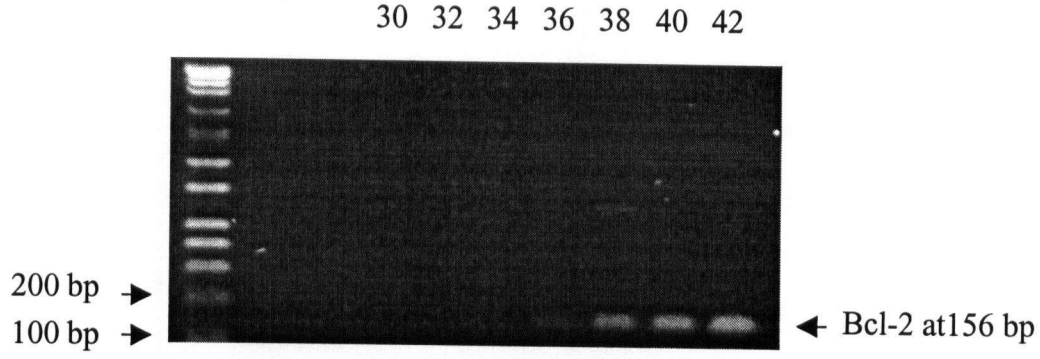


Plate 5.1. Fluorescence microscopic images of bovine morula **A) & B)** to blastocyst **C) & D)** stage embryos stained by annexin V, propidium iodide and bis-benzamide. The photographs were taken at 400x magnification using UV-2A (excitation filter of 330 – 380 nm and barrier filter of 420 nm), B-2A (excitation filter of 450 – 490 nm and barrier filter of 520 nm) and G-2A (excitation filter of 510 – 560 nm and barrier filter of 590 nm) filter combination. The live, apoptotic, and dead cells show blue, green, and red or red & green fluorescence, respectively.

A)



B)

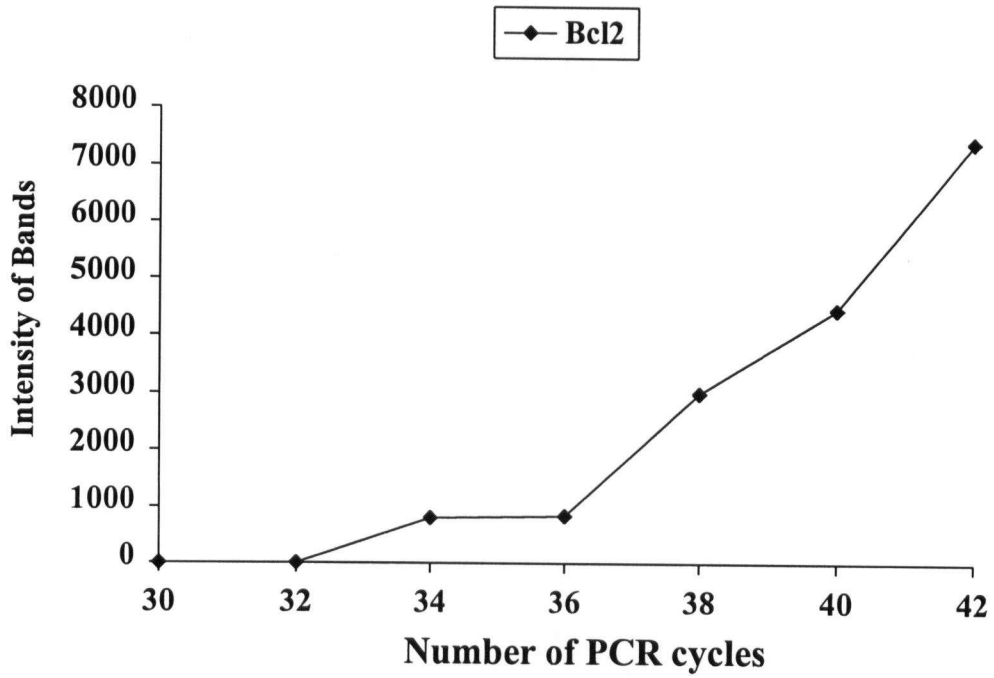


Figure 5.1. Characterization of semi-quantitative RT-PCR for Bcl-2 mRNA transcripts from bovine in-vitro produced embryos. The total RNA in the experimental embryo samples was reverse transcribed and amplified using a thermal-cycler as described in the Materials and Methods. The PCR products were resolved on ethidium bromide stained 2% agarose gel and photographed **A**. The optical density of the bands on the inverse image was measured. The linear relationship between PCR products and the number of amplification cycles is shown in **B.**

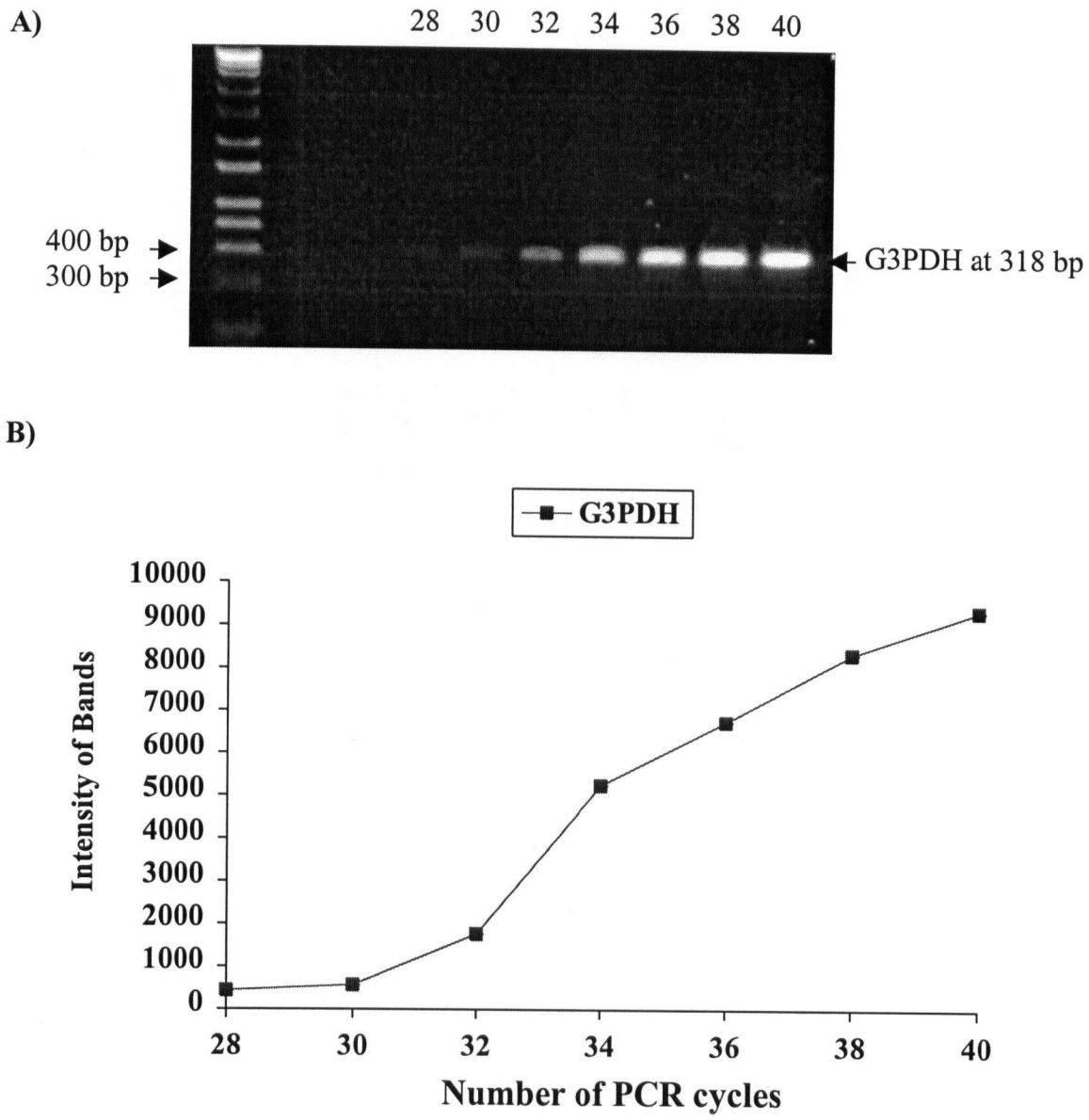


Figure 5.2. Characterization of semi-quantitative RT-PCR for G3PDH mRNA transcripts from bovine in-vitro produced embryos. The total RNA in the experimental embryo samples was reverse transcribed and amplified using a thermal-cycler as described in the Materials and Methods. The PCR products were resolved on ethidium bromide stained 2% agarose gel and photographed **A**. The optical density of the bands on the inverse image was measured. The linear relationship between PCR products and the number of amplification cycles is shown in **B.**

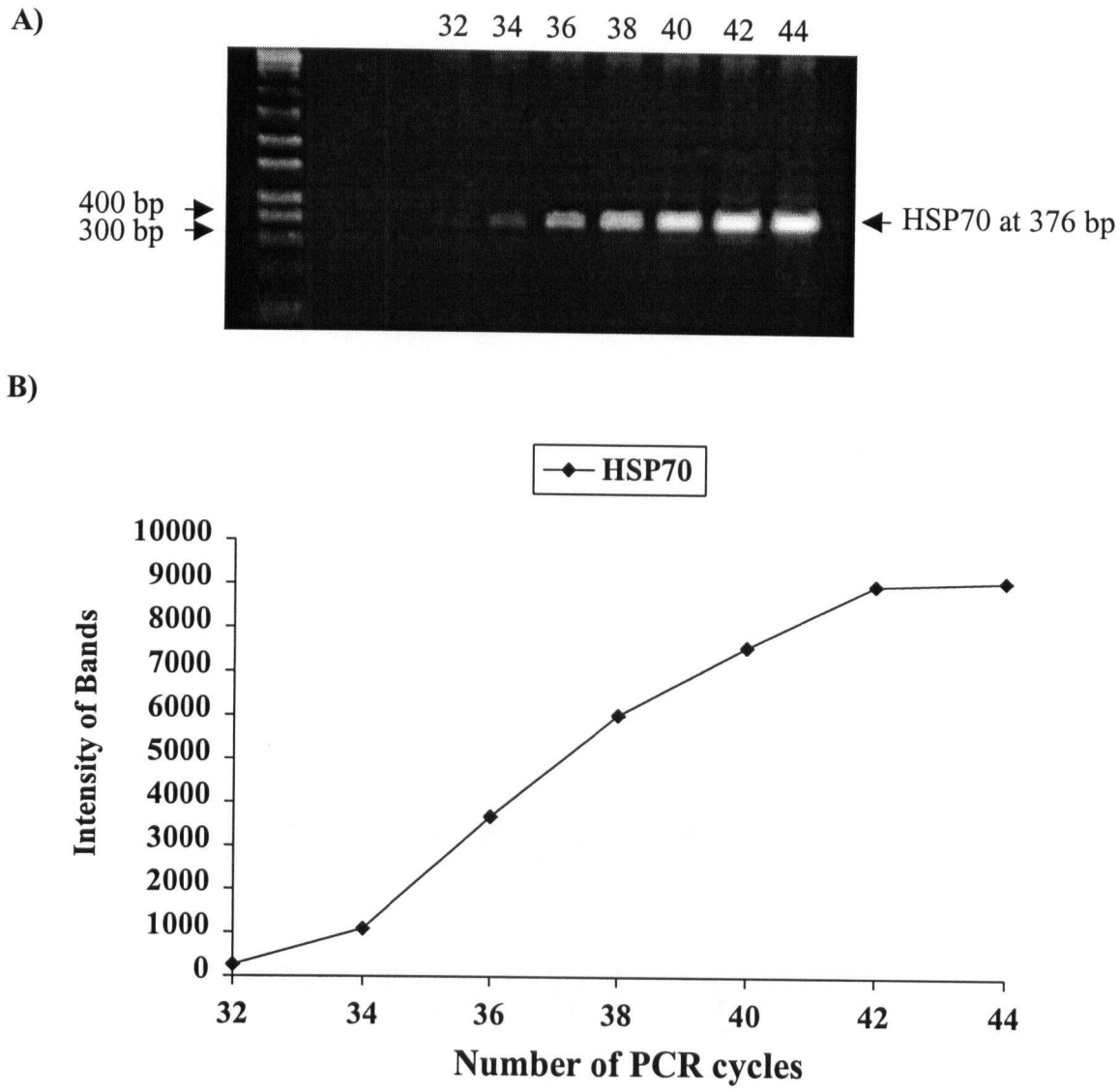


Figure 5.3. Characterization of semi-quantitative RT-PCR for HSP70 mRNA transcripts from bovine in-vitro produced embryos. The total RNA in the experimental embryo samples was reverse transcribed and amplified using a thermal-cycler as described in the Materials and Methods. The PCR products were resolved on ethidium bromide stained 2% agarose gel and photographed **A**. The optical density of the bands on the inverse image was measured. The linear relationship between PCR products and the number of amplification cycles is shown in **B.**

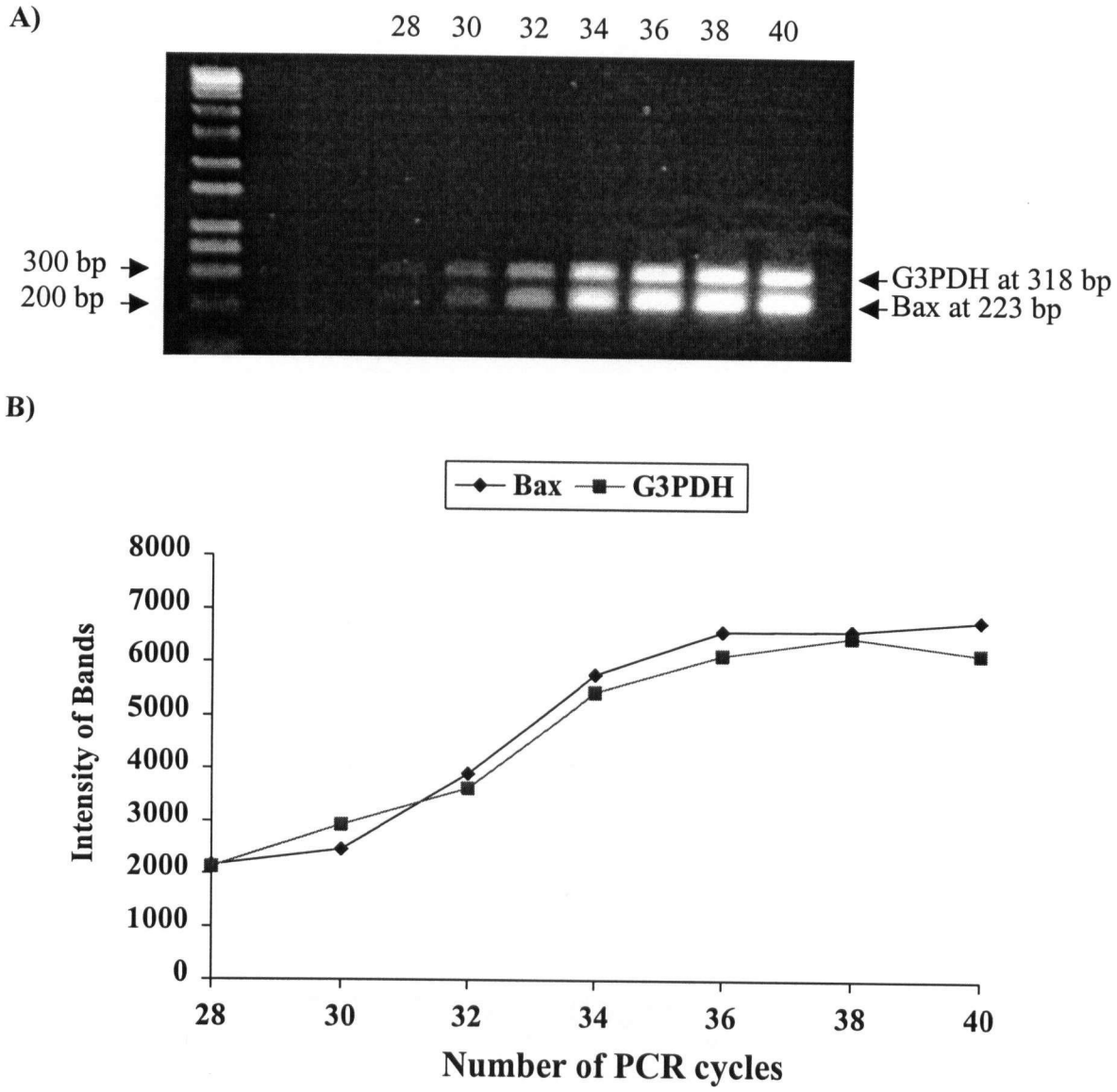


Figure 5.4. Characterization of semi-quantitative RT-PCR for Bax and G3PDH mRNA transcripts from bovine in-vitro produced embryos. The total RNA in the experimental embryo samples was reverse transcribed and amplified using a thermal-cycler as described in the Materials and Methods. The PCR products were resolved on ethidium bromide stained 2% agarose gel and photographed **A**. The optical density of the bands on the inverse image was measured. The linear relationship between PCR products and the number of amplification cycles is shown in **B.**

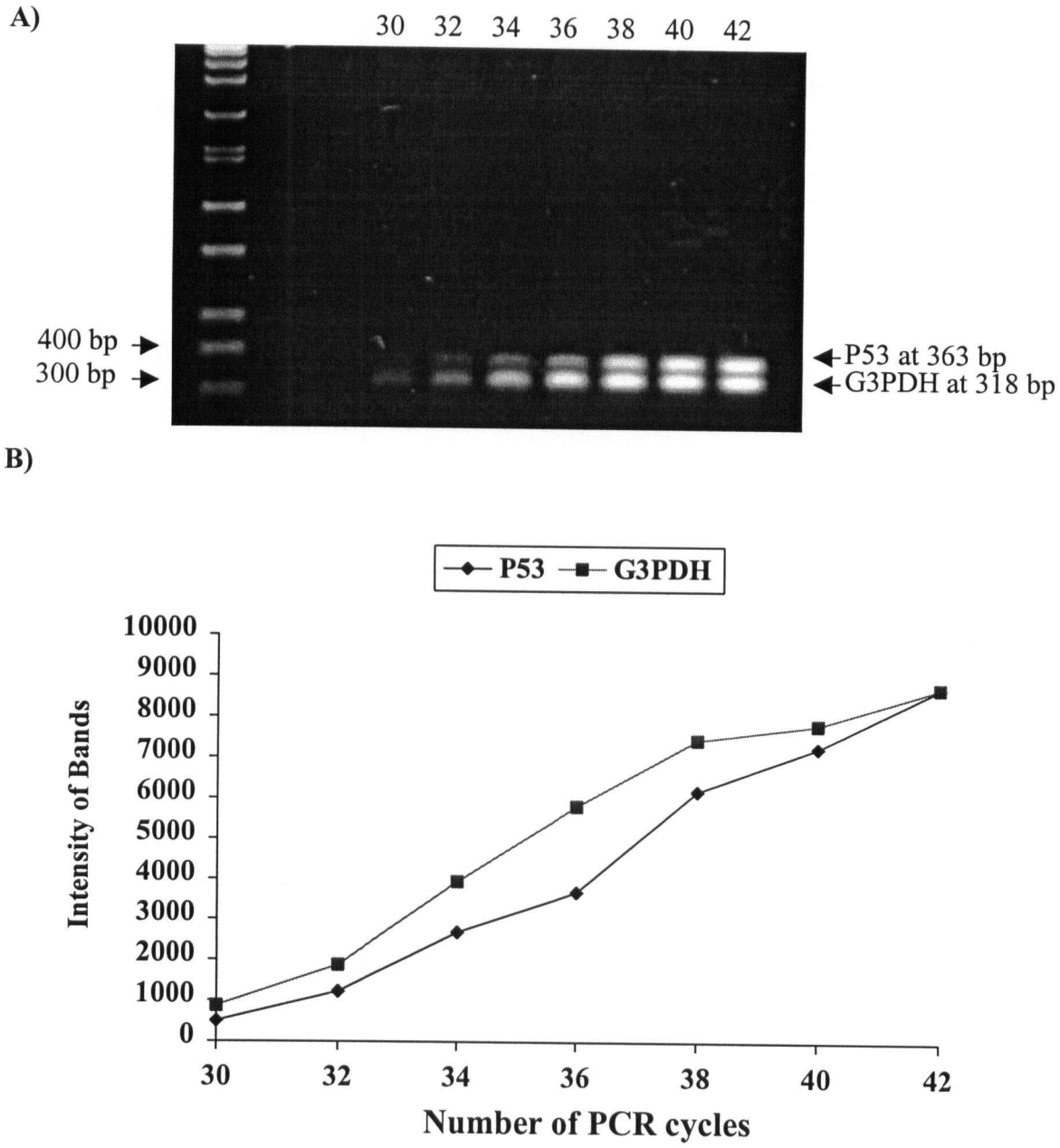
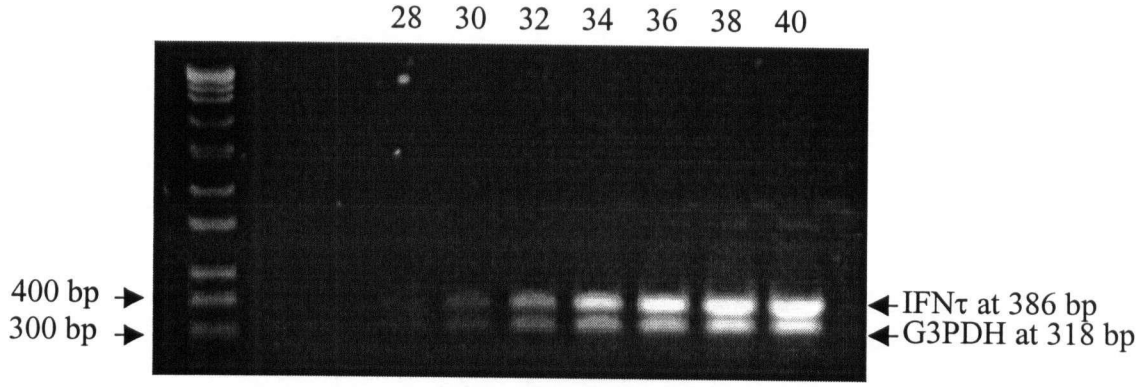


Figure 5.5. Characterization of semi-quantitative RT-PCR for P53 and G3PDH mRNA transcripts from bovine in-vitro produced embryos. The total RNA in the experimental embryo samples was reverse transcribed and amplified using a thermal-cycler as described in the Materials and Methods. The PCR products were resolved on ethidium bromide stained 2% agarose gel and photographed **A**. The optical density of the bands on the inverse image was measured. The linear relationship between PCR products and the number of amplification cycles is shown in **B.**

A)



B)

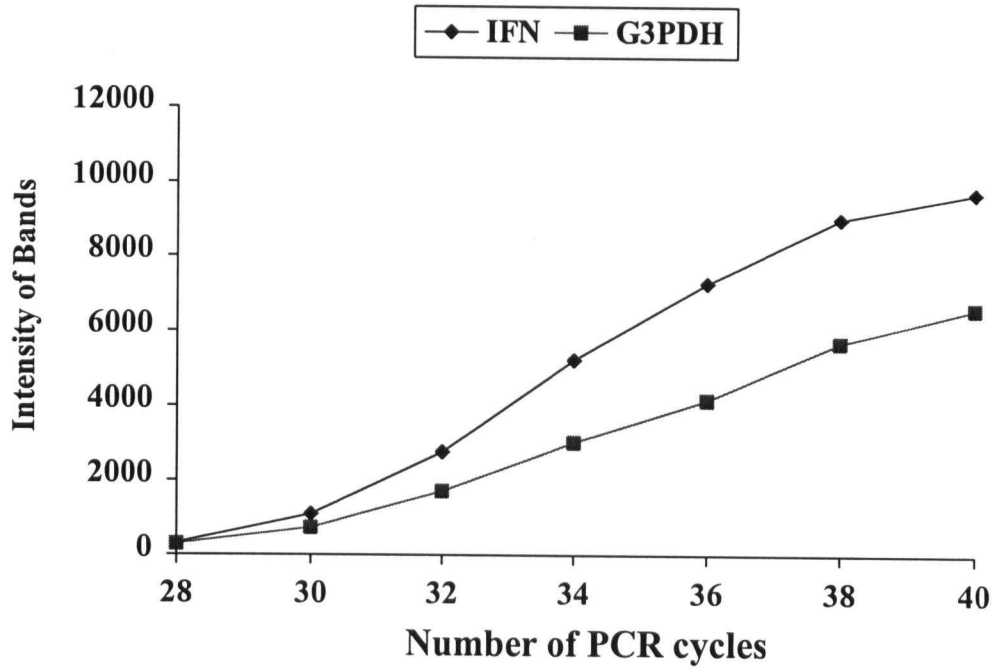


Figure5.6. Characterization of semi-quantitative RT-PCR for interferon tau and G3PDH mRNA transcripts from bovine in-vitro produced embryos. The total RNA in the experimental embryo samples was reverse transcribed and amplified using a thermal-cycler as described in the Materials and Methods. The PCR products were resolved on ethidium bromide stained 2% agarose gel and photographed **A**. The optical density of the bands on the inverse image was measured. The linear relationship between PCR products and the number of amplification cycles is shown in **B.**

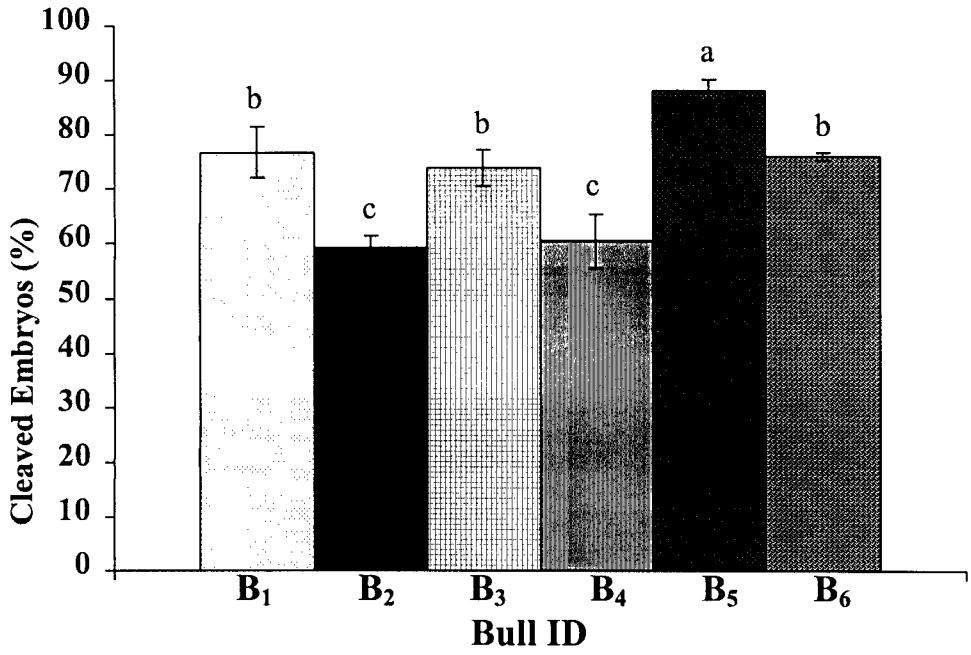


Figure 5.7. Percentage of cleaved embryos produced by fertilization of oocytes with spermatozoa from six experimental bulls (B_1-B_6) 72 h post insemination. The in-vitro fertilization procedure was repeated four times for each bull with a total of 480 oocytes. a,b,c – Bars with different superscripts differ significantly ($P<0.01$).

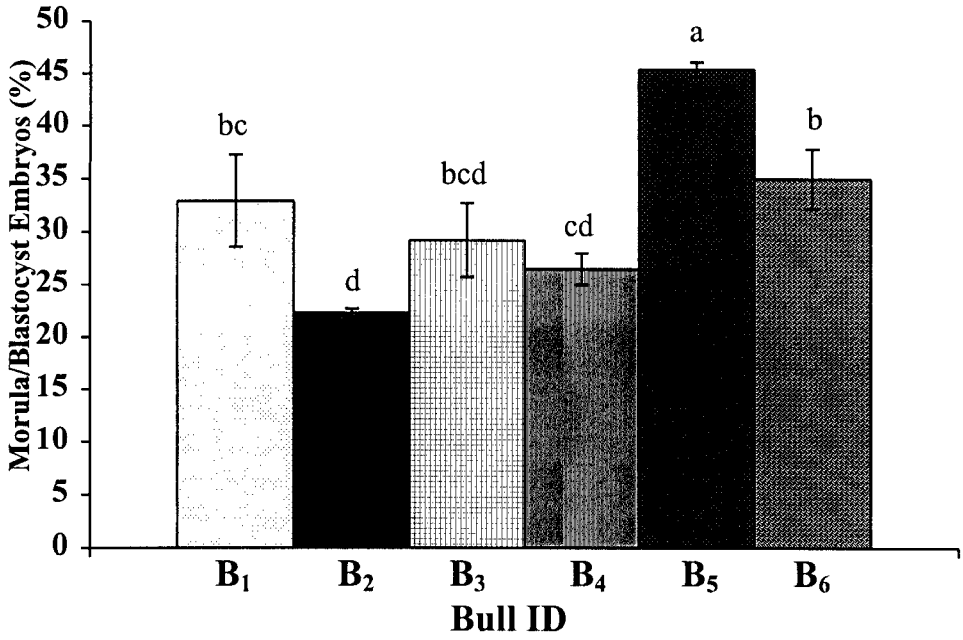


Figure 5.8. Percentage of morula to blastocyst stage embryos produced by fertilization of oocytes with spermatozoa from six experimental bulls (B_1-B_6) 168 h post insemination. The in-vitro fertilization and embryo production procedure was repeated four times for each bull with a total of 480 oocytes. a,b,c – Bars with different superscripts differ significantly ($P<0.01$).

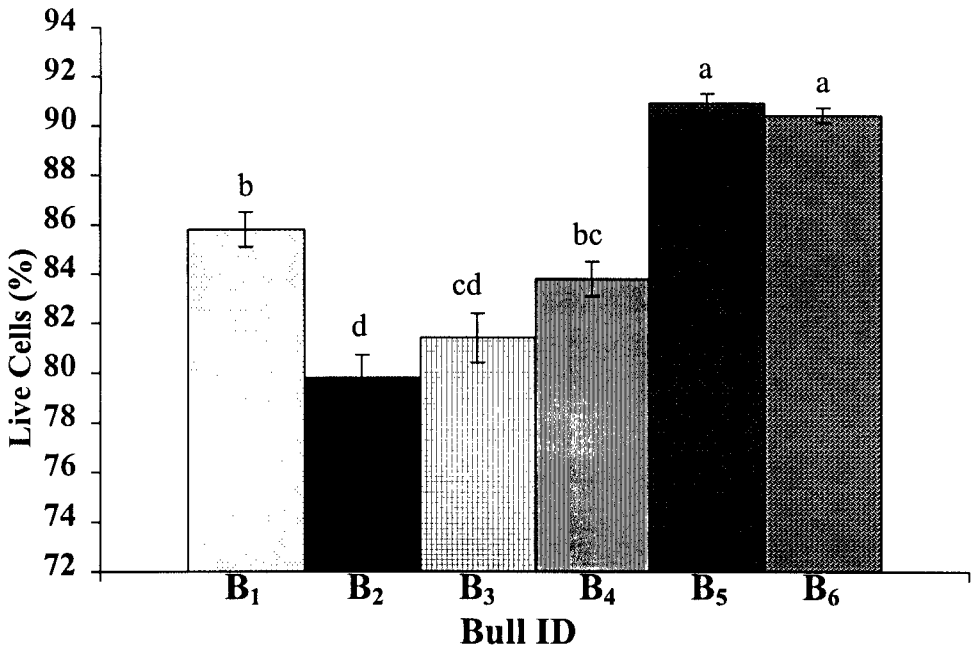


Figure 5.9. Percentage of live blastomeres in morula to blastocyst embryos produced by fertilization of oocytes with spermatozoa from six experimental bulls (B_1-B_6). The embryos were stained by bis-benzamide, annexin V, and propidium iodide and the live (blue), apoptotic (green) and dead (red) blastomeres were counted under a fluorescence microscope. The staining procedure was repeated four times with different sets of embryos and a total of 40 embryos were used for each bull. a,b,c – Bars with different superscripts differ significantly ($P<0.01$).

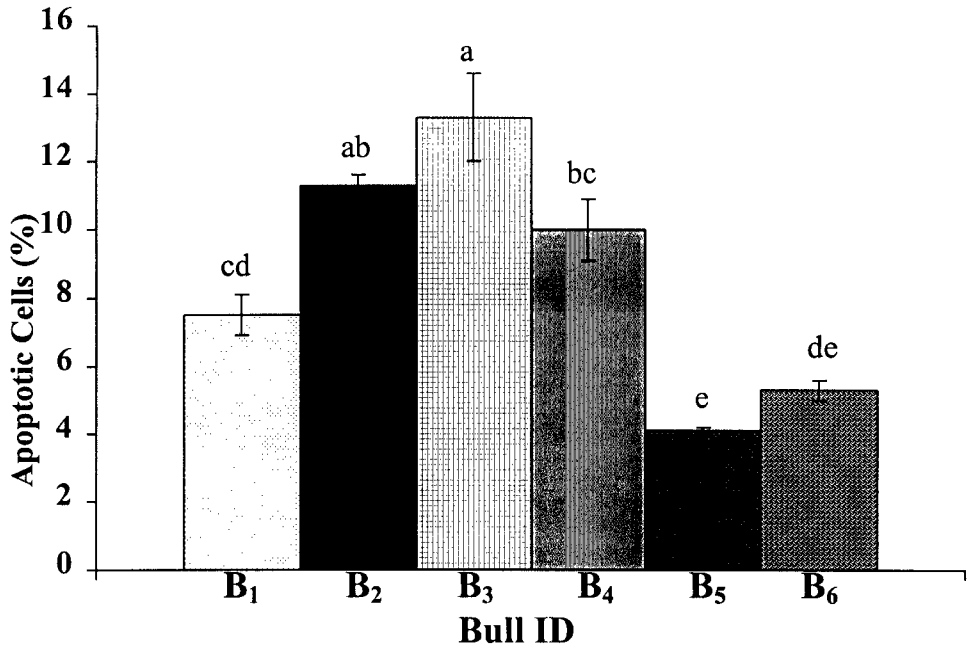


Figure 5.10. Percentage of apoptotic blastomeres in morula to blastocyst embryos produced by fertilization of oocytes with spermatozoa from six experimental bulls (B_1-B_6). The embryos were stained by bis-benzamide, annexin V, and propidium iodide and the live (blue), apoptotic (green) and dead (red) blastomeres were counted under a fluorescence microscope. The staining procedure was repeated four times with different sets of embryos and a total of 40 embryos were used for each bull. a,b,c – Bars with different superscripts differ significantly ($P<0.01$).

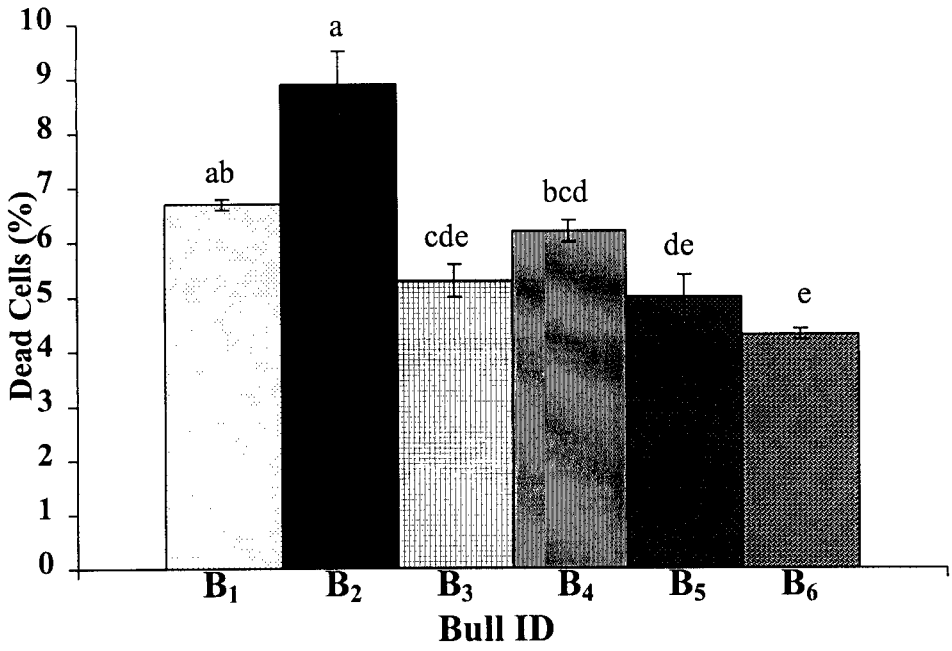
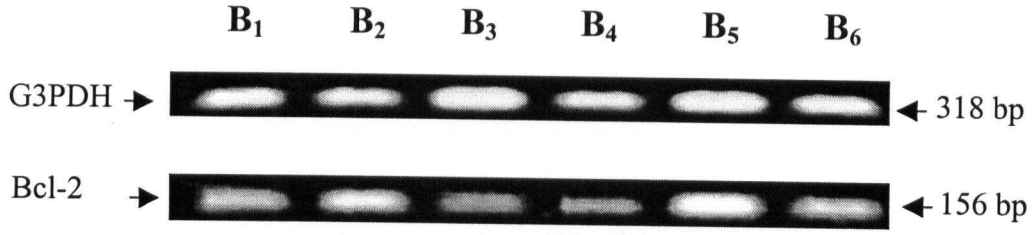


Figure 5.11. Percentage of dead blastomeres in morula to blastocyst embryos produced by fertilization of oocytes with spermatozoa from six experimental bulls (B_1-B_6). The embryos were stained by bis-benzamide, annexin V, and propidium iodide and the live (blue), apoptotic (green) and dead (red) blastomeres were counted under a fluorescence microscope. The staining procedure was repeated four times with different sets of embryos and a total of 40 embryos were used for each bull. a,b,c – Bars with different superscripts differ significantly ($P<0.01$).

A)



B)

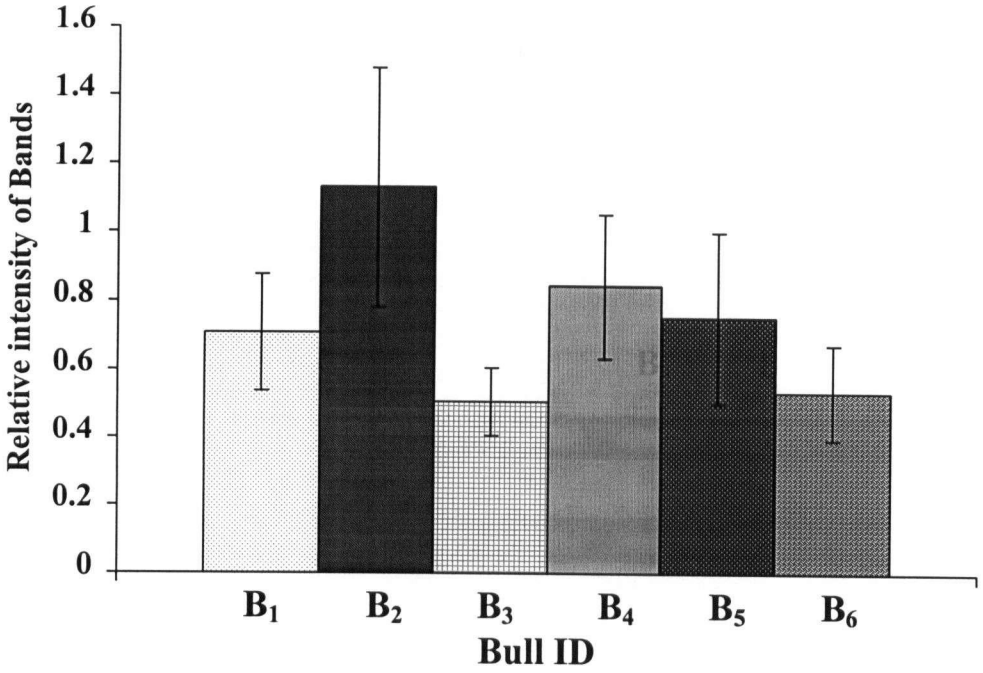
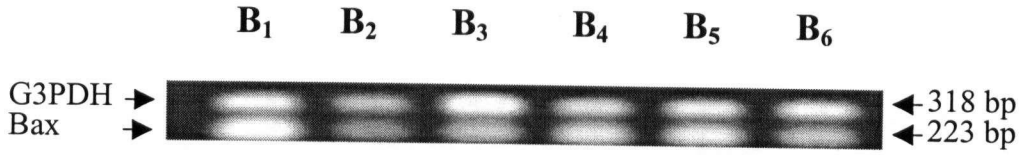


Figure 5.12. Relative abundance of Bcl-2 mRNA transcripts in morula to blastocyst embryos produced by fertilization of oocytes with spermatozoa from six experimental bulls (B_1-B_6). The total RNA in the experimental embryo samples was reverse transcribed and amplified using a thermal-cycler as described in the Materials and Methods. The PCR products were resolved on ethidium bromide stained 2% agarose gel and photographed. The optical density of the bands on the inverse image was measured. The RT-PCR was repeated four times with separate sets of embryos, the representative gel photograph and the average ratio of Bcl-2 to G3PDH band density (mean ±SEM) are shown in **A)** and **B)**, respectively.

A)



B)

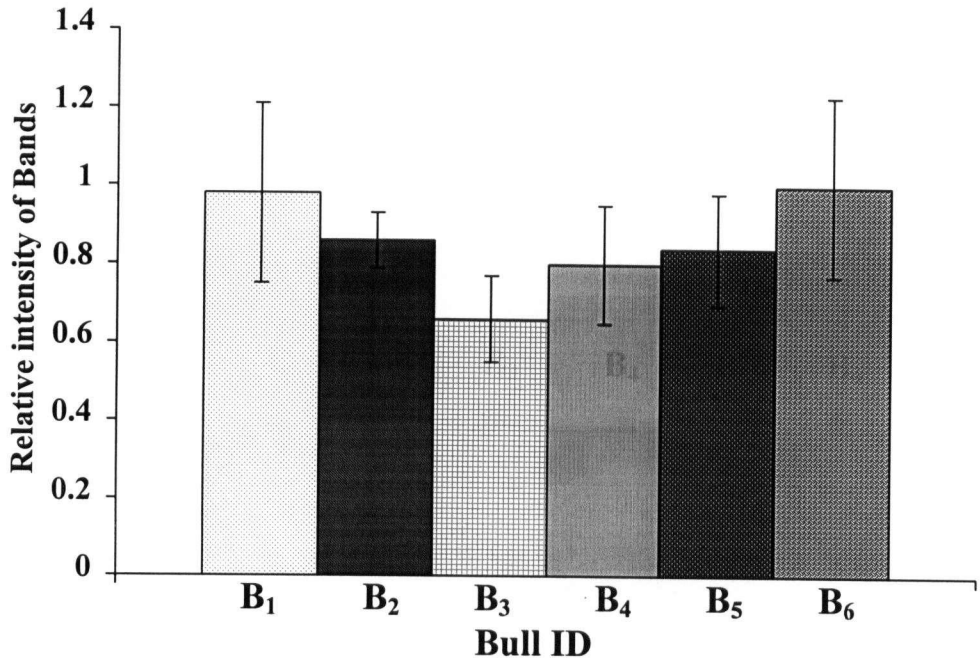
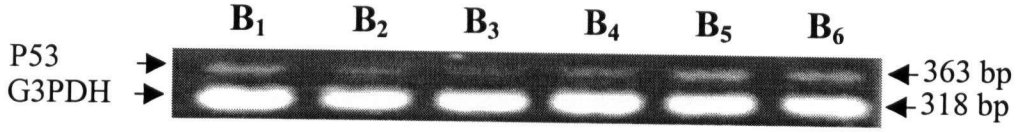


Figure 5.13. Relative abundance of Bax mRNA transcripts in morula to blastocyst embryos produced by fertilization of oocytes with spermatozoa from six experimental bulls (B_1-B_6). The total RNA in the experimental embryo samples was reverse transcribed and amplified using a thermal-cycler as described in the Materials and Methods. The PCR products were resolved on ethidium bromide stained 2% agarose gel and photographed. The optical density of the bands on the inverse image was measured. The RT-PCR was repeated four times with separate sets of embryos, the representative gel photograph and the average ratio of Bax to G3PDH band density (mean ±SEM) are shown in **A)** and **B)**, respectively.

A)



B)

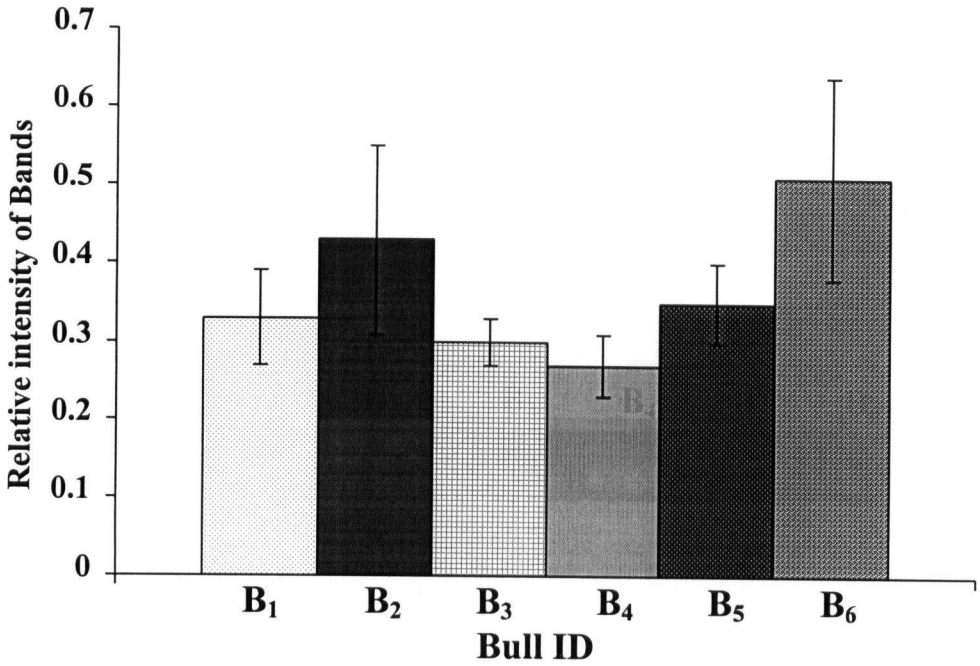
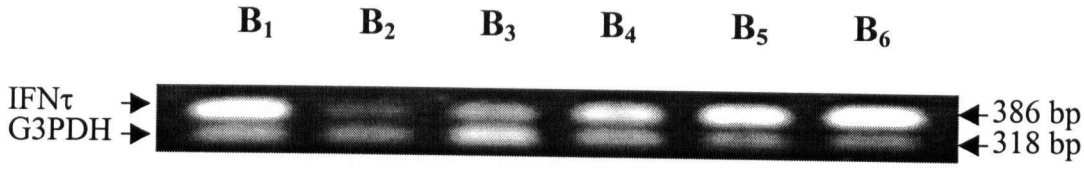


Figure 5.14. Relative abundance of p53 mRNA transcripts in morula to blastocyst embryos produced by fertilization of oocytes with spermatozoa from six experimental bulls (B_1-B_6). The total RNA in the experimental embryo samples was reverse transcribed and amplified using a thermal-cycler as described in the Materials and Methods. The PCR products were resolved on ethidium bromide stained 2% agarose gel and photographed. The optical density of the bands on the inverse image was measured. The RT-PCR was repeated four times with separate sets of embryos, the representative gel photograph and the average ratio of p53 to G3PDH band density (mean ±SEM) are shown in **A)** and **B)**, respectively.

A)



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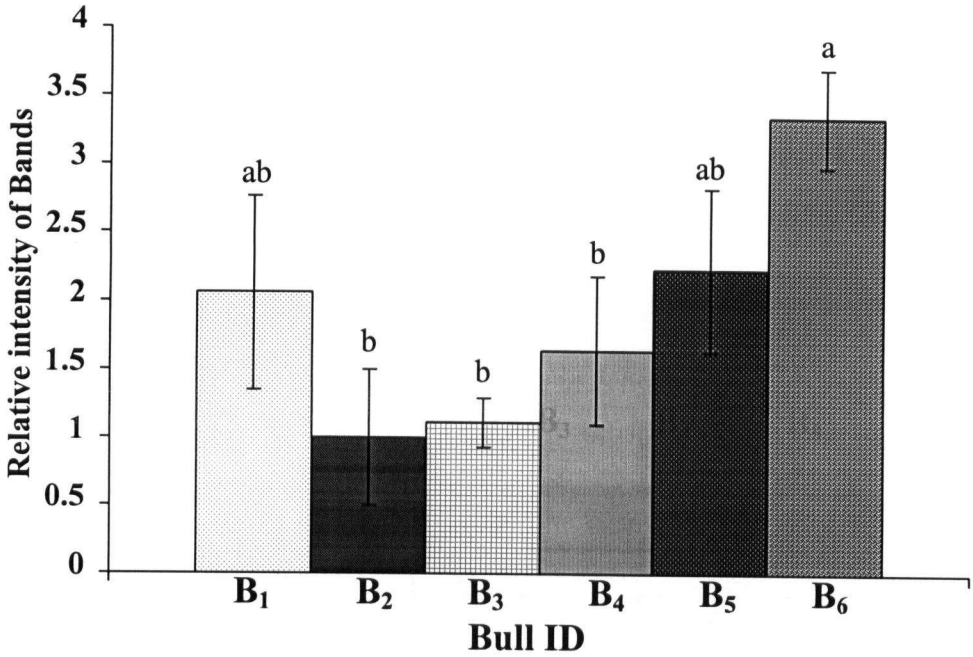
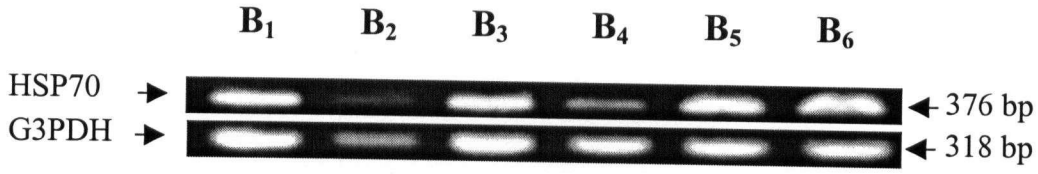


Figure 5.15. Relative abundance of interferon tau mRNA transcripts in morula to blastocyst embryos produced by fertilization of oocytes with spermatozoa from six experimental bulls (B_1-B_6). The total RNA in the experimental embryo samples was reverse transcribed and amplified using a thermal-cycler as described in the Materials and Methods. The PCR products were resolved on ethidium bromide stained 2% agarose gel and photographed. The optical density of the bands on the inverse image was measured. The RT-PCR was repeated four times with separate sets of embryos, the representative gel photograph and the average ratio of IFNτ to G3PDH band density (mean ±SEM) are shown in **A)** and **B)**, respectively. a,b,c – Bars with different superscripts differ significantly ($P<0.05$).

A)



B)

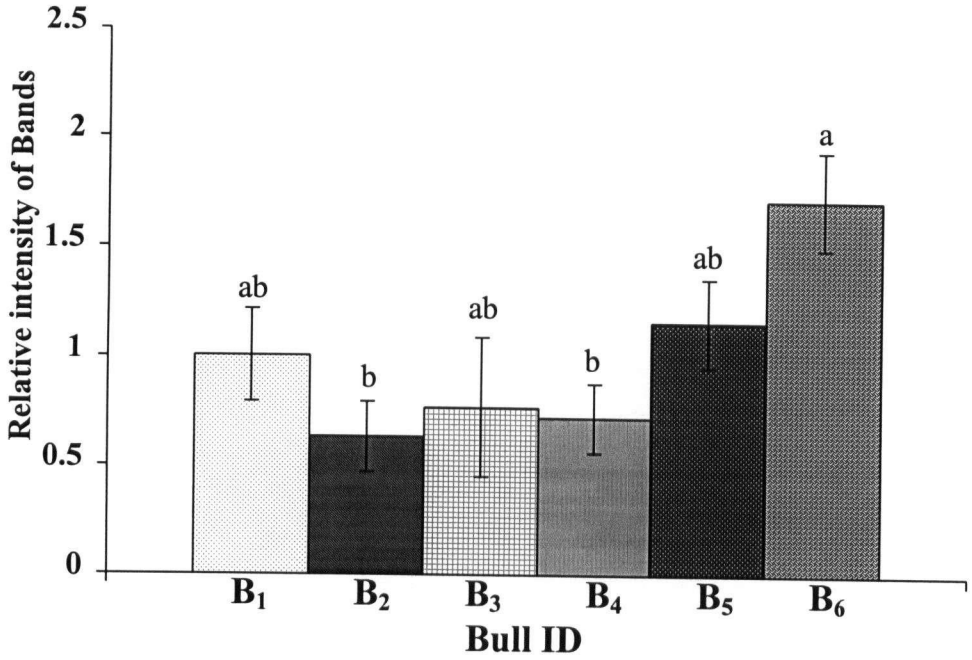


Figure 5.16. Relative abundance of HSP70 mRNA transcripts in morula to blastocyst embryos produced by fertilization of oocytes with spermatozoa from six experimental bulls (B_1-B_6). The total RNA in the experimental embryo samples was reverse transcribed and amplified using a thermal-cycler as described in the Materials and Methods. The PCR products were resolved on ethidium bromide stained 2% agarose gel and photographed. The optical density of the bands on the inverse image was measured. The RT-PCR was repeated four times with separate sets of embryos, the representative gel photograph and the average ratio of HSP70 to G3PDH band density (mean ±SEM) are shown in **A)** and **B)**, respectively. a,b,c – Bars with different superscripts differ significantly ($P<0.05$)

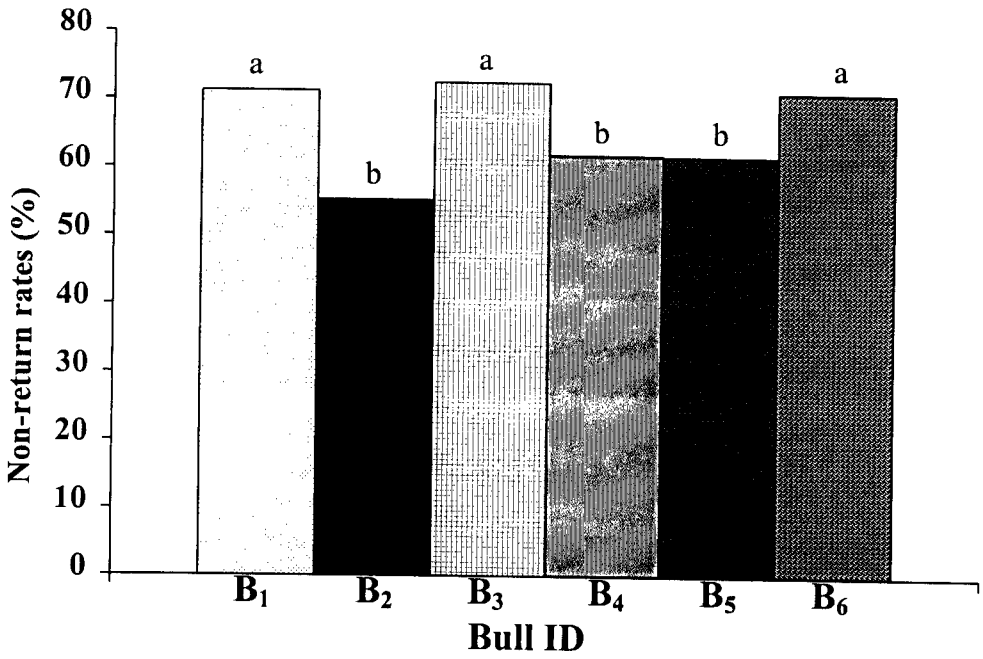


Figure 5.17. The field fertility of six experimental bulls measured by 60-90 day non-return rates. a, b - Bars with different superscripts differ significantly (P<0.01).

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CHAPTER 6 – GENERAL DISCUSSION AND CONCLUSIONS

Artificial insemination is the most extensively used technology in the dairy industry to improve reproductive and production efficiency (reviewed by Foote 2003). Huge expenditures are incurred by AI organizations in terms of time, labour and management costs to prove bulls for their fertility and genetic merit (reviewed by Rodriguez-Martinez and Larsson, 1998; Giritharan et al., 2004). This indicates that there will be significant advantages to the cattle industry as well as to the AI industry, if simple laboratory tests are made available to predict fertility of young bulls recruited for progeny testing programs. Although several laboratory tests for semen evaluation have been developed in the past, most of them lack repeatability or precise prediction of bull fertility in the field (reviewed by Larsson and Rodriguez-Martinez, 2000; Foote, 2003; Rodriguez-Martinez, 2003). The aim of this thesis was to further address the problem of prediction of bull fertility in the field by analyzing sperm related functions such as pre-freeze motility, acrosome reaction, sperm-zona binding, fertilization, embryo gene expression, apoptosis and development in-vitro. The possible implications of these in-vitro sperm functions on the prediction of in-vivo fertility were also examined.

In the first experiment (Chapter 3), the bull effects on sperm acrosome reaction, sperm-zona binding and in-vitro embryo production, and the correlation of these parameters to pre-freeze motility and field fertility measured by 60-90 day non-return rate were investigated. Bull effects were observed in pre-freeze motility, acrosome reaction at 0 h, increase in acrosome reaction at 4 h and sperm-zona binding. The pre-freeze motility was correlated negatively with acrosome reaction at 0 h, and positively with the increase in acrosome reaction at 4 h, and sperm-zona binding. Although

acrosome reaction at 0 h was negatively correlated to sperm-zona binding and cleavage rate, the increase in acrosome reaction at 4 h was positively correlated with sperm-zona binding and cleavage rate. None of these tests was correlated with non-return rates (field fertility). The results of this experiment showed bull variations in sperm pre-freeze motility, acrosome reaction 0 h after thawing, spontaneous acrosome reaction after 4 h incubation in sperm capacitation medium and sperm-zona binding. When checking the correlations, although there was no relationship between in-vitro sperm functions and 60-90 day non-return rates, individual sperm functions showed relationships among them. This indicates that the accuracy of the non-return rate measurement may be questionable. Because, some of the routinely measured sperm functions, such as sperm pre-freeze motility, showed either positive correlation (Januskauskas et al., 2001) or no correlation with field fertility measured by 60-90 day non-return rate (Bailey et al., 1994; Zhang et al., 1999). In addition, most of the sperm functions examined in this study were correlated with pre-freeze motility. Many factors determine the accuracy of the non-return rate, and non-return rates corrected for these factors such as ejaculate, season, inseminator and parity are considered very accurate and have shown high correlation with in-vitro tests (Zhang et al., 1999). On the other hand, since several factors determine in-vivo fertility, thousands of inseminations should be performed to get the accurate estimate of in-vivo fertility of a bull (reviewed by Foote, 2003; Rodriguez-Martinez, 2003). However, less than one thousand inseminations were performed to get the field fertility of the experimental bulls used in the present study. Getting field fertility data from a larger sample may give a definitive answer for the relationship of these sperm functions to field fertility (reviewed by Foote,

2003; Rodriguez-Martinez, 2003). The results of this experiment indicated that bulls did not affect the in-vitro embryo development measured by cleavage and blastocyst production rates. Whereas, the result of the third experiment (chapter 5), in which the bulls were selected with a wide range of in-vivo fertility, revealed that bull affects embryo development as measured by cleavage and morula to blastocyst production rates. This finding indicates that the bull effect on in-vitro embryo development can be clearly shown by selecting the bulls with a wide range of in-vivo fertility, as previous findings showed high degrees of correlation between in-vitro tests and the field fertility using bulls with wide range of non-return rates (Linford et. al., 1976; Zhang et. al., 1999).

The other interesting finding in the first experiment was that a very high percentage of sperm, on average 30-40%, showed acrosomal membrane damage due to the freezing procedure and low fertility bulls showed higher acrosomal membrane damage. This indicates that some of the fertility problems associated with bulls may be overcome by either improving cryopreservation procedure with the same insemination dose or increase the dose with the same cryopreservation procedure. This observation supports the findings of Saacke et al. (2000) who also showed that some of the compensable fertility deficiencies could be corrected by increasing the concentration of sperm in the insemination dose. In addition, results of the second experiment, in which increased sperm concentration yielded high fertility, support this concept.

This was the first study in which the zona pellucida of the 18-24 h matured oocyte was used for sperm-zona binding assay with the assumption that the zona from mature oocytes would reflect a very accurate relationship with in-vitro as well as in-

vivo fertility. However, the results revealed that, although bulls affected the sperm-zona binding, the sperm-zona binding showed a poor relationships with in-vitro as well as with in-vivo fertility due to unknown reasons. This poor relationship may be attributable to either the use of bulls with a narrow range of non-return rates (64.9 to 71.9 %) or the high concentrations of sperm used in this experiment. Using bulls with a wide range of non-return rates and a lower insemination dose may yield a definitive relationship between sperm-zona binding and field fertility.

In the second experiment (Chapter 4), a very efficient and less time consuming nuclear staining technique was used to determine the effect of bull on in-vitro fertilization (including both normal and polyspermic) using short and long time pre-incubated sperm in normal and high sperm concentrations. The implications of this bull effect on the prediction of in-vivo fertility, and the relationships to sperm acrosome reaction, sperm-zona binding and in-vitro fertilization were also examined. In addition, the effect of sperm pre-incubation time and concentration on in-vitro fertilization was investigated. Using both normal (25,000:1) or higher (50,000:1) sperm:oocyte ratios with shorter (0 h) sperm pre-incubation time in the in-vitro fertilization process, the bull affected the percentage of zygotes (including both normal and polyspermic) and normally fertilized zygotes formed. When using the higher sperm:oocyte ratio with longer (6 h) sperm pre-incubation, the bull affected the percentage of zygotes formed, but not the percentage of normally fertilized zygotes. However, when using a normal sperm:oocyte ratio with longer sperm pre-incubation time, the bull affected only the percentage of normally fertilized zygotes, but not the percentage of zygotes. In addition, using a higher sperm:oocyte ratio with a longer sperm pre-incubation time, the bull

affected the percentage of polyspermic zygotes. When data from all the bulls were pooled, the sperm concentration and pre-incubation time affected the percentage of zygotes and normally fertilized zygotes formed. When using a normal sperm:oocyte ratio, the difference in the percentage of normally fertilized zygotes between shorter and longer sperm pre-incubation times showed high degree of correlation with non-return rates of the experimental bulls. A high degree of correlation was also observed between sperm pre-freeze motility, percentage of zygotes, and normally fertilized zygotes with normal sperm:oocyte ratio and longer sperm pre-incubation time. Increase in acrosome reaction at 4 h was highly correlated with the percentage of polyspermic zygotes, with a normal sperm:oocyte ratio, and a long sperm pre-incubation time. The cleavage rate showed a high degree of correlation with percentage of zygotes with normal sperm:oocyte ratio and long sperm pre-incubation time. When using the high sperm:oocyte ratio, the difference in the percentage of normally fertilized zygotes between short and long sperm pre-incubation times showed high degree of correlation with blastocyst production rate of the experimental bulls.

The findings of this experiment indicated that bulls showing higher non-return rates exhibited a significant reduction in their in-vitro fertilizing ability within 6 h of pre-incubation in capacitation medium. Although, the bulls showing higher non-return rates produced higher percentages of zygotes in both short and long pre-incubation times with the normal sperm:oocyte ratio, surprisingly, their in-vitro fertility was significantly reduced from short to longer pre-incubation time than bulls showing lower non-return rates. Hence, evaluation of the reduction in in-vitro fertility in combination with other routinely used semen evaluation methods may be useful in predicting bull

field fertility. Another interesting finding in this experiment is that increasing the sperm:oocyte ratio from 25,000:1 to 50,000:1 using sperm concentrations of 5 million/mL and 10 million/mL resulted in an increase in the percentage of zygotes and normally fertilized zygotes. In contrast to the findings of this experiment, previous studies using sperm:oocyte ratio of 5,000:1 to 50,000:1 in a sperm concentrations of 0.5 to 4 million/mL reported that increasing sperm:oocyte ratio above 5,000:1 either did not affect or reduced the fertilization rate (Kurtu et al., 1996; Camargo et al., 2000; Tanghe et al., 2000).

The third experiment (Chapter 5) investigated bull effects on embryo viability at various levels such as in-vitro embryo development, apoptosis, and gene expression. Significant bull effects were observed on in-vitro embryo development as measured by cleavage and morula to blastocyst development rates, embryo apoptosis as measured by percentage of apoptotic, live and dead blastomeres in morula to blastocyst stage embryos, and the expression levels of HSP70 and IFNτ genes in morula to blastocyst stage embryos. However, bulls did not affect the expression levels of Bax, Bcl-2 and p53 genes in morula to blastocyst stage embryos produced in-vitro. Percentage of live cells was positively correlated with cleavage rate, morula to blastocyst development rate, and IFNτ and HSP70 gene expression levels in morula to blastocyst stage embryos produced in-vitro. Percentage of apoptotic cells was negatively correlated with cleavage rate, morula to blastocyst development rate, and IFNτ and HSP70 gene expression levels in morula to blastocyst stage embryos. The field fertility measured by 60-90 day non-return rate was correlated negatively with relative abundance of Bcl-2 mRNA

transcripts and positively with the ratio of Bax to Bcl-2 gene expression. None of the other in-vitro embryo parameters tested was correlated with 60-90 day non-return rate.

During the past decade, several studies have reported the regulation of embryo development by apoptosis and gene expression (Yang and Rajamahendran, 2002; Knijn et al., 2003; Lonergan et al., 2003; Rizos et al., 2003). However, very few studies have investigated the bull effects on these developmentally essential cell processes. The present study is the first, which investigated bull effects on these processes in bovine embryos. The findings of this experiment showed that the bull influences development, apoptosis, and expression of IFNτ and HSP70 genes in in-vitro produced morula to blastocyst stage embryos. Another interesting finding in this experiment was that bulls affected embryo apoptosis and development in-vitro. Since apoptosis, IFNτ and HSP70 regulate cell proliferation, maternal recognition of pregnancy and protection, respectively, to ensure survival of embryo (Edwards et al., 1997; Godkin et al., 1997; Hardy 1997; Paula-Lopes and Hansen, 2002; Wolf et al., 2003), it was hypothesized that the in-vivo fertility of the bulls is better reflected by expression of IFNτ and HSP70 genes, and apoptosis in the pre-implantation embryos. However, the results revealed that although in-vitro fertility was related to expression of IFNτ and HSP70 genes, and apoptosis in morula to blastocyst stage embryos, in-vivo fertility did not show a relationship with expression of these genes and apoptosis. This was due to an opposite trend in the expression of these genes, and apoptosis in the morula to blastocyst embryos produced from the sperm of one bull in each of the low fertility and high fertility groups. This could be due to the small sample number used in this study in which, by chance, one bull in the low fertility group and one bull in the high fertility

group might have been allocated very poor and good quality oocytes in all four replications, respectively. In addition, it has been shown that there is a high variation in the quality of ovaries and oocytes in the day-to-day collection from slaughterhouse. Using either oocytes collected repeatedly by ultrasound guided ovum pick up from same animals for in-vitro fertilization with sperm from all experimental bulls or a higher sample number might reveal a definite answer for the relationship of vivo fertility with the expression of these genes.

Irregular or imbalanced expression patterns of Bax, Bcl-2 and p53 genes induce apoptotic cell death and embryo mortality during early stages of development (Korsmeyer et al., 1993; Hardy, 1997; Haupt et al., 2003). Hence, it was hypothesized that the fertility of bulls could be reflected by the expression of Bcl-2, Bax and p53 genes in the morula to blastocyst stage embryos. However, the results showed that although there is no relationship between expression levels of these genes and percentage of apoptotic cells, surprisingly, there is a negative relationship between expression of Bcl-2 gene and in-vivo fertility. Since Bcl-2 is an anti-apoptotic protein and expressed to ensure the survival or viability of cells, the negative relationship of in-vivo fertility with the expression level of Bcl-2 gene in the embryo might be due to compensatory expression of Bcl-2 in the remaining live cells of the embryos, which were produced using sperm from low fertility bulls and undergoing apoptosis.

This study demonstrated that there is a paternal influence on apoptosis and expression levels of IFNτ and HSP70 genes in bovine preimplantation embryos. This is a strong indication that the paternal influence is also on the expression of other essential genes involved in the pre-implantation embryo development. Based on these finding,

future studies could be designed to evaluate expression levels of multiple genes using gene microarray technique. The outcome of these future studies might be usefull for the evaluation of bull field fertility.

This study concluded that the new functional assays such as sperm acrosome reaction, sperm zona-binding and in vitro fertilization tests might be useful with routine semen analysis tests in the prediction of bull fertility in the field. The fertile lifespan of sperm from bulls showing higher field fertility measured by 60-90 day non-return rates reduces with in-vitro pre-incubation in capacitation medium and this measure may be also useful for the prediction of bull fertility in the field. The bull affects preimplantation embryo development, apoptosis, and expression levels of HSP70 and IFNτ genes. Measurement of either the Bcl-2 gene or a ratio of Bax to Bcl-2 gene expression levels in morula to blastocyst stage embryos produced in-vitro may be useful in predicting bull field fertility. Getting a fertility index from the outcome of a combination of these tests might help in the accurate prediction of field fertility. Such a combination of assays, however, remains yet to be determined.

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