CONSEQUENCES OF POST-EJACULATORY STRESS ON THE BOVINE SPERMATOZOA FOR THE RESULTANT EMBRYO

By

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To my husband, Gregory Hendricks, for all the love, laughter and tears that we have shared together through this adventure and to my mother, Elsa-May, who believed in me and taught me that you can do anything you put your mind to.

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The ability of the preimplantation embryo to complete its developmental program is determined in large part by its acquisition of genetic and non-genetic components from the sperm and oocyte. While the role of damage to the oocyte on embryo competence has been well established, it is not clear whether damage to the sperm after ejaculation results in formation of an embryo with reduced competence for development. Accordingly, a series of experiments were conducted to determine whether this was the case. Stresses examined were aging, heat shock, irradiation, and oxidative stress. The experimental approach involved evaluation of oocytes inseminated with sperm exposed to stress to cleave and develop to the blastocyst stage of development.

Aging of sperm by incubation for 4 h at a temperature characteristic of normal body temperature (38.5 degree Celsius) or hyperthermia (40 degree Celsius) reduced cleavage, but did not alter embryo competence as measured by the percent of cleaved embryos that became blastocysts. Similarly, exposure of sperm to X-irradiation characteristic of airport screening devices for checked or carry-on luggage had no effect on embryo competence. Cleavage rate was reduced by X-irradiation after multiple exposures at the checked luggage dose but there was no reduction in the percent of cleaved embryos becoming blastocysts at any exposure.

In contrast, oxidative stress did affect embryo competence to develop to the blastocyst stage. Oocytes inseminated with sperm treated with menadione or *tert*-butyl hydroperoxide had reduced cleavage and a reduced percentage of cleaved embryos that became blastocysts.

One potential mechanism for sperm damage affecting the resultant embryo is the initiation of DNA damage during sperm cell apoptosis. To test whether apoptosis could be induced in ejaculated spermatozoa, bull and stallion sperm were subjected to heat shock and aging and features of apoptosis evaluated. Endpoints included terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), mitochondrial membrane potential, and presence and activation of procaspase-9 and procaspase-3. There was only a slight increase in TUNEL labeling after 24 h aging and the increase was not blocked with a caspase inhibitor. Moreover, procaspase-9 was detected in bovine sperm but not activated by 4 h aging at 38.5, 40 and 41 degree Celsius and procaspase-3 was not detected.

Taken together, these results indicate that specific types of stress occurring after ejaculation can negatively impact the ability of the subsequent embryo to develop. Understanding these effects on early embryonic development may lead to new approaches for reducing early embryonic loss.

CHAPTER 1
LITERATURE REVIEW

> It is conceivable, and indeed probable, that every part of the adult contains molecules derived from the male and from the female parent; and that, regarded as a mass of molecules, the entire organism may be compared to a web of which the warp is derived from the female and the woof from the male.--Huxley and Sully, 1888

The ability of the preimplantation embryo to complete its developmental program is determined in large part by its acquisition of genetic and non-genetic components from the sperm and oocyte. While the role of damage to the oocyte on embryo competence has been well established (Sirard et al., 2006), it is not clear whether damage to the sperm after ejaculation results in formation of an embryo with reduced competence for development.

Spermatozoal abnormalities which lead to failure in embryogenesis prior to maternal recognition are defined as 'uncompensable' since these cannot be minimized or eliminated by increasing sperm dosage alone. Several examples in the literature illustrate the importance of the sperm for embryo quality. For example, fertilization with sperm produced from bulls with thermally-insulated testis leads to a delay in pronuclear formation (Walters et al., 2006), reduction in embryo cleavage rates, blastocyst development, production of a higher frequency of low quality embryos, and increased early embryo losses *in vivo* (Walters et al., 2005ab) and *in vitro* (Saacke et al, 1994). Similarly, fertilization with sperm produced by low fertility bulls results in a reduction in early cleavage rates and pronuclear formation (Eid et al., 1994; Saacke et al., 1994) and reduction in embryo development (Saacke et al., 1994).

Most research on contributions of the sperm to embryonic competence has focused on effects of stress on the male gamete that occurred during spermatogenesis. Little is known regarding whether stress of sperm occurring after ejaculation (while sperm are in the female reproductive tract or cryopreserved) leads to decreased embryonic potential for development. In the past 50 years the interest in post-ejaculatory sperm damage has increased, especially in light

of improvements in artificial reproductive technology (ART). Today, a single sperm can be selected based on morphological qualities to be used for intra-cytoplasmic sperm injection (ICSI), yet a morphologically normal sperm does not always give rise to a competent embryo (Gómez et al., 1997). The view that sperm were simply a DNA bullet is changing as more focus is being placed on the paternal contributions to the embryo.

Sire Effects on Embryonic Development

Differences in embryonic development can be attributed in part to differences in spermatozoal contribution to the embryo. Despite a group of bulls producing embryos having similar cleavage rates, the ability of the embryos produced by individual bulls to develop can be drastically different (Shi et al., 1990; 1991; Saacke et al., 1994). This phenomenon, the 'bull or sire effect' is due to differences in sperm quality rather than quantity and has been termed 'uncompensable'. These uncompensable differences reflect spermatozoal abnormalities which lead to failure of fertilization or sustained embryogenesis (Saacke et al., 1994). This paternal effect has also been noted in other species, including the ram (Morris et al., 2003) and men (Tesarik et al., 2002).

There are genes that control embryonic development. One of the best known is the *Ped* gene in the mouse. The product of the Ped gene is Qa-2, a cell surface protein and mice are defined as either *Ped fast* (expressing Qa-2 protein) or *Ped slow* (Qa-2 is not expressed). When exposed to anti-Qa-2 monoclonal Ab at the 2-cell stage cross-linking of the protein occurs and results in a significant increase in blastocyst development in the embryos expressing Qa-2 (McElhinny and Warner, 2000). There appears to be a bovine homolog (bovine MHC I 4221.1/Ped gene; Fair et al., 2004), however it is not known whether this gene is maternally imprinted and hence paternally expressed. Fibroblast growth factor 2 (FGF2) gene has also been associated with embryonic survival and fertilization rate in cattle; however, FGF2 seems to be

maternally governed (Khatib et al., 2008a). However, there is evidence to suggest that the paternal inherited signal transducer and activator of transcription 5A (STAT5A) gene plays a role in fertilization rates and embryonic survival in cattle (Khatib et al., 2008b).

The sire effect extends to the ability of males to fertilize the oocyte and may be reflected for example, in a group of bulls by their non-return rates. This parameter for the evaluation of reproductive performance of bulls gives the percent of cows/heifers that did not return for breeding within a specific time after insemination. In one study where bulls were divided into two groups based on their lifetime non-return rates as either low fertility (66±1) or high fertility bulls (78±1), sperm from low fertility bulls were less able to penetrate the oocyte and to sustain embryonic development to the morula and blastocyst stage (Hillery et al., 1990). Further, bulls differ in their ability to form the sperm aster following successful penetration and sperm incorporation into the oocyte (Navara et al., 1996). In this study sire effects on sperm aster size and microtubule organization during bovine fertilization was investigated using three bulls of known field and/or *in vitro* fertility – high, medium and low. It was demonstrated that the bull with the highest field fertility (79.5% nonreturn rate) and development after *in vitro* fertilization had the largest and most organized sperm aster, while the bull with the poorest development after *in vitro* fertilization (IVF) had the smallest and least organized aster (Navara et al., 1996).

Earlier cleaving embryos are more likely to develop to the blastocyst stage (Longerhan et al., 1999; Dinnyés et al., 1999; Ward et al., 2001) and sperm could affect an embryo's competence for development if the timing of first cleavage is delayed. The timing of first cleavage following insemination differ between bulls (Ward et al., 2001) and embryos produced by high-fertility bulls entered S-phase of the first cell cycle earlier and had a longer S-phase than

those produced by low-fertility bulls (Eid et al., 1994; Comizzoli et al., 2000) which may influence the timing of first cleavage and hence embryo competence for development.

Sire effects extend to differences in an embryo's potential to develop under heat stress conditions. In one study, lactating Holstein cows inseminated with Gyr semen during the Brazilian summer were more likely to be diagnosed pregnant 80 days after artificial insemination (AI) when compared to cows inseminated with Holstein semen (Pegorer et al., 2007). Moreover, Pegorer et al. (2007) demonstrated that even within breed (Gyr) sires can affect embryonic loss, where one Gyr bull had lower embryo/fetal loss between the first and second pregnancy diagnosis (30 to 40 days and 60 to 80 days after AI) than other Gyr bulls and even between breeds (Holstein bulls). It has been suggested that the oocyte has a more crucial role in the genetic ability of an embryo to resist effects of heat shock than the contribution of the spermatozoa (Block et al., 2002). However, other studies have indicated that the breed of the sire plays just as much a crucial role in embryo thermotolerance both in *in vivo* derived (Pegorer et al., 2007) and *in vitro* derived embryos (Barros et al., 2006). Eberhardt et al. (2005) demonstrated that both the oocyte and the sperm contribute to thermotolerance where embryos that had a predominant *Bos indicus* genotype were more likely to develop to the blastocyst stage following heat shock at 41°C for 12 h at 48 h post-insemination.

Fertilization

Following capacitation within the female reproductive tract, spermatozoa utilize enzymatic and mechanical (forward propulsion-hyperactivation) action to penetrate the cumulus cells of the cumulus oocyte complex. Once in contact with the zona pellucida the spermatozoon binds to the zona by receptor-ligand interaction. In the bovine this is achieved by interactions between galactosyltransferase (GalT) on the sperm membrane and N-acetylglucosamine residues on the ZP3 (Tengowski et al., 2001). Interaction with the ZP3 receptor stimulates the acrosome

reaction - plasma membrane and outer acrosomal membrane fusion, vesiculation and release of the acrosomal content. Forward motion and acrosomal enzymes help the spermatozoon to create a tract through the zona pellucida. Once through the zona, sperm bind and fuse to the plasma membrane of the oocyte. Sperm–oocyte fusion begins from the equatorial segment (between the inner acrosomal and plasma membranes overlying the nucleus in the posterior region of the sperm head (Yanagimachi and Noda, 1970, Bedford et al., 1979) and is thought to be mediated by a group of proteins which include the dimeric sperm glycoprotein – fertilin (Kaji and Kudo, 2004).

Upon fusion of the sperm with the plasma membrane of the oocyte, the oocyte becomes activated (intracellular calcium [Ca^{2+}] oscillations, completion of the second meiotic division and the oocyte undergoes the cortical reaction – exocytosis of cortical granules; Sutovsky et al., 2003b). The cortical reaction leads to hardening of the zona pellucida, loss of sperm receptors and constitutes the major block to polyspermy (Sun, 2003).

Fusion of the sperm membrane with the oocyte membrane and interaction with oocyte microvilli leads to engulfment of the entire sperm by the ooplasm (Sutovsky et al., 1996). Once within the oocyte the nuclear envelope of the sperm disperses and nuclear decondensation occurs; mitochondria within the connecting piece are displaced, the sperm proximal centriole is exposed to the ooplasm and formation of the sperm aster occurs (Sutovsky et al., 1996). The paternal mitochondria are targeted for destruction by ubiquitin (Sutovsky et al., 1999) and the remains of the sperm principal sheath are destroyed (Sutovsky et al., 1996; 2003a).

One of the first steps to male pronucleus formation involves desolution of the sperm nuclear envelope and the reduction of inter- and intraprotamine disulphide bonds formed during sperm maturation between cystein residues by oocyte derived glutathione. These events normally

occur as the fertilized oocyte transits from metaphase II to telophase II (Adenot et al., 1991). As telophase II advances sperm chromatin re-condenses and decondense once more concomitant with female pronucleus development (Adenot et al., 1991). The final steps of fertilization involve apposition of the male and female pronuclei and syngamy, mediated by the sperm aster and associated cytoskeletal structures.

Paternal Contributions to the Embryo

Paternal investments in development of the embryo consist of genetic and non-genetic factors. The genetic factors incorporated into the oocyte include the paternal DNA and mitochondrial DNA (mtDNA). However, paternal mitochondria are selectively ubiquitinated (Sutovsky et al., 1996) and shunted to the lysosomal apparatus for destruction in the bovine oocyte (Sutovsky et al., 2000). Nevertheless, paternally derived mitochondria are found in the bovine embryo as late as 2 to 4 cell stage (Sutovsky et al., 1996). It is possible that the paternal mtDNA might contribute to early embryonic development, but this is unlikely in the pig since complete degradation of sperm mitochondria in the cytoplasm of fertilized porcine oocytes occurs within 20 to 30 h after insemination prior to the first cleavage event (Sutovsky et al., 2003c). Others would argue that paternal mitochondria inheritance does occur. It has been demonstrated that a small amount of paternal mtDNA can survive in the mouse embryos of interspecific mitochondrial congenic mice and that 0.1 to 0.01% mtDNA in all tissues is of paternal origin (Gyllensten et al., 1991). However, Shitara and colleagues demonstrated that in interspecific hybrids, paternal mtDNA was not distributed to all tissues and was not transmitted from the females to the following generation (Shitara et al., 1998) and that sperm mitochondria were uniquely selected for elimination from mouse embryos when compared to liver mitochondria (Shitara et al., 2000).

The inheritance of paternal mtDNA has been associated with disease. The New England Journal of Medicine reported the case of a 28-year-old man with mitochondrial myopathy due to a 2-bp mtDNA deletion in the *ND2* gene. It was determined that the mutated mtDNA was paternal in origin and accounted for 90% of the patients muscle mtDNA (Schwartz and Vissing, 2002).

How does one account for the finding of paternal mtDNA in the offspring? Perhaps, the ubiqitination process of sperm is a species-specific phenomenon. Cross-breed embryos from interspecific mice; hybrid embryos formed from domestic cow eggs and sperm of wild cattle (gaur; Sutovsky et al., 2000) and hybrid birds formed from major and minor subspecies of the Great Tit (*Parus major*; Kvist et al., 2003) have been shown to contain paternally inherited mtDNA, and this may reflect the inability of the maternal ubiqitin-associated degradation machinery to recognize and eliminate paternal mitochondria with an inter-species ubiquitin tag.

The most important contribution of the sperm to the embryo is its DNA. Paternal DNA must undergo morphological and biochemical changes such that the chromatin structure is compatible with that of the oocyte for DNA replication, transcription and mitosis. Mammalian sperm chromatin structure and composition differs from that of somatic cell chromatin and that of the oocyte (Clarke, 1992). The basic unit is the toroid consisting of 50 kb of DNA wrapped around highly basic proteins – protamines (Hud et al., 1993). Protamines consist largely of arginine and cysteine residues which allow for strong DNA binding and for disulphide cross-linkage (Brewer et al., 2003) during sperm nuclear maturation in the epididymis. There are two types of protamines, P1 and P2. Bull sperm contains P1 only (Lee and Cho, 1999) and may have residual histones. Human sperm contain some of their histones (Zalensky et al., 1997; Gineitis et al., 2000) usually associated with the nuclear periphery and telomeric regions (Zalensky et al.,

1997; Gineitis et al., 2000). Since the DNA associated with histones is less tightly wound than DNA associated with protamines it is postulated that these areas may be the first structures to respond to oocyte signals for pronucleus formation (Gineitis et al., 2000).

The non-genetic factors incorporated into the oocyte upon fertilization include the oocyte-activating factor, centriole, sperm nuclear matrix and RNAs. During fertilization the sperm perinuclear theca is solubilized in the oocyte cytoplasm and releases a number of sperm factors into the oocyte (Sutovsky et al., 1997; 2003b). One such factor is the sperm oocyte-activating factor (SOAF), which triggers Ca^{2+} oscillations and a block to polyspermy (Sutovsky et al., 2003b). Several candidates for SOAF have been extracted from sperm. However, phospholipase C-zeta (PLCζ) seems to be the likely candidate for SOAF (Swann et al., 2006; Saunders et al., 2007). Studies using oocytes and oocyte extracts established that microinjection of PLCζ complementary RNA (Saunders et al., 2002; Yoneda et al., 2006) and recombinant PLCζ (Kouchi et al., 2004) trigger Ca^{2+} oscillations similar to those seen at fertilization. Phospholipase C-zeta-immunodepleted soluble sperm extracts are incapable of triggering Ca^{2+} oscillations (Saunder et al., 2002) and RNAi knockdown of PLCζ reduces the number of Ca^{2+} oscillations and the activation rates in oocytes fertilized by transgenic sperm (Knott et al., 2005). Phospholipase C-zeta has been identified in the soluble sperm extracts of the mouse, pig, hamster and man (Saunders et al., 2002; Fujimoto et al., 2004; Kurokawa et al., 2005; Young et al., 2008; Grasa et al., 2008) and localized to the equatorial area of bull sperm and to the post-acrosomal region of mouse sperm (Yoon and Fissore, 2007). Importantly, the quantity of PLCζ required to activate the oocyte is in the range of that carried by a single sperm (Saunders et al., 2002).

Boveri first recognized (in 1901) that the egg typically losses the centrosome during oogenesis and that the sperm introduces this structure at fertilization (Schatten, 1994). In most

mammals with exception of some rodents, the centrioles are lost during oogenesis and partially degraded during spermatogenesis (Schatten, 1994; Manandhar et al., 2005). Concurrently, the pericentriolar matrix is retained in the oocyte but lost in the sperm. During fertilization mammalian embryos inherit the proximal centriole from the sperm, which is responsible for aster formation during the first cell-cycle (Navara et al., 1994; 1995; Palermo et al., 1997). Proper sperm aster formation is required for successful fertilization as sperm aster microtubules provide the tracks for pronuclear migration and apposition of the male and female pronuclei facilitating syngamy (Navara et al., 1996; Sutovsky et al., 1996). The paternally derived centriole duplicates during interphase concurrent with the pronuclear stage and the centrosome initially organizes a sperm aster concurrent with male and female pronuclei breakdown (prometaphase). The sperm aster now containing two centrioles split and moves to opposite poles of a bipolar spindle to establish bipolarization (anaphase), and paternal and maternal chromosomes organize on the equator of a metaphase spindle, at syngamy (Schatten, 1994; Sutovsky et al., 1996; Palermo et al., 1997). During cytokinesis the centrioles are at opposite ends and will form the centrosome of the daughter blastomeres (Schatten, 1994; Navara et al., 1995).

The sperm nuclear matrix is also inherited by the fertilized oocyte and there is some evidence that the sperm nuclear matrix is essential for events during fertilization (Ward et al., 1999; Shaman et al., 2007). In mice, removal of the sperm nuclear matrix from the DNA prior to injection into mouse oocytes results in failure of paternal DNA replication and pronuclear formation (Shaman et al., 2007) without derailment of maternal DNA replication and pronuclear formation. Injection of isolated sperm nuclear matrix and isolated matrix associated DNA does not reconstitute the ability for paternal DNA replication (Shaman et al., 2007). While injection of matrix-associated regions (areas where the DNA loop domain attach to the sperm nuclear matrix)

into mouse oocytes without large portions of the DNA loop initiate DNA replication (Shaman et al., 2007).

Ribonucleic acids (RNAs; messenger RNAs, microRNAs and antisense RNAs) have been localized within ejaculated sperm (Ostermeier et al., 2002; Miller and Ostermeier, 2006; Amanai et al., 2006) and unique paternal mRNA have been found to persist in the embryo up to 3 h post fertilization (Ostermeier et al., 2004). However, the role of paternal RNAs in the developing embryo remains a mystery (Lalancette et al., 2008).

Post-ejaculatory Sperm Damage

Investigations into post-ejaculatory sperm damage have been limited to studies designed to evaluate the effect of cryopreservation, short term storage, heat stress or the female tract on sperm. While much emphasis has been placed on effects of stress on the fertilizing capability of sperm, few studies have suggested that damage to sperm after ejaculation may compromise the resultant embryo.

Heat Shock

Evidence to support the idea that sperm damage after ejaculation can affect embryo competence of an embryo formed from post-ejaculatory damaged sperm includes work done utilizing heat shock as a model. Burfening and Ulberg (1968) demonstrated that *in vitro* heat shock of ejaculated sperm decrease embryo survival as determined by the number of implantation sites noted on day 9 or 12 post insemination per number of cleaved ova 30 h post coitus in rabbits. Moreover, post-ejaculatory sperm damage *in utero* can result in the formation of an embryo with reduced competence for development (Howarth et al., 1965). This phenomenon has also been confirmed by work done more recently by Cozzi et al. (2001) where epididymal sperm harvested from male mice and heat shocked (56°C for 30 min) prior to ICSI

resulted in embryos produced that failed to develop to the blastocyst stage compared to controls (16% vs 62%, respectively).

Cryopreservation

Since the advent of artificial reproductive technologies (ARTs) in the late 1940s by the accidental freezing and recovery of live motile sperm cells in the presence of glycerol (Polge et al., 1949; Lovelock and Polge, 1954) the practice of freezing sperm for later use in artificial insemination (AI) programs in the livestock industry has become common practice. This technique has been adopted in companion animal and human reproductive medicine and in wildlife conservation and preservation programs (Wildt, 2000; Pukazhenthi et al., 2006; Swanson et al., 2007).

Frozen-thawed mammalian spermatozoa however differ from freshly ejaculated spermatozoa in many ways. These differences include a shorter lifespan (Gillian and Maxwell, 1999; Sankai et al., 1994; Rodríguez-Martínez et al., 2008), decreased motility (Gandini et al., 2006; Jin et al., 2008; Rodríguez-Martínez et al., 2008), a higher degree of membrane damage or membrane alterations (Hammerstedt et al., 1990; De Leeuw et al., 1990; Gillian et al., 1997; Gillian and Maxwell, 1999; Pegg, 2002; Nishizono et al., 2004), increased incidence of acrosome reacted sperm (Gillian et al., 1997; Gillian and Maxwell, 1999; Gillian et al., 1999), increase in chromatin abnormalities and DNA fragmentation in some studies (Horse: Baumber et al., 2003; Ram: Peris et al., 2004, 2007; Human: Gandini et al., 2006; Boar: Fraser and Strzezek, 2007; Mouse: Yildiz et al., 2007, 2008) but not in others (Humans – Chernos and Martin, 1989; Martin et al., 1991; Okada et al., 1995) and decreased fertility following intrauterine or cervical insemination (Dogs: Gill et al., 1970. Ewes: Jabbour and Evans, 1991; Maxwell et al., 1993; Gillian et al., 1997; Gómez et al., 1997; Donovan et al., 2004; Nizański, 2006. Mouse: Nishizono et al., 2004.) and even after intracytoplasmic sperm injection (ICSI; Gómez et al., 1997).

Cryopreservation results in formation of intracellular ice crystals (Pegg, 2002), and an increase in osmotic pressure, that is, the remaining solutes become more concentrated as water is removed from solution (Watson and Duncan, 1988; Pegg, 2002) consequently leading to mechanical and osmotic damage of membranes (plasma and nuclear) and cellular organelles (Hammerstedt et al., 1990; Pegg, 2002; Nishizono et al., 2004). Cryopreservation induces oxidative damage to sperm membrane lipids (mid-piece) and reduces the ability of frozen-thawed sperm to withstand further oxidative stress (Neild et al., 2005), and induces lateral phase separation in plasma membranes of bovine and porcine sperm (De Leeuw et al., 1990). Cryopreservation also results in the production of reactive oxygen species (Bilodeau et al., 2000; Ball et al., 2001; Chatterjee et al., 2001), redistribution of the antioxidant defense system (Marti et al., 2008) and a reduction in glutathione in sperm (Bilodeau et al., 2000; Gadea et al., 2004).

Cryopreservation results in sperm damage and embryos formed from cryo-damaged sperm have reduced developmental competence. For example, mouse oocytes fertilized with frozen-thawed epididymal sperm have reduced fertilization (as assessed by synchronous pronuclei at 7.5 hours post insemination), reduced cleavage rates and hatching rates when compared to oocytes produced from fresh epididymal sperm (Songsasen et al., 1997). This observation is not unique to frozen-thawed mouse sperm, as ewes undergoing cervical insemination of frozen-thawed semen have reduced pregnancy rates and produced smaller litter size (Donovan et al., 2004) when compared to fresh semen. Furthermore, the use of frozen-thawed semen for vaginal insemination in the dog results in reduced pregnancy rates, whelping rates and litter size (Nizański, 2006). Moreover, deposition of frozen-thawed dog semen into the uterus does not improve pregnancy rates over deposition of fresh semen into the cranial vagina (Linde-Forsberg and Forsberg, 1989). In horses, pregnancy rates following hysteroscopic insemination with

frozen-thawed sperm tends to be lower than with fresh semen (Lindsey et al., 2002). Even following ICSI embryo quality, pregnancy and delivery rates of human embryos produced with frozen-thawed testicular sperm is lower than in embryos produced with fresh testicular sperm (Aoki et al., 2004) and spontaneous abortion rates are higher when embryos are produced from frozen-thawed sperm (Aoki et al., 2004). Additionally, human embryos produced by ICSI from frozen-thawed testicular sperm have lower implantation rates (measured as the number of gestational sacs observed by ultrasound at 6 weeks of pregnancy divided by the number of embryos transferred) when compared to embryos produced with fresh testicular sperm (De Croo et al., 1998). Even in domestic animals, such as the horse, ICSI performed with frozen-thawed ejaculated spermatozoa yielded zygotes with reduced pronuclear formation, and embryos with reduced cleavage rate and a reduction in the average number of nuclei at 96 h when cultured either *in vitro* or *in vivo* (Choi et al., 2002). However, these reductions in embryo development parameters were not statistically significant when compared to ICSI performed with fresh ejaculated equine spermatozoa (Choi et al., 2002). However, it is possible that developmental differences may have become significant had the embryos been allowed to develop further as in the human studies.

Interestingly, frozen-thawed epididymal boar sperm as opposed to frozen-thawed ejaculated sperm retains motility similar to fresh ejaculated boar sperm and frozen-thawed epididymal sperm show enhanced fertilizing capacity as measured by pronuclear formation (Rath and Niemann, 1997). Furthermore oocytes fertilized with frozen-thawed epididymal boar sperm have increased cleavage rates, with significantly more embryos developing to the 2- and 4-cell stages compared with oocytes fertilized with fresh or with frozen-thawed ejaculated semen (Rath and Niemann, 1997).

Hydrogen Peroxide (H_2O_2)

The effect of hydrogen peroxide on ejaculated sperm is two fold. At physiological concentrations H_2O_2 facilitates sperm hyperactivation and acrosome reaction (Griveau et al., 1994), capacitation (Rivlin et al., 2004; de Lamirande and O'Flaherty, 2008), zona pellucida binding and penetration thus improving *in vitro* fertilization (Ford, 2004). The deleterious effects of H_2O_2 typically occur at high concentrations (100 μM to 1.5 mM H_2O_2) but may also depend on the species involved. In human ejaculated sperm, concentrations ranging from 230 μM to 1.5 mM reduce motility (Chen et al., 1997; Armstrong et al., 1999; Kemal Duru et al., 2000) and are associated with decreased mitochondrial transmembrane potential ($\Delta\Psi_m$) and reduced ATP sperm content (Armstrong et al., 1999). Furthermore, 100 to 230 μM H_2O_2 induces the formation of toxic lipid peroxide and DNA damage in human ejaculated sperm (Chen et al., 1997; Kemal Duru et al., 2000) and 200 μM H_2O_2 reduces sperm-zona pellucida binding (Oehninger et al., 1995).

Ram sperm however exhibit a reduction in motility when exposed to lower concentrations (50 to 300 μM H_2O_2) and exhibit DNA damage at H_2O_2 concentration similar to human sperm (150 to 300 μM H_2O_2; Peris et al., 2007). Interestingly, the reduction in motility has not been associated with increased lipid peroxidation in ram sperm (50 to 300 μM; Peris et al., 2007). Similarly, ejaculated ram sperm exposed to 0.0375 and 0.375% H_2O_2 exhibit no increase in lipid peroxidation as measured by fluorescence recovery after photobleaching (FRAP) to monitor 5-(N-octadecanoyl)-aminofluorescein (ODAF; lipid reporter probe) diffusion (Christova et al., 2004). In addition exposure to 150 and 300 μM H_2O_2 prevents the acrosome reaction in ram sperm (Peris et al., 2007).

In the boar, incubation with 300 μM H_2O_2 reduces motility, but is not associated with decreased $\Delta\Psi_m$ or sperm ATP content (Guthrie et al., 2008) in contrast to human ejaculated

sperm (Armstrong et al., 1999). Interestingly, even lower concentration of H_2O_2 reduced motility (25 μM) and 10 to 250 μM H_2O_2 reduced the capacity of bovine frozen-thawed ejaculated spermatozoa to undergo capacitation, however an increase in acrosome reacted sperm was noted at 25 μM H_2O_2 as detected by the chlortetracycline assay (O'Flaherty et al., 1999).

Hydrogen peroxide results in sperm damage and embryos formed from damaged sperm have reduced developmental competence. For example, bovine frozen-thawed ejaculated sperm exposed to 500 μM H_2O_2 or a cocktail (100 mM ascorbic acid, 20 mM $FeSO_4$ and 500 mM H_2O_2) produced embryos with reduced capacity to cleave by Day 5 post insemination and to develop to the blastocyst stage by Day 9 (Silva et al., 2007). The proportion of Day 5 cleaved embryos that had ≥ 8 blastomeres was reduced in embryos produced by bovine sperm exposed to 500 μM H_2O_2 and no 8-cell or greater embryos were formed from sperm treated with the cocktail (Silva et al., 2007). Human ejaculated sperm exposed to media containing 0.23 mM H_2O_2, 1.8 mM ADP and 2.7 mM $FeSO_4$ have increased levels of 8-hydroxydeoxyguanosine (8-OH-dG; Chen et al., 1997) and embryos formed from sperm containing 8-OH-dG demonstrate fragmentation and overall reduced embryo quality (Meseguer et al., 2008).

Menadione

Menadione (2-methyl-1,4-naphthoquinone) is a polycyclic aromatic ketone precursor to vitamin K_2. Menadione is metabolized by flavoprotein reductase to a semiquinone, which can be oxidized back to a quinone in the presence of molecular oxygen. In this redox cycle, the superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and other reactive oxygen species are generated (Monks et al., 1992). As such menadione has been utilized to generate reactive oxygen species (ROS) in order to study oxidative damage *in vitro* on fresh and frozen-thawed ejaculated

sperm (Guthrie and Welch, 2006, 2007; Guthrie et al., 2008). Menadione reduces sperm motility, depresses $\Delta\Psi_m$ within 30 min and will eventually reduce ATP content (Guthrie et al., 2008).

Gossypol

Gossypol, a polyphenolic yellow pigment found in cotton plants of the genus, *Gossypium* has been implicated in reducing or obliterating male reproductive function in a number of species (reviewed in Randel et al., 1992). Uptake of gossypol by sperm may be due to interactions of gossypol with membrane phospholipids (Ueno et al., 1988). Work with human and hamster ejaculated sperm indicate that gossypol prevents capacitation and penetration of zona-free oocytes (Kennedy et al., 1983; Aitken et al., 1983) as a result of a reduction in acrosin activity by preventing the conversion of proacrosin to acrosin (Kennedy et al., 1983; Yuan and Shi, 2000) and in the inactivation of other acrosomal enzymes (Yuan et al., 1995). Gossypol also induces uncoupling of the respiratory chain and oxidative phosphorylation in boar ejaculated sperm (Tso and Lee, 1982).

Other mechanisms of action of gossypol include inhibition of anion exchange (Haspel et al., 1985). In isolated plasma membranes of human ejaculated sperm, gossypol inhibits Ca^{2+} transport and Ca^{2+}-activated ATPase activity (Kanwar et al., 1989). It also inhibits the membrane bound Mg^{2+}- and Na^{+}-K^{+} dependent ATPases, 5'-nucleotidase and alkaline phosphatase systems (Kanwar et al., 1989). Similar findings have been noted in mouse, ram and bull ejaculated sperm (Shi et al., 2003; Breitbart et al., 1984). Further, gossypol inhibits glucose uptake in human ejaculated sperm, increases thiobarbituric acid reaction products and decreases total phospholipids establishing the lipid peroxidative effect of gossypol on sperm plasma membranes (Kanwar et al., 1990). Kanwar et al. (1990) speculated that inhibition of glucose uptake was linked to lipid peroxidation of the plasma membranes and subsequent membrane damage.

The mechanism of action of gossypol and their effects on cells are varied and complicated, because gossypol can be metabolized to yield several derivatives – aldehyde, enol, hemiacetal forms and gossypolone. Gossypol has the ability to generate reactive oxygen species (Kovacic, 2003) and has been investigated for its anti-oxidant (Dodou et al., 2005), contraceptive (Coutinho, 2002), anticancer (Balakrishnanet al., 2008), antiprotozoan, antiparasitic and carcinogenic properties (Kovacic, 2003).

Gossypol induced sperm damage reduces fertilizing ability and could have a negative impact on embryo competence. For example, treatment of bovine ejaculated sperm with gossypol has been reported to reduce cleavage rate when sperm are used for *in vitro* fertilization and lead to the formation of embryos with reduced probability of developing to the blastocyst stage (Brocas et al., 1997). Precoital intrauterine administration of gossypol reduces the number of ejaculated spermatozoa reaching the ampullae assessed the morning after mating and the number of penetrated oocytes assessed on Day 2 and 3 postcoitus in rats (Moore et al., 1988). Furthermore, precoital intrauterine administration of gossypol inhibited implantation, however it is difficult to ascertain if this was a direct result of gossypol-induced sperm damage alone, or in combination with gossypol-induced oocyte or zygote and uterine damage (Moore et al., 1988). Hernández-Cerón et al. (2005) demonstrated that gossypol reduces the ability of one-cell bovine embryos to develop to the blastocyst stage but has no effect on later stages of development.

X-irradiation

X-irradiation causes DNA strand breaks and chromosomal aberrations in somatic cells (Haines et al., 2001, 2002; Cordelli et al., 2003), oocytes (Matsuda et al., 1985b; Griffin et al., 1990), ejaculated sperm (Kamiguchi et al., 1990) and embryos (Matsuda et al., 1985b). Mechanism through which this damage occur are either the direct effect of ionizing radiation (Cadet et al., 2003) or through the indirect effects – generation of free radicals from surrounding

water molecules (Cadet et al., 2003). The generation of $^{\bullet}$OH via radiolysis of water account for 60-70% of cellular DNA damaged produced by ionizing radiation (Ward, 1988). Damage to DNA by $^{\bullet}$OH include oxidation of bases, abasic sites, DNA-DNA interstrand adducts, DNA single and double strand breaks and oxidation of DNA-proteins cross-links (Cadet et al., 2004). Oxidative damage to the sugar-phosphate backbone of DNA leads to single- and double-strand breaks (Cadet et al., 2004).

The dose at which X-irradiation causes DNA damage in ejaculated sperm varies from one species to another. McKelvey-Martin et al. (1997) exposed human ejaculated sperm to increasing doses to X-rays (5, 10 and 30 Gy) and found that only when sperm were exposed to the highest dose of radiation (30 Gy) increased DNA degradation as measured by the alkaline comet assay. However, Fatehi et al. (2006) demonstrated that there was a significant increase in DNA damage at much lower doses of X-irradiation in bovine sperm (0.6 Gy) using the TUNEL assay in conjunction with flow cytometry and this increased with increasing radiation dose (1.25, 2.5, 5.0 and 10 Gy).

X-irradiation results in sperm damage (DNA damage) and embryos formed from damaged sperm have reduced developmental competence. For example, exposure of bovine ejaculated sperm to X-irradiation (1.25, 2.5 and 5.0 Gy) reduces the proportion of oocytes that cleave after insemination and the proportion of cleaved embryos that became blastocysts (Fatehi et al., 2006). Furthermore, exposure of caudal epididymal mouse sperm to X-irradiation (50 to 400 cGy) does not affect fertilization (Matsuda et al., 1985a; Pampfer et al., 1989) and there is a dose-dependent increase in the proportion 2-cell embryos containing fragmented DNA (Matsuda et al., 1985a; Pampfer et al., 1989) despite the ability of the oocyte to repair DNA damage (Matsuda et al., 1989). Additionally, the consequences of DNA damage in the male gamete are low fertilization

rates (Sakkas et al., 1996; Høst et al., 2000a,b; Bakos et al., 2007), reduced embryo development (Morris et al., 2002; Tomsu et al., 2002; Seli et al., 2004; Fatehi et al., 2006; Paul et al., 2008) and increased embryo and fetal loss (Tomlinson et al., 2001; Tomsu et al., 2002; Virro et la., 2004; Waterhouse et al., 2006; Paul et al., 2008). Sperm DNA damage has also been associated with an increased incidence of cancer in offspring produced from such damaged sperm (Lord, 1999; Lewis and Aitken, 2005).

Pathways of Apoptosis

The typical response to DNA damage in somatic cells is arrest of the cell cycle and activation of DNA repair mechanisms (Friedberg, 2003). In some cases the damage is tolerated (Goodman, 2002) or repaired (Friedberg, 2003). However, in the event that the DNA cannot be repaired the cell undergoes programmed cell death – apoptosis (Friedberg, 2003). It is possible that one mechanism by which sperm damage compromises the competence of the resulting embryo to develop is activation of apoptosis responses in the sperm cell.

Apoptosis may be an important mechanism for sperm damage and experimental elevation in testicular temperature causes germ cell apoptosis via the intrinsic pathway (Hikim et al., 2003; Vera et al., 2004; 2005). Furthermore, apoptosis or programmed cell death appears to be a normal phenomenon during spermatogenesis and has been proposed to maintain homeostasis in the testes (Blanco-Rodriguez, 1998). There are two well-known pathways involved in apoptosis in mammalian cells; the intrinsic or mitochondrial pathway and the extrinsic or receptor-mediated pathway.

The intrinsic pathway is depicted in Figure 1-1. It involves the release of cytochrome c from the mitochondrial intermembrane space into the cytosol. The molecular events which lead to the formation of pores in the mitochondrial outer membrane and release of cytochrome c in response to stress are not well understood. It is believed that stresses such as, heat shock (Chung

et al., 2003), infections with *S. aureus* (Esen et al., 2001), *N. gonorrhoeae* (Grassme et al., 1997), or treatments with gamma-irradiation (Santana et al., 1996) and UV light (Zhang et al., 2001) can induce the activation of sphinogomyelinase (SMase) which cleaves plasma membrane sphinogomyelin (SM) to ceramide. It has been shown that approximately 13% of bovine sperm plasma membrane is made up of sphinogomyelin, which is concentrated in the acrosomal membrane (Parks et al., 1987). Ceramide then triggers the activation of signal transduction pathways which activate pro-apoptotic proteins of the Bcl-2 family, Bax and Bad. Activated Bax translocates from the cytosol to the mitochondria where they form pores in the outer mitochondrial membrane and cytochrome c is released into the cytosol (Zamzami and Kroemer, 2001; Fumarola and Guidotti, 2004). Cytosolic cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1), inducing oligomerization and exposure of the caspase recruitment domain (CARD; Riedl and Salvesen, 2007) resulting in the recruitment of procaspase 9 and the formation of the apoptosome (Fumarola and Guidotti, 2004; Riedl and Salvensen, 2007). Procaspase 9 undergoes activation via proteolytic autoactivation (Fumarola and Guidotti, 2004). The active caspase 9 then activates the down stream executioner caspases (caspase 3 and 7) (Fumarola and Guidotti, 2004). Along with cytochrome c, Smac/Diablo, apoptosis-inducing factor (AIF) and endonuclease G are released from the intermembrane space (Fumarola and Guidotti, 2004). The pro-apoptotic factor Smac/Diablo binds to IAPs (inhibitors of apoptosis proteins) promoting caspase activity and hence apoptosis (Fumarola and Guidotti, 2004). Inhibitors of apoptosis proteins bind to procaspases and activated caspases inhibiting there activation and activity respectively (Riedl and Shi, 2004). Apoptosis-inducing factor and endonuclease G translocate to the nucleus where they induce caspase-independent DNA damage (Fumarola and Guidotti, 2004).

The receptor mediated pathway, involves the interaction of death receptor and its ligand, such as Fas–FasL interaction (Waring and Müllbacher, 1999; Sharma et al., 2000). In this case, binding of FasL to Fas induces trimerization of the Fas receptor and recruitment of the adapter molecule Fas-associated death domain (FADD) to the cytoplasmic tail of Fas to form the death-inducing signaling complex (DISC; Sharma et al., 2000). Fas-associated death domain binds and activates procaspase 8 (Sharma et al., 2000). Active caspase 8 either goes on to activate the executioner caspase, caspase 3 or it may cleave Bid, a proapoptotic Bcl-2 family member, to form a truncated form of Bid, tBid (Li et al., 1998). The truncated form of Bid, tBid, facilitates the cross-talk between the receptor mediated and mitochondrial pathways, by stimulating the release of cytochrome c via Bax and/or Bak oligomerization and insertion into the mitochondrial membrane with the resultant release of cytochrome c (Korsmeyer et al., 2000).

The overall importance of exposure of ejaculated sperm to hydrogen peroxide, menadione, and X-irradiation is that once the sperm DNA is damaged, unlike in somatic cell or even the oocyte, the DNA damage is not repaired as sperm lack DNA repair mechanisms. Secondly, failure to undergo complete apoptosis implies that these defective sperm remain in the fertilizing population increasing the possibility that oocytes will become fertilized with damaged sperm. This is especially true in assisted-reproductive technology (ART), where the mechanisms which have evolved to ensure selection of high quality sperm for fertilization have been bypassed.

Synopsis

It is possible that post-ejaculatory sperm damage can compromise the ability of embryos formed from damaged sperm to fulfill their developmental potential, and this could involve damage to paternally inherited macromolecules. Moreover, damage to paternally inherited macromolecules may occur by apoptosis. Using ejaculated bull sperm as a model this thesis will examine the effects of aging and heat shock, airport security X-irradiation and oxidative stress on

embryo competence. These will be addressed in order and Chapter 5 will investigate the role of apoptosis in aging and heat shock as a possible mechanism of sperm damage using ejaculated bull and stallion sperm as a model. Finally, the General Discussion in Chapter 6 will provide an overview of the findings and possible mechanisms for the reduction in the developmental competence of embryos formed from damaged ejaculated sperm.

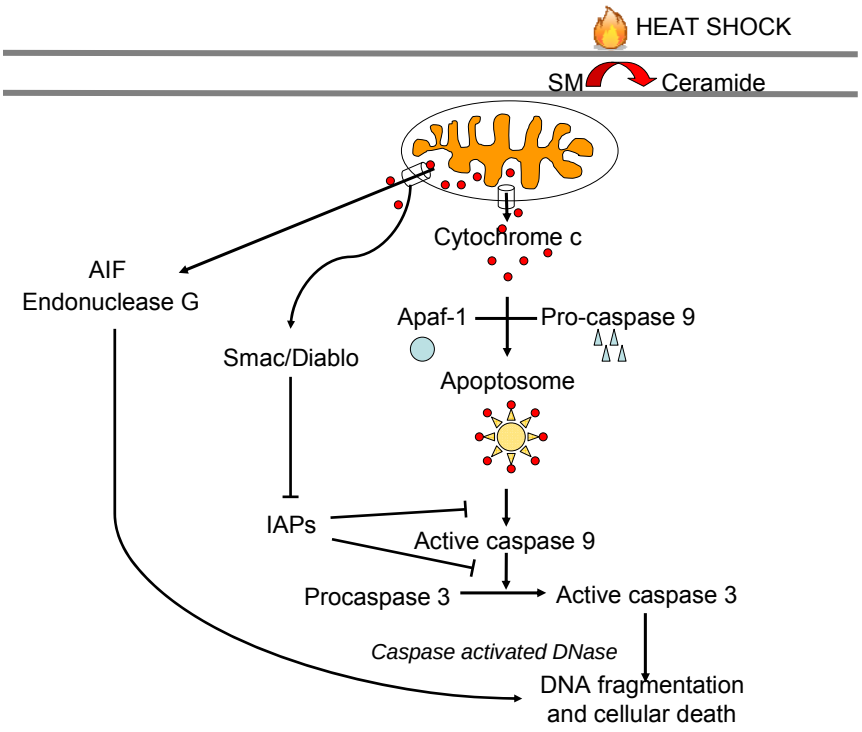

Figure 1-1. Simplification of induction of apoptosis by heat shock with emphasis on the mitochondrial or intrinsic apoptotic pathway. Other pathways controlling apoptosis are not shown here.

CHAPTER 2
CONSEQUENCES FOR THE BOVINE EMBRYO OF BEING DERIVED FROM A SPERMATOZOAN SUBJECTED TO POST-EJACULATORY AGING AND HEAT SHOCK: DEVELOPMENT TO THE BLASTOCYST STAGE AND SEX RATIO

Introduction

Developmental competence of the mammalian embryo is dependent on genetic and non-genetic contributions from its parents (Warner et al., 1998; Sirard et al., 2006; Ménézo, 2006; Baumann et al., 2007; Khatib et al., 2008ab). Sperm could affect an embryo's competence for development if the timing of fertilization or early cleavage is delayed. For example, embryos produced by high-fertility bulls entered S-phase of the first cell cycle earlier and had a longer S-phase than those produced by low-fertility bulls (Eid et al., 1994). In another study, spermatozoa from 50% of bulls identified as being of low fertility in artificial insemination studs experienced premature capacitation (Kuroda et al., 2007). Damage to the macromolecular portions of the sperm that are incorporated by the embryo could also result in formation of embryos with reduced developmental competence. Among these sperm contributions are DNA, the centriole (Sutovsky and Schatten, 2000), and RNA (Ostermeier et al., 2004). Embryos fertilized with semen containing a high proportion of sperm with extensive DNA damage have reduced competence for development (Virro et al., 2004; Seli et al., 2004; Muriel et al., 2006), but the importance of damage to the centriole or sperm RNA is not known.

Damage to sperm can occur in the male reproductive tract or after deposition of sperm in the female. In bulls, for example, thermal stress of the scrotum leads to production of sperm that produce embryos with delayed or reduced pronuclear formation (Walters et al., 2006), a reduced ability of cleaved embryos to become blastocysts for some bulls (Walters et al., 2004; 2005a), and increased embryo apoptosis (Walters et al., 2005b). Sperm from diabetic mice have reduced capacity to fertilize oocytes and for the resultant embryos to give rise to blastocysts (Kim and

Moley, 2008). There is also evidence that sperm can be damaged after ejaculation by stresses that lead to reduced embryo competence after fertilization. Irradiation of mouse sperm did not affect fertilizing ability but did reduce rates of blastocyst development and implantation (Ahmadi and Ng, 1999ab). Exposure of ejaculated frozen-thawed bull spermatozoa to gossypol (Brocas et al., 1997) or reactive oxygen species reduced the percent of cleaved embryos that developed to the blastocyst stage (Silva et al., 2007).

For the current study, we used bull spermatozoa to test the hypothesis that aging of ejaculated sperm for 4 h after freeze-thawing would damage sperm and lead to embryos with reduced developmental competence after fertilization. The term 'aging' was used to represent incubation *in vitro* since this treatment can cause a reduction in sperm motility after 3 h (Monterroso et al., 1995). A second hypothesis was that sperm damage would be enhanced if aging occurred at elevated temperatures. The heat-stress temperature used, 40ºC, is characteristic of rectal temperatures of lactating cows exposed to heat stress (Elvinger et al., 1992; de Castro e Paula et al., 2008) and effects of aging sperm at this temperature could be relevant to understanding causes of reduced fertility of dairy cows during heat stress (Hansen, 2007). Exposure of human ejaculated sperm to mild heat shock caused DNA damage (Mann et al., 2002) and studies in the rabbit indicate that fertilization with sperm incubated at elevated temperature *in vitro* or in the female rabbit results in embryos with reduced implantation rates (Burfening and Ulberg, 1968; Howarth et al., 1965).

Materials and Methods

Materials

The media HEPES-Tyrodes Lactate (HEPES-TL), IVF-TL, and Sperm-TL were purchased from Caisson (Sugar City, ID, USA) and used to prepare HEPES-Tyrodes albumin lactate pyruvate (TALP), IVF-TALP, and Sperm-TALP as previously described (Parrish et al., 1986).

Oocyte collection medium (OCM) was Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (Atlanta Biologicals, Norcross, GA, USA) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR) containing 2 U/mL heparin, 100 U/mL penicillin-G, 0.1 mg/mL streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) was Tissue Culture Medium 199 (Gibco®, Invitrogen, Carlsbad, CA, USA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 μg/mL estradiol 17-β, 20 μg/mL bovine FSH (Folltropin-V; Bioniche Animal Health, London, ON, Canada), 22 μg/mL sodium pyruvate, 50 μg/mL gentamicin sulfate, and 1 mM glutamine. Percoll was from GE Healthcare (Uppsala, Sweden). Potassium simplex optimized medium (KSOM) containing 1 mg/mL BSA was obtained from Caisson. Essentially fatty-acid free (EFAF) BSA was from Sigma (St. Louis, MO). On the day of use, KSOM was modified for bovine embryos to produce KSOM-BE2 as described elsewhere (Soto et al., 2003). Hoechst 33342 was from Calbiochem (San Diego, CA, USA). PCR oligonucleotide primers were obtained from Integrated DNA Technology (Coralville, IA, USA). *Taq* DNA polymerase and 100 mM dNTPs were from Invitrogen. All other reagents were purchased from Sigma or Fisher Scientific (Pittsburgh, PA, USA) unless otherwise stated.

Sperm Preparation

Extended and frozen semen from Holstein bulls was obtained from Select Sires Inc. (Plain City, OH, USA) and ABS Global (Deforest, WI, USA). Semen was thawed, subjected to Percoll gradient purification to obtain motile spermatozoa (Parrish et al., 1986), diluted in Sperm-TALP medium to 20 x 10^6 spermatozoa/mL, and aged by incubation at 38.5°C or 40°C in air for 4 h using a water bath. Additional semen was thawed to prepare a non-incubated spermatozoa control. In this case, semen was thawed, subjected to Percoll purification, and diluted to 20 x 10^6 spermatozoa/mL at a time to coincide with the end of the incubation period for aged sperm.

Sperm Motility

The percent of sperm exhibiting motility was assessed by visual examination. Briefly, 20 µL of sperm suspension were placed on a glass slide pre-warmed at 37ºC and examined under 200x magnification. Motility was estimated for 100 spermatozoa located in 10 different fields.

In Vitro Production of Embryos

Embryo production was performed as previously described (Soto et al., 2003) except that sperm were subjected to incubation prior to fertilization as described above and oocytes in a single replicate were inseminated with semen from a single bull. Briefly, cumulus-oocyte complexes (COCs) were obtained by slicing 2 to 10 mm follicles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from a local abattoir. Cumulus-oocyte complexes containing at least one layer of compact cumulus cells were selected for maturation and fertilization. They were washed twice in OCM and placed in groups of 10 in 50 µL drops of OMM overlaid with mineral oil and matured for 22 h at 38.5°C, 5% CO_2 in humidified air. Matured oocytes were then washed once in HEPES-TALP and transferred in groups of 30 to 4-well-plates containing 600 µL IVF-TALP per well and 25 µL PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 µM epinephrine in 0.9% [w/v] NaCl) per well and fertilized with 25 µL (5×10^5) Percoll-purified spermatozoa from a single Holstein bull. After 8 h of co-incubation at 38.5°C, 5% CO_2 in humidified air, putative zygotes were removed from fertilization wells and denuded of cumulus cells by vortexing in 100 µL hyaluronidase (1000 U/mL in approximately 0.5 mL HEPES-TALP). Denuded putative zygotes were cultured in groups of 25 to 30 in 50-µL drops of KSOM-BE2 overlaid with mineral oil at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 and the balance nitrogen. Fertilization was assessed at 18 h post-insemination (hpi), cleavage was assessed on Day 3 after insemination and presence of blastocysts was determined on Day 7 and/or Day 8 after insemination.

Determination of Fertilization

Inseminated oocytes were transferred at 18 hpi from KSOM-BE2, washed in 10 mM KPO_4 (pH 7.4) containing 0.9% (w/v) NaCl (PBS) and 1 mg/mL polyvinylpyrollidone (PVP) (PBS-PVP) and transferred onto poly-L-lysine coated slides. Slides were allowed to air dry and fixed overnight in 100% ethanol and then stained with Hoechst 33342 (1 µg/mL in PBS-PVP) for 10 min in the dark at room temperature. Slides were washed three to four times with PBS-PVP, and cover slips mounted using 5 µL mounting medium containing ProLong® Gold antifade reagent (Invitrogen). Pronuclei of inseminated oocytes were identified by fluorescence using a Zeiss Axioplan 2 fluorescence microscope (Carl Zeiss, Inc., Göttingen, Germany) with an ultraviolet excitation filter. Oocytes were classified in four groups: X, unknown, unable to assess presence of pronuclei; M, unfertilized oocyte in Metaphase II with one polar body visible; 1PN, presence of a single decondensed pronucleus; 2PN, presence of two pronuclei, indicative of fertilization; and PPN; presence of 3 or more pronuclei. Oocytes with two pronuclei (2PN) were considered as fertilized and those with more than 2PN were considered as fertilized but polyspermic.

Embryo Sex Determination

Blastocysts were removed from culture drops, washed in PBS-PVP and transferred into a solution of 0.1% (wt/vol) protease from *Streptomyces griseus* (Sigma) in PBS for 1.5 min. Embryos were then washed 3 times in 150 µl PBS-PVP, collected individually in 10 µl drops of 0.1% (wt/vol) diethylpyrocarbonate in water, transferred into 0.2 mL PCR tubes and stored at -20ºC until analysis. To prepare samples for PCR, tubes were thawed at room temperature and centrifuged at 2000 x g for 5 s, heated to 98°C for 10 min and centrifuged at 2000 x g for 5 s prior to addition of PCR reagents.

Two sets of PCR primers were used to determine embryo sex: Y-chromosome specific primers that amplify a 141 bp product and auotosomal bovine-specific satellite sequence primers

that amplify a 216 bp product (Park et al., 2001). The amplification reactions were conducted in a total volume of 20 µl. The first amplification consisted of 10x PCR buffer, 2.5 mM dNTPs, 50 mM $MgCl_2$, 1 units of Taq DNA polymerase and 10 µM Y-specific forward primer (5´-GATCACTATACATACACCACT-3´) and 10 µM Y-specific reverse primer (5´-GCTATGCTAACACAAATTCTG-3´). The first PCR was programmed for an initial denaturation at 95ºC for 7 min followed by 10 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s; after the 10 cycles the reaction mixtures were kept at 72ºC for 7 min. Tubes were centrifuged at 2000 x g for 5 s prior to addition of the second PCR mix for autosomal primers containing 10 µM forward (5´-TGGAAGCAAAGAACCCCGCT-3´) and 10 µM reverse primers (5´-TCGTCAGAAACCGCACACTG-3´). The second PCR was programmed for initial denaturation at 95ºC for 7 min, 30 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30 s, and a final step at 72ºC for 7 min. PCR amplification products were separated by electrophoresis on 3% (w/v) agarose gels in a 1 x TBE buffer (89 mM Tris, 88.9 mM boric acid, 2.2 mM EDTA, pH 8.3) containing 10 µg/mL ethidium bromide.

Experiments

The first experiment tested the effects of aging on sperm motility. Sperm motility was assessed for non-incubated sperm immediately following Percoll purification and for sperm at the end of the incubation period at 38.5°C or 40°C in air for 4 h using a water bath. The experiment was replicated a total of 21 times with sperm from 17 bulls.

The second experiment tested effects of aging on cleavage rate and development to the blastocyst stage when oocytes were fertilized for 8 h. Oocytes were fertilized with unincubated control sperm, sperm aged at 38.5ºC or sperm aged at 40ºC. Another group of oocytes remained unfertilized (i.e., incubation of oocytes in fertilization medium without sperm) to determine

parthenogenesis. After fertilization for 8 h, oocytes (fertilized, i.e., putative zygotes, and unfertilized, possible parthenotes) were placed in groups of 25 to 30 in 50 µl microdrops of KSOM-BE2 at 38.5ºC in a humidified atmosphere of 5% CO_2, 5% O_2 and the balance nitrogen. Cleavage was accessed on Day 3 after insemination and blastocyst development on Day 8 after insemination. The experiment was replicated a total of 11 times with a different bull for each replicate and with a total of 226 to 853 oocytes/group. For 10 of these replicates, blastocysts were harvested at Day 7 and again at Day 8 for determination of embryo sex.

A third experiment was designed to determine the effect of aging on fertilization. Oocytes were fertilized with unincubated control sperm, sperm aged at 38.5ºC or sperm aged at 40ºC. After fertilization for 8 h, oocytes (i.e., putative zygotes) were placed in groups of 25 to 30 in drops of KSOM-BE2 medium until processing for fertilization determination at 18 hpi at 38.5^{o}C in a humidified atmosphere of 5% CO_2, 5% O_2 and the balance nitrogen (vol/vol). The experiment was replicated three times using a different bull for each replicate and with a total of 59 to 72 inseminated oocytes/group COCs.

Statistical Analyses

For each replicate, percent sperm that were motile, percent of oocytes that were fertilized, cleaved or developed to the blastocyst stage, percent of cleaved embryos that became blastocysts, and percent of blastocysts that were male were calculated for all oocytes or embryos within the same treatment. Thus, the group of embryos treated alike within each replicate was the experimental unit. Data were subjected to least squares analysis of variance using the GLM procedure of the Statistical Analysis System (SAS for Windows, Release 9.0, SAS Institute, Inc., Cary, NC). Data were analyzed without transformation and again after arcsin transformation to correct for any non-normality associated with percentage data. The mathematical model included

effects of bull, sperm treatment and treatment x bull (i.e., error). Data are reported as least-squares means ± SEM from the analysis of the untransformed data while probability values are derived from analyses of transformed data. The CONTRAST statement of SAS was utilized to compare individual treatments.

Treatment effects on sex ratio were determined by logistic regression using the logistic procedure of SAS. Two comparisons were made: between control sperm and sperm aged at 38.5ºC and between control sperm and sperm aged at 40ºC.

Results

Sperm Motility

As compared to nonincubated control sperm (79.3 ± 1.8%), a smaller percent of sperm exhibited motility after aging for 4 h at either 38.5ºC or 40ºC ($P<0.001$). Moreover, motility was lower ($P<0.01$) for sperm aged at 40ºC than for sperm aged at 38.5ºC (38.3 ± 1.8% vs 46.6 ± 1.8%).

Cleavage and Development to the Blastocyst Stage

As compared to oocytes inseminated with control sperm, cleavage rate was lower for oocytes inseminated with sperm aged at 40°C ($P<0.05$) and tended to be lower ($P=0.08$) for oocytes inseminated with sperm aged at 38.5°C (Table 2-1). However, there was no significant difference in cleavage rates between oocytes produced from sperm pre-incubated at 38.5°C vs 40°C. There was no effect of aging at either temperature on the percent of oocytes that became blastocysts or on the percent of cleaved embryos that became blastocysts (Table 2-1).

Fertilization

The effects of aging on the proportion of oocytes fertilized after 8 h was evaluated by counting the number of pronuclei at 18 hpi (Table 2-2). Overall fertilization rate, as determined

by the proportion of embryos with at least two pronuclei, was not affected by aging of sperm at 38.5°C or at 40°C. Similarly, the percent of oocytes that were fertilized with a single spermatozoon (i.e., those with two pronuclei) was similar for all three groups and not affected by treatment. The percent of oocytes undergoing polyspermy (i.e., those with more than two pronuclei) tended (P=0.08) to be lower for oocytes fertilized with sperm aged at 40°C as compared to the controls. Aging at 38.5°C did not affect rate of polyspermy.

Sex of Blastocysts

A total of 375 blastocysts were produced and 367 of these were successfully sexed. PCR reactions in which there were two amplicons (for Y-specific primers and for autosomal primers) were classified as males while those exhibiting an autosomal amplicon only were classified as female (Figure 2-1). The effect of sperm treatment on the proportion of blastocysts at Day 7 and 8 that were male is presented in Table 2-3. For embryos produced from oocytes inseminated with non-incubated sperm, there was a preponderance of male blastocysts at both Day 7 (57.5%) and Day 8 (59.6%) after insemination. The percent of blastocysts that were male was reduced for embryos produced with sperm aged at 38.5°C (P=0.08) but not for embryos produced with sperm aged at 40°C.

Discussion

Aging of sperm after freeze-thawing reduced motility but had no effect on the fertilizing ability of bovine spermatozoa, a slight effect only on the proportion of oocytes cleaving after insemination, and no effect on the competence of the resultant embryo to develop to the blastocyst stage. Lack of effect of aging on embryo competence was true even when spermatozoa were incubated at a temperature of 40°C that is characteristic of heat-stressed cows.

Aging did affect sperm function, however, since sperm motility and cleavage rate was reduced and the blastocyst sex ratio tended to be altered by aging of sperm at 38.5°C.

Sperm survival after ejaculation is transient unless sperm are maintained with oviductal cells (Pollard et al., 1991), cryopreserved or suspended in diluents designed to prolong viability while stored cold. Bull sperm incubated for 24 h at 39°C had reduced competence for fertilizing oocytes as determined by subsequent cleavage (Pollard et al., 1991; Lechniak et al., 2003). Even short-term aging can compromise sperm function. In this study, motility of bull sperm was decreased by 4 h incubation at 38.5ºC and slightly more so by incubation at 40ºC. Previous work indicates bull sperm motility was decreased by as little as 3 h incubation at 39°C (Monterroso et al., 1995). The reduction in cleavage rate when oocytes were fertilized with sperm aged at 38.5°C or 40°C in the current study could reflect decreased sperm motility and fertilizing capacity. However, examination of pronuclear formation after fertilization failed to indicate a decline in fertilization rate in oocytes inseminated with aged sperm. It may be, therefore, that the reduction in cleavage rate in oocytes inseminated with aged sperm reflects a delay in fertilization and aging of the oocyte (Agung et al., 2006). It is also possible that aging damaged the sperm centriole so that syngamy was compromised.

The lack of effects of aging on the proportion of inseminated oocytes or cleaved embryos that became blastocysts agrees with other studies finding no effect of aging sperm for 3 to 6 h at 39°C on cleavage rate or on the proportion of oocytes becoming blastocysts (Kochhar et al., 2003; Lechniak et al., 2003). Embryos produced by sperm aged for 24 h did exhibit reduced competence for development however (Lechniak et al., 2003). Aging *in vivo* is likely to result in less steep decline in sperm function than seen here because oviductal epithelial cells can maintain fertilizing capacity of bull spermatozoa for up to 30 h (Pollard et al. 1991).

Lactating dairy cows exposed to heat stress often have rectal temperatures that reach or exceed 40ºC (Elvinger et al., 1992; de Castro e Paula et al., 2008). There is the potential, therefore, for sperm in the reproductive tract to be damaged by exposure to elevated temperature. Most of the data presented here are not supportive of such a hypothesis. As compared to aging at 38.5ºC, there was no effect of 40ºC on the fertilizing capacity of sperm as measured by pronuclear formation or on cleavage rate of oocytes at Day 3 after insemination. As compared to sperm at 38.5ºC, aging at 40ºC did not reduce the proportion of oocytes and cleaved embryos becoming blastocysts. Aging of sperm at 40ºC also reduced the rate of polyspermy. This effect might reflect a reduction in motility or ability of sperm to attach to and penetrate the zona pellucida of the oocyte.

Developmental competence in the present study was evaluated to the blastocyst stage. One cannot rule out effects of sperm aging on embryo competence for development to later stages of embryogenesis. Studies in the rabbit using sperm exposed to heat shock *in vitro* (Burfening and Ulberg, 1968) or *in vivo* (Howarth et al., 1965) indicate increased embryonic loss at Day 9 or 12 after insemination.

One effect of aging sperm was on blastocyst sex ratio. In the absence of sperm aging, the sex ratio of blastocysts was skewed to males and aging at 38.5ºC resulted in a sex ratio close to an equal number of males and female embryos. A similar effect of sperm aging on the sex ratio of the resultant blastocysts has been seen elsewhere (Kochhar et al., 2003; Lechniak et al., 2003; Iwata et al., 2008).

A preponderance of male blastocysts is a characteristic of the *in vitro* embryo production system in our laboratory (Block et al., 2003; Block and Hansen, 2007; Franco et al., 2006) and other laboratories (Lechniak et al., 2003; Agung et al., 2006; Kochhar et al., 2003; Iwata et al.,

2008, King et al., 1991, Hasler et al., 2002). The reason for this bias in sex gender is not clearly understood. It has been reported that male embryos develop faster in KSOM than female embryos (Nedambale et al., 2004), making it more likely that the first emerging blastocysts would be male. However, the increased proportion of males for embryos produced with control sperm in the present study was seen for embryos becoming blastocysts by Day 7 and between Day 7 and 8. Kimura et al. (2005) demonstrated that glucose in excess of 1 mM is toxic to female bovine embryos but the concentrations of glucose in embryo culture medium in the present experiment (0.2 mM) was too low to be toxic (Soto et al., 2003). It seems mostly likely that the gender bias is due to differential fertilizing ability of Y-bearing vs X-bearing spermatozoa. In support of this are the findings that the sex bias occurs as early as the 4 to 8 cell stage (Kochhar et al., 2003) and that lengthening fertilization time beyond 5 to 6 h eliminated the male bias in the sex ratio of embryos (Kochhar et al., 2003; Iwata et al., 2008). Iwata et al. (2008) speculates that the more rapid fertilization achieved with Y-bearing sperm reflects earlier capacitation for Y-bearing sperm. Thus, it is likely that the reduction in the proportion of blastocysts that were male caused by aging of sperm at 38.5ºC reflects differential effects of aging on fertilizing ability of Y-bearing and X-bearing sperm. Energy store depletion, free radical damage, membrane changes or other aging-associated changes (Vishwanath and Shannon, 1997; Krzyzosiak et al., 2000, 2001) could occur more rapidly for Y-bearing sperm, particularly if they are more active because of earlier capacitation.

The reduction in male bias in sex ratio caused by aging at 38.5ºC was not significant when sperm were aged at 40ºC. Possibly, aging at 38.5ºC affects Y-bearing sperm preferentially while aging at a higher temperature results in aging-associated changes in both Y- and X-bearing sperm.

The observation that aging of sperm can lead to changes in sex ratio of the resultant embryo points out the potential for changes in sperm function to effect the embryo formed by fertilization with that sperm. Nonetheless, despite nuclear and non-nuclear contributions of the sperm to the embryo (Sutovsky and Schatten, 2000), there was no evidence that the competence of the embryo to develop to the blastocyst stage was determined by aging at temperatures characteristic of normothermia or hyperthermia. Thus, at least under the conditions tested, damage to the sperm is more likely to lead to a reduction in fertilizing ability than to the cellular characteristics of the resultant embryo that determine its developmental potential.

Table 2-1. Effect of sperm aging on cleavage rate and blastocyst development following insemination of matured oocytes with sperm for 8 h

Treatment	*n* [a]	Cleavage (%)	Blastocysts/oocyte (%)	Blastocyst/cleaved embryo (%)
Control	367	65.7 ± 4.7	30.4 ± 3.0	46.6 ± 3.6
38.5ºC	845	56.6 ± 2.7 †	28.9 ± 1.7	50.9 ± 2.1
40ºC	853	52.2 ± 2.7*	25.1 ± 1.7	46.9 ± 2.1
Parthenogenesis	226	3.5 ± 2.7***	-0.3 ± 1.7***	-0.3 ± 2.1***

Data are least-squares means ± SEM of the percent cleaved or percent blastocysts for each of 11 replicates using a separate bull for each replicate. Means that differ from controls are indicated by superscripts: † P=0.08; * P<0.05; *** P<0.001. There were no differences between 38.5 and 40ºC. [a] *n:* total number of embryos evaluated per treatment.

Table 2-2. Fertilization rate at 18 h post-insemination as affected by aging of sperm at 38.5 or 40ºC for 4 h

		Percent Fertilized		Percent Polyspermy
Treatment	*n* [a]	≥2PN	2PN	>2PN
Control	64	79.8 ± 8.8	63.0 ± 8.6	16.8 ± 3.5
38.5ºC	72	74.0 ± 8.8	60.1 ± 8.6	13.9 ± 3.5
40ºC	59	66.3 ± 8.8	61.3 ± 8.6	5.0 ± 3.5[†]

Data are least-squares means ± SEM of the percent fertilized or percent polyspermy for each of three replicates using a separate bull for each replicate. There were no differences between 38.5 and 40ºC. [a] *n:* total number of oocytes evaluated per treatment; *≥2PN*: embryos having 2 or more pronuclei; *2PN*: embryos having 2 pronuclei. [†] Differs from control (P=0.08).

Table 2-3. Sex of blastocysts at Day 7 and Day 8 after insemination as affected by sperm aging at 38.5 or 40ºC for 4 h

Sperm	Blastocysts at Day 7			Blastocysts at Day 8[b]			Day 7 and 8 combined		
treatment	*n*	M	% Male	*N*	M	% Male	*n*	M	% Male
Control	78	45	57.7	47	28	59.6	125	73	58.4
38.5ºC	77	35	45.5	48	23	47.9	125	58	46.4 †
40ºC	80	42	52.5	37	19	51.4	117	61	52.1

n: total number of blastocysts evaluated per treatment; M: total number of embryos that were males. Blastocysts at Day 8 represent embryos that were not blastocysts at Day 7 but which became blastocysts by Day 8.† Differs from control (P=0.08).

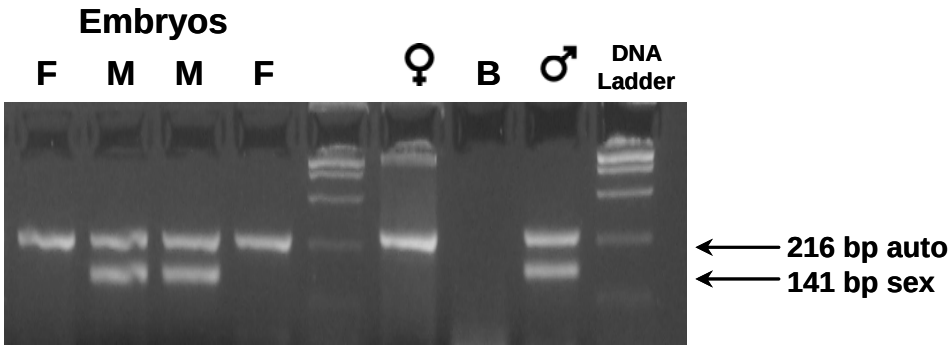

Figure 2-1. Representative results for analysis of embryo sex by PCR. (♀)=female bovine DNA isolated from whole blood. (♂)=male bovine DNA isolated from whole blood. B= blank-PCR reaction mixture without embryo. Amplificons for the Y-specific primer (141 bp) and autosomal primer (216 bp) are indicated by arrows. Note that embryos that produced both the 216 and 141 bp amplicons were classified as male (M), while those with only the 216 bp product were classified as female (F).

CHAPTER 3
CONSEQUENCES FOR THE BOVINE EMBRYO OF BEING DERIVED FROM A SPERMATOZOAN SUBJECTED TO X-IRRADIATION CHARACTERISTIC OF AIRPORT SCREENING DEVICES

Introduction

X-irradiation has been shown to cause DNA strand breaks and chromosomal aberrations in somatic cells (Dobrzyńska, 2007; Liang et al., 2007), germ cells (Griffin et al., 1990; Haines et al., 2002; Cordelli et al., 2003), ejaculated sperm (Matsuda et al., 1985; Kamiguchi et al., 1990) and embryos (Molls and Streffer, 1984; Streffer et al., 1993). DNA damage in the male gamete has been attributed to low fertilization rates (Sakkas et al., 1996; Høst et al., 2000ab; Waterhouse et al., 2006), reduced embryo development (Seli et al., 2004; Fatehi et al., 2006) and increased embryo and fetal loss (Virro et la., 2004; Paul et al., 2008). Sperm DNA damage has also been associated with an increased incidence of cancer in offspring produced from such damaged sperm (Lord, 1999; Lewis and Aitken, 2005).

Worldwide and inter-continental transportation of biological material such as sperm, ova and embryos has become more common with the advent in artificial reproductive technologies. Transportation of these biological materials may require that they be exposed to X-irradiation due to airport security at ports of entry and exit. Currently, it is not known whether the levels of X-irradiation employed at airport security checkpoints adversely affect sperm or the embryos produced from sperm. It was hypothesized that exposure of frozen bovine sperm to x-rays generated by airport security x-ray machines for a) checked luggage and b) carry-on luggage would induce changes in sperm DNA and cause a reduction in competence of the resultant embryos to develop to the blastocyst stage.

Materials and Methods

Materials

The media HEPES-Tyrodes Lactate (HEPES-TL) and IVF-TL were purchased from Caisson (Sugar City, ID, USA) and used to prepare HEPES-Tyrodes albumin lactate pyruvate (TALP) and IVF-TALP as previously described (Parrish et al., 1986). Oocyte collection medium (OCM) was Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (Atlanta Biologicals, Norcross, GA, USA) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR) containing 2 U/mL heparin, 100 U/mL penicillin-G, 0.1 mg/mL streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) was Tissue Culture Medium 199 (Gibco®, Invitrogen, Carlsbad, CA, USA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 μg/mL estradiol 17-β, 20 μg/mL bovine FSH (Folltropin-V; Bioniche Animal Health, London, ON, Canada), 22 μg/mL sodium pyruvate, 50 μg/mL gentamicin sulfate, and 1 mM glutamine. Percoll was from GE Healthcare (Uppsala, Sweden). Potassium simplex optimized medium (KSOM) containing 1 mg/mL BSA was obtained from Caisson. Essentially fatty-acid free (EFAF) BSA was from Sigma (St. Louis, MO). On the day of use, KSOM was modified for bovine embryos to produce KSOM-BE2 as described elsewhere (Soto et al., 2003). Extended and frozen semen from nine bulls was obtained from Genex Cooperative, Ithaca, NY and exposed to x-irradiation under the supervision of Dr. Linda Penfold, White Oak Conservation Center, Yulee, FL.

Sperm Treatment and Preparation

X-irradiated bovine semen straws were donated by Dr. Linda Penfold, White Oak Conservation Center, Yulee, FL. Straws of semen extened with a milk-based extender from known fertile bulls (n= 9) were stored in a dry shipper at -196ºC and processed through airport security x-ray machines. Straws were x-rayed 0, 1, 2, or 3 times using a checked luggage x-ray

machine or a carry-on luggage x-ray machine. The zero group was passed through the x-ray machine without exposure to X-irradiation.

In Vitro Production of Embryos

Embryo production was performed as previously described (Soto et al., 2003) except that oocytes in a single replicate were inseminated with semen from a single bull. Briefly, cumulus-oocyte complexes (COCs) were obtained by slicing 2 to 10 mm follicles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from a local abattoir. COCs containing at least one layer of compact cumulus cells were selected for maturation and fertilization. They were washed twice in OCM and placed in groups of 10 in 50 μL drops of OMM overlaid with mineral oil and matured for 20 to 22 h at 38.5°C, 5% CO_2 in humidified air. Matured oocytes where then washed once in HEPES-TALP and transferred in groups of 30 or 50 to 4-well-plates containing 425 μL IVF-TALP per well and 20 μL PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% [w/v] NaCl) per well and fertilized with 30 μL (5×10^5) Percoll-purified spermatozoa from a single bull. External controls were used to monitor the IVF procedure and consisted of a pool of three bulls (a different pool of bulls was used for each replicate). Sperm used for the external control was extended in an egg yolk based extender prior to cryopreservation. After 8 to 9 h of co-incubation at 38.5°C, 5% CO_2 in humidified air, putative zygotes were removed from fertilization wells and denuded of cumulus cells by vortexing in 100 μL hyaluronidase (1000 U/mL in approximately 0.5 mL HEPES-TALP). Denuded putative zygotes were cultured in groups of 25 to 30 in 50-μL drops of KSOM-BE2 overlaid with mineral oil at 38.5ºC in a humidified atmosphere of 5% CO_2, 5% O_2 and the balance nitrogen. Cleavage was assessed on Day 3 after insemination and presence of blastocysts was determined on Day 7 and/or Day 8 after insemination. The experiment for sperm exposed

to checked luggage x-ray machine was replicated a total of 9 times with 9 bulls and with a total of 652 to 821 oocytes/group. The experiment for sperm exposed to carry-on luggage x-ray machine was replicated a total of 5 times with 5 bulls and with a total of 421 to 510 oocytes/group.

Statistical Analyses

For each replicate, percent of presumptive zygotes that cleaved, percent of embryos that developed to the blastocyst stage and percent of cleaved embryos that became blastocysts were calculated for all embryos within the same treatment. Thus, the group of embryos treated alike within each replicate was the experimental unit. Data were subjected to least squares analysis of variance using the GLM procedure of the Statistical Analysis System (SAS for Windows, Release 9.0, SAS Institute, Inc., Cary, NC). Percentage data were analyzed without transformation and again after arcsin transformation to correct for any non-normality associated with percentage data. All main effects and interactions were included in the mathematical models for ANOVA. Replicate was considered random for analysis of the developmental parameters (cleavage rate, blastocyst/total and blastocyst/cleaved) and other main effects were considered fixed. Hence, treatment × replicate was the error term for treatment. The CONTRAST statement of SAS was utilized to compare each irradiation dose to the zero dose and to compare all treatments (0, 1, 2 and 3 exposures) to the external control.

Results

Results for carry-on luggage doses are shown in Table 3-1 and results for checked luggage doses are shown in Table 3-2. As compared to the 0 dose, there was no effect of 1, 2 or 3 exposures to the carry-on luggage dose on the proportion of oocytes that cleave after fertilization or on blastocyst development rates (expressed as percent of oocytes becoming blastocysts or percent of cleaved embryos becoming blastocysts). For the checked luggage dose, exposure of

sperm 1 or 2 times did not affect cleavage or blastocyst development. There was, however, a tendence towards a reduction in the proportion of oocytes that cleaved (P=0.07) and the percent of oocytes becoming blastocysts (P=0.06) when sperm received 3 doses of X-irradiation using checked x-ray machine. The percent of cleaved embryos becoming blastocysts (a measure of embryo competence for development) was not significantly affected by any checked luggage exposure.

Overall, cleavage rates (P<0.001) and the percent of oocytes becoming blastocysts (P<0.001) were low compared to the external control. The percent of oocytes that cleaved for the external control was 77.7 ± 4.7% for the carry-on luggage experiment and 82.7 ± 3.7% for the checked luggage experiment. The percent of oocytes that became blastocysts was 37.7 ± 3.5% and 32.6 ± 2.0% for the carry-on and checked luggage experiments, respectively. The proportion of cleaved embryos that developed to the blastocyst stage was similar for the external control and treated sperm (P>0.10). The percent of cleaved embryos that became blastocysts was 48.4 ± 6.5% and 37.6 ± 6.7% for the carry-on and checked luggage experiments, respectively.

Discussion

Except at the highest cumulative dose (exposure 3 times to checked luggage X-irradiation), exposure of frozen semen straws in dry shippers to airport security x-ray machines did not induce changes in sperm that were reflected as a reduction in oocyte cleavage rate or embryo competence to develop to the blastocyst stage. Exposure to 3 doses of checked luggage irradiation induced changes in sperm that tended to cause a reduction in cleavage rate and resulted in reduced blastocyst development. Even at this high cumulative dose, however, embryo competence for development as measured by the proportion of cleaved embryos that became blastocysts was not different from results for control sperm exposed to the 0 dose or for external control sperm. Taken together, results indicate that fertilizing ability of sperm could be damaged

by repeated exposure to checked luggage doses of irradiation employed in airport security x-ray machines. There is no evidence to suggest that competence of the resultant embryos to develop to the blastocyst stage is reduced by exposure to X-irradiation doses characteristic of airport a-ray machines.

The tendency for the reduction in cleavage rate for oocytes inseminated with sperm exposed three-times to checked luggage X-irradiation is most likely a reflection of a reduction in sperm fertilizing capacity. One cannot rule out, however, that fertilizing ability was not affected by irradiation but that some zygotes formed from X-irradiated sperm had defects, such as in the spindle apparatus, that precluded completion of the first cleavage division. The fact that embryos formed from X-irradiated sperm did not have a reduced capacity for development to the blastocyst stage is consistent with an earlier study (Faheti et al., 2006) where there was no adverse effect of exposure of bovine sperm to a single low dose of X-irradiation (0.6 Gy). In that study, exposure to higher doses (1.25, 2.5 and 5.0 Gy) reduced the proportion of oocytes that cleaved after insemination and the proportion of cleaved embryos that became blastocysts (Fatehi et al., 2006).

The resiliency of ejaculated sperm DNA to low dose irradiation can be attributed to sperm chromatin structure and composition. Unlike somatic cells and spermatogonia, the majority of the histones in spermatozoa have been replaced by protamines (Poccia 1986) and the DNA is organized into toroids (Ward and Coffey 1991; Ward 1993). In addition, protamines are cross-linked by disulphide bonds and together these changes cause sperm DNA to become compacted into one-sixth the volume of a somatic cell nucleus (Ward and Coffey 1991). The resilience of sperm DNA is reflected by the high levels of irradiation required to damage sperm DNA compared with somatic cells. McKelvey-Martin et al. (1997) exposed human ejaculated sperm to

increasing doses of X-irradiation (5, 10 and 30 Gy) and found that DNA degradation as measured by the alkaline comet assay occurred only when sperm were exposed to the highest dose of radiation. In contrast, exposure of mice to 0.1 Gy whole-body X-irradiation caused DNA damage in peripheral blood cells as determined by the comet assay (Giovanetti et al., 2008).

It is possible that radiation damage sustained by sperm DNA was repaired following fertilization by the oocyte DNA repair machinery since the oocyte contains the requisite DNA repair machinery and is capable of initiating and carrying out repair of damaged paternal DNA prior to syngamy (Brandriff and Pedersen, 1981; Ashwood-Smith and Edwards, 1996). The DNA damage caused to spermiogenic cells by X-irradiation that persist in the spermatozoon can be repaired by the oocyte in the mouse (Matsuda et al., 1989).

The sperm used for the X-irradiation experiments resulted in significantly reduced proportions of oocytes that cleaved and that became blastocysts when compared to sperm used as external controls. This may be a reflection of the extender used – the experimental sperm were in a milk extender while the external control sperm were in an egg yolk extender. Lonergan et al. (1994) demonstrated that performing IVF with bovine sperm extended in a milk-based extender results in lower cleavage rates than when IVF is performed with sperm in an egg-yolk-based extender.

Despite the possibility that X-ray induced DNA damage may have been limited or repaired, one must take into consideration the potential for epigenetic and genetic changes to the original DNA in the embryo formed from X-irradiated sperm. These changes may not be exhibited as a reduction in embryo competence to the blastocyst stage but as a reduced implantation rate, or increased rates of mid and late gestational abnormalities, death or increased

susceptibility to disease in the offspring (Brinkworth, 2000; Anderson, 2005; Dobrzyńska and Czajka, 2005; Cordier, 2008).

Table 3-1. Effect of sperm irradiation with a carry-on X-ray machine on cleavage rate, blastocyst development and embryo competence (blastocyst/cleaved) following insemination of matured oocytes

Treatment	Cleavage (%)	Blastocysts/oocytes (%)	Blastocysts/cleaved (%)
0 exposure	24.5 ± 4.7	13.0 ± 3.5	40.3 ± 6.5
1X exposure	31.3 ± 4.7	12.8 ± 3.5	34.0 ± 6.5
2X exposure	31.1 ± 4.7	14.0 ± 3.5	34.4 ± 6.5
3X exposure	30.6 ± 4.7	13.5 ± 3.5	34.7 ± 6.5

Data are least-squares means ± SEM for five replicates involving five bulls. The total number of oocytes inseminated varied from 412 to 510 per treatment. There was no difference between 0 exposure and all other doses ($P>0.10$).

Table 3-2. Effect of sperm irradiation with a checked luggage x-ray machine on cleavage rate, blastocyst development and embryo competence (blastocyst/cleaved) following insemination of matured oocytes

Treatment	Cleavage (%)	Blastocysts/oocytes (%)	Blastocysts/cleaved (%)
0 exposure	29.4 ± 3.1	13.8 ± 1.7	45.4 ± 5.7
1X exposure	24.9 ± 3.1	11.5 ± 1.7	40.4 ± 5.7
2X exposure	25.7 ± 3.3	12.6 ± 1.9	46.4 ± 6.1
3X exposure	21.6 ± 3.1[a]	9.0 ± 1.7 [b]	41.8 ± 5.7

Data are least-squares means ± SEM for nine replicates involving nine bulls. The total number of oocytes inseminated varied from 652 to 821 per treatment. [a] Different from 0 at P=0.07. [b] Different from 0 at P=0.06.

CHAPTER 4
CONSEQUENCES FOR THE BOVINE EMBRYO OF BEING DERIVED FROM A SPERMATOZOAN SUBJECTED TO POST-EJACULATORY STRESS: OXIDATVE STRESS

Introduction

Developmental competence of the mammalian embryo is dependent on genetic and non-genetic contributions from its parents (Warner et al., 1998; Sirard et al., 2006; Ménézo, 2006; Baumann et al., 2007; Khatib et al., 2008ab). Individual males having similar *in vitro* cleavage rates can have different abilities to produce embryos competent for continued development (Shi et al., 1990; Saacke et al., 1994; Tesarik et al., 2002; Morris et al., 2003). The sperm contribution to the embryo includes the paternal DNA, plasma membrane, centriole (Sutovsky and Schatten, 2000) and RNAs (Ostermeier et al., 2004). Damage to these components could theoretically compromise the developing embryo. Sperm DNA damage has been associated with failure of spermatozoal pronuclear decondensation, reduction in embryo developmental potential and reduced implantation rates (Tomsu et al 2002; Seli et al., 2004).

The degree to which damage to sperm after ejaculation actually reduces embryo competence is not clear. In the previous chapters (Chapter 2 and 3) aging, heat shock and X-irradiation from airport x-ray machines did not damage post-ejaculated sperm in a manner that reduced embryo competence to develop to the blastocyst stage. There is some evidence, however, that molecules that increase sperm oxidative stress can reduce embryo competence. The proportion of cleaved bovine embryos that developed to the blastocyst stage was reduced for embryos treated with the pro-oxidants: gossypol (Brocas et al., 1997) and hydrogen peroxide (Silva et al., 2007).

Spermatozoa are extremely sensitive to oxidative damage owing to their high polyunsaturated fatty acid content (Poulos et al., 1986; Alvarez and Storey, 1995) and exposure

to pro-oxidants have been shown to increase oxidation of molecular components of the sperm's plasma membrane, mitochondria, cytosol and DNA (Silva et al., 2007; Peris et al., 2007). For the current study, we used bull spermatozoa to test the hypothesis that oxidative stress of ejaculated sperm after freeze-thawing would damage sperm in a manner that leads to the formation of embryos with reduced developmental competence. Two pro-oxidant chemicals were examined – menadione and *tert*-butyl hydroperoxide. Menadione is a vitamin K_2 precursor that generates superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and other reactive oxygen species in its conversion from a quinone to a semiquinone and back to a quinone in the presence of molecular oxygen (Monks et al., 1992). Menadione reduces sperm motility, depresses mitochondrial transmembrane potential and reduces ATP content (Guthrie et al., 2008). Finally, *tert*-butyl hydroperoxide is an organic peroxide, which has been reported to decrease sperm count, sperm motility and reduce litter size following intra-peritoneal injection in male mice (Kaur et al., 2006) and mid-piece lipid peroxidation in fresh and frozen-thawed stallion sperm (Neild et al., 2005).

Materials and Methods

Materials

The media HEPES-Tyrodes Lactate (HEPES-TL), IVF-TL, and Sperm-TL were purchased from Caisson (Sugar City, ID, USA) and used to prepare HEPES-Tyrodes albumin lactate pyruvate (TALP), IVF-TALP, and Sperm-TALP as previously described (Parrish et al., 1986). Oocyte collection medium (OCM) was Tissue Culture Medium-199 (TCM-199) with Hank's salts without phenol red (Atlanta Biologicals, Norcross, GA, USA) supplemented with 2% (v/v) bovine steer serum (Pel-Freez, Rogers, AR) containing 2 U/mL heparin, 100 U/mL penicillin-G, 0.1 mg/mL streptomycin, and 1 mM glutamine. Oocyte maturation medium (OMM) was Tissue

Culture Medium 199 (Gibco®, Invitrogen, Carlsbad, CA, USA) with Earle's salts supplemented with 10% (v/v) bovine steer serum, 2 μg/mL estradiol 17-β, 20 μg/mL bovine FSH (Folltropin-V; Bioniche Animal Health, London, ON, Canada), 22 μg/mL sodium pyruvate, 50 μg/mL gentamicin sulfate, and 1 mM glutamine. Percoll was from GE Healthcare (Uppsala, Sweden). Potassium simplex optimized medium (KSOM) containing 1 mg/mL BSA was obtained from Caisson. Essentially fatty-acid free (EFAF) BSA was from Sigma (St. Louis, MO). On the day of use, KSOM was modified for bovine embryos to produce KSOM-BE2 as described elsewhere (Soto et al., 2003).

Menadione (2-methyl-1,4-naphthoquinine) was purchased from Sigma (St. Louis, MO) and dissolved in absolute ethanol to make a 10 mM stock solution. Fresh stock solutions of menadione were made every two weeks. *Tert*-butyl hydroperoxide solution (TBHP) was purchased from Sigma (St. Louis, MO) and diluted to 20 nM [0.1% (v/v) ethanol] where the effect of low concentrations of menadione and TBHP were assessed in the same experiment or 300 and 600 μM [0 % (v/v) ethanol] in Sp-TALP on the day of use from the original solution. Hoechst 33342 was from Calbiochem (San Diego, CA, USA). All other reagents were purchased from Sigma or Fisher Scientific (Pittsburgh, PA, USA) unless otherwise stated.

Sperm Preparation

Extended and frozen semen from bulls was obtained from Select Sires Inc. (Plain City, OH, USA), Southeast Semen (Wellborn, FL, USA) and ABS Global (Deforest, WI, USA). Semen was thawed, subjected to Percoll gradient purification to obtain motile spermatozoa (Parrish et al., 1986), diluted in Sperm-TALP to 40 x 10^6 spermatozoa/mL, and mixed 1:1 with Sp-TALP containing treatment chemicals prior to incubation at 38.5°C in air for 3 h using a water bath. Treatments were designed so that the final concentration of ethanol was the same for

all treatments (including the vehicle control). Depending on the experiment, the final concentration of ethanol varied from 0 to 0.3%. At the end of the incubation period, treated sperm were washed and resuspend in Sp-TALP.

In Vitro Production of Embryos

Embryo production was performed as previously described (Soto et al., 2003). Briefly, cumulus-oocyte complexes (COCs) were obtained by slicing 2 to 10 mm follicles on the surface of ovaries (a mixture of beef and dairy cattle) obtained from a local abattoir. COCs containing at least one layer of compact cumulus cells were selected for maturation and fertilization. They were washed twice in OCM and placed in groups of 10 in 50 μL drops of OMM overlaid with mineral oil and matured for 20 to 22 h at 38.5°C, 5% CO_2 in humidified air. Matured oocytes where then washed once in HEPES-TALP and transferred in groups of 30 or 60 to 4-well-plates containing 425 μL IVF-TALP per well and 20 μL PHE (0.5 mM penicillamine, 0.25 mM hypotaurine, and 25 μM epinephrine in 0.9% [w/v] NaCl) per well and fertilized with 30 μL (6×10^5) Percoll-purified spermatozoa from a single bull. After 8 to 9 h of co-incubation at 38.5°C, 5% CO_2 in humidified air, putative zygotes were removed from fertilization wells and denuded of cumulus cells by vortexing in 100 μL hyaluronidase (1000 U/mL in approximately 0.5 mL HEPES-TALP). Denuded putative zygotes were cultured in groups of 25 to 35 in 50-μL drops of KSOM-BE2 overlaid with mineral oil at 38.5^{o}C in a humidified atmosphere of 5% CO_2, 5% O_2 and the balance nitrogen. Cleavage was assessed on Day 3 after insemination and presence of blastocysts was determined on Day 7 and Day 8 after insemination.

Experiments

Effects of menadione and TBHP were evaluated in three experiments. In the first, oocytes were fertilized with sperm that had been incubated in vehicle [0.05% (v/v) ethanol in Sp-TALP),

sperm that had been incubated in Sp-TALP containing 5 μM menadione or sperm that had been incubated in Sp-TALP containing 10 nM TBHP. The experiment was replicated seven times with a total of five bulls (i.e., two bulls were used twice) and with a total of 445 to 682 oocytes/group. Subsequently, experiments were conducted to evaluate effects of higher concentrations of menadione and TBHP. In one experiment, oocytes were fertilized with sperm that had been incubated in vehicle [0.3% (v/v) ethanol in Sp-TALP], or sperm that had been incubated with either 15 or 30 μM menadione. The experiment was replicated a total of four times with a separate bull for each replicate and with a total of 278 to 347 oocytes/group. In the other experiment, oocytes were fertilized with sperm that had been incubated with vehicle (Sp-TALP) or sperm that had been incubated in Sp-TALP containing 150 or 300 μM TBHP. The experiment was replicated a total of five times with a different bull for each replicate and with a total of 266 to 464 oocytes/group.

Statistical Analyses

For each replicate, the percent of oocytes that cleaved, percent of oocytes that became blastocysts and the percent of cleaved embryos that became blastocysts were calculated for all embryos within the same treatment. Thus, the group of embryos treated alike within each replicate was the experimental unit. Data were subjected to least squares analysis of variance using the GLM procedure of the Statistical Analysis System (SAS for Windows, Release 9.0, SAS Institute, Inc., Cary, NC). Data were analyzed without transformation and again after arcsin transformation to correct for any non-normality associated with percentage data. All main effects and interactions were included in the mathematical models. Replicate was considered random and other main effects were considered fixed. Hence, treatment × replicate was the error term for treatment. The CONTRAST statement of SAS was utilized to compare treatments (TBHP and menadione) against their respective vehicle controls. Data reported for P values are

from the analysis of transformed data while least-squares means are from the analysis of non-transformed data.

Results

Menadione

In a preliminary experiment, incubation of sperm with 5 μM menadione had no effect on the cleavage rate or blastocyst development rate as compared to sperm incubated with vehicle (Table 4-1). Subsequently, higher concentrations of menadione were evaluated (Table 4-2). Cleavage of oocytes following fertilization with sperm preincubated with either 15 or 30 μM menadione was reduced ($P<0.001$) as compared to cleavage of oocytes fertilized with sperm with 0 μM dose. At 15 μM, there was no significant reduction in the proportion of cleaved embryos that became blastocysts although values were numerically lower than for oocytes inseminated with control sperm. At 30 μM, the percent of cleaved embryos that became blastocyts was lower ($P<0.01$) than for controls; in fact, no cleaved embryo derived from sperm treated with 30 μM menadione became a blastocyst.

Tert-Butyl Hydroperoxide

When tested at a concentration of 10 nM, *tert*-butyl hydroperoxide treatment of sperm had a positive effect on the percentage of oocytes that were blastocysts at Day 7 and 8 ($P<0.05$) after insemination (Table 4-1). In addition, competence of embryos formed from treated sperm tended to be increased as determined by a greater proportion of cleaved embryos that became blastocysts at Day 8 after insemination ($P=0.07$).

At higher concentrations, however, treatment of sperm with TBHP had deleterious effects on oocyte cleavage and development (Table 4-3). In particular, cleavage of oocytes following insemination with sperm preincubated with 300 μM TBHP was reduced ($P<0.05$) as compared to cleavage of oocytes fertilized with sperm preincubated with 0 μM dose. Moreover, the

proportion of oocytes that were blastocysts at Day 7 or 8 after insemination was less for oocytes inseminated with sperm treated with 150 or 300 μM TBHP ($P<0.05$ or less) than for oocytes inseminated with sperm incubated with 0 μM dose. The proportion of cleaved embryos that were blastocysts at Day 7 or 8 after insemination was also less for oocytes inseminated with sperm treated with 150 μM ($P<0.05$ or less) or 300 μM TBHP ($P<0.01$).

Discussion

Exposure of frozen-thawed ejaculated sperm to the pro-oxidants used in this study – menadione and *tert*-butyl hydroperoxide reduced cleavage rate. Such an effect, which is most likely due to damage to the fertilizing ability of sperm, is not surprising because similar effects have been seen for other molecules causing oxidative stress such as hypoxanthine-xanthine oxidase system (Blondin et al., 1997), hydrogen peroxide (Hsu et al., 1999; Silva et al., 2007) and gossypol (Kennedy et al., 1983; Aitken et al., 1983). Menadione reduces sperm motility, mitochondrial membrane potential and ATP content (Guthrie et al., 2008) while *tert*-butyl hydroperoxide causes mid-piece lipid peroxidation in fresh and frozen-thawed stallion sperm (Neild et al., 2005). The present results extend previous observations found with hydrogen peroxide treatment of bull sperm (Silva et al., 2007) that high concentrations of oxidative stress to ejaculated sperm also can cause the formation of embryos with reduced competence for development to the blastocyst stage. Thus, damage to the sperm not only reduces sperm fertilizing ability but also causes changes in the resultant embryo that reduces embryonic function. An unexpected result was the observation that low concentrations of *tert*-butyl hydroperoxide tended to improve embryo competence. This result implies that alterations in the sperm can also result in beneficial changes to the resultant embryo.

There are several potential causes for reduced developmental competence of embryos derived from sperm exposed to oxidative stress. Perhaps, oxidative stress caused damage to

paternal DNA. There is some evidence to support the idea of limited capacity for the bovine oocyte to repair damaged paternal DNA (Generoso et al., 1979; Brandriff and Pedersen, 1981; Fatehi et al., 2006) and DNA damage introduced in to the embryo by sperm can lead to genomic instability affecting the maternally derived genome in zygotes and in embryos (Niwa and Kominami, 2001) and may reduce embryo competence. X-irradiation induced sperm DNA damage does not cause arrest at the G_1/S border in mouse zygote, but retards S-phase by 2 h (Shimura et al., 2002) and such a delay in cell cycle could conceivable disrupt embryonic development. Bovine zygotes that demonstrate a delayed cleavage following *in vitro* fertilization have a reduced competence for development to blastocyst stage (Lonergan et al., 1999). *Tert*-butyl hydroperoxide causes mid-piece lipid peroxidation in fresh and frozen-thawed stallion sperm (Neild et al., 2005) and it is possible that lipid peroxidation in this region of the sperm cell may result in damage to the centriole which is inherited by the zygote and is essential for pronuclear apposition and syngamy (Sutovsky et al., 1996). The role of paternal RNAs in early embryo development is not known. However oxidative damage to these may play some role in reducing embryo competence as oxidized bases in mRNAs cause ribosome to stall on the transcripts and leads to a decrease in protein expression (Shan et al., 2007).

Another possibility is that damage to sperm reduces fertilizing ability and could lead to aging of the oocyte and reduced developmental competence. That aging can compromise oocytes is indicated by the observation that inseminated bovine oocytes matured for 28 or 34 h are less likely to produce cleaved embryos that develop to the blastocyst stage than inseminated oocytes aged for 22 h (Agung et al., 2006). Aged oocytes have been linked to asynchrony in pronuclear formation (Goud et al., 1999) and in alterations in methylation patterns of maternally imprinted genes (Liang et al., 2008).

It is possible that the beneficial effects of low concentrations of *tert*-butyl hydroperoxide on embryo competence also results from a change in oocyte aging before fertilization – in this case, accelerated fertilization and reduced oocyte aging. A low level of reactive oxygen species can facilitate sperm functions directly involved in fertilization (Sengoku et al., 1998; Aitken et al., 1989; de Lamirande et al., 1997; Ford, 2004). Sengoku et al. (1998) demonstrated that low concentrations of nitric oxide enhance sperm capacitation and zona pellucida binding while de Lamirande et al. (1997) reviewed the enhancement in spermatozoa hyperactivation, capacitation and acrosomal reaction in response to low levels of superoxide anion, hydrogen peroxide and nitric oxide in human sperm. Similarly, hydrogen peroxide and nitric oxide are essential for sperm capacitation and the acrosome reaction in cattle (O'Flaherty et al., 1999, 2003; Rodriguez et al., 2005ab).

The observation that oxidative stress can lead to a reduction in fertilization and in embryo competence demonstrates that change in sperm function can impact the embryo formed from that sperm. Such effects are likely to be physiologically relevant because sperm may encounter oxidative stress in the female reproductive tract (Agarwal et al., 2005; Agarwal and Prabakaran, 2005), male reproductive tract (Tremellen, 2008) or during processing (Agarwal et al., 2005; Agarwal and Prabakaran, 2005).

Table 4-1. Effect of treatment of sperm with menadione (5 μM) or *tert*-butyl hydroperoxide (TBHP; 10 nM) on cleavage and blastocyst formation following insemination of matured oocytes

Treatment	n [a]	Cleavage (%)	Day 7		Day 8	
			Blastocysts/oocyte (%)	Blastocysts/cleaved (%)	Blastocysts/oocyte (%)	Blastocysts/cleaved (%)
Vehicle control	7 (483)	48.3 ± 3.9	13.4 ± 1.3	30.0 ± 3.9	20.4 ± 1.9	43.6 ± 3.0
Menadione, 5 μM	7 (668)	43.6 ± 3.9	12.7 ± 1.3	27.1 ± 3.9	16.9 ± 1.9	38.6 ± 3.0
TBHP, 10 nM	7 (682)	53.9 ± 3.9	18.4 ± 1.3 *	34.7 ± 3.9	27.4 ± 1.9 *	51.6 ± 3.0 †

Data are least-squares means ± SEM. Means that differ from vehicle control are indicated by superscripts: † P=0.07; * P<0.05.
[a] number of replicates (total number of embryos evaluated per treatment).

Table 4-2. Effect of treatment of sperm with menadione (0, 15 and 30 μM) on cleavage and blastocyst formation following insemination of matured oocytes

Menadione (μM)	n [a]	Cleavage (%)	Day 7		Day 8	
			Blastocysts/oocyte (%)	Blastocysts/cleaved (%)	Blastocysts/oocyte (%)	Blastocysts/cleaved (%)
0	4 (301)	77.4 ± 3.1	26.0 ± 1.9	34.2 ± 8.4	34.7 ± 1.3	45.4 ± 9.6
15	4 (347)	7.4 ± 3.1***	2.6 ± 1.9***	22.8 ± 8.4	3.2 ± 1.3***	29.5 ± 9.6
30	4 (285)	3.9 ± 3.1***	0.0 ± 1.9***	0.0 ± 8.4*	0.0 ± 1.3***	0.0 ± 9.6*

Data are least-squares means ± SEM. Means that differ from 0 μM are indicated by superscripts: * P<0.05, ** P<0.01; *** P<0.001.
[a] number of replicates (total number of embryos evaluated per treatment)

Table 4-3. Effect of treatment of sperm with *tert*-butyl hydroperoxide (TBHP; 0, 150 and 300 μM) on cleavage and blastocyst formation following insemination of matured oocytes

TBHP (μM)	n [c]	Cleavage (%)	Day 7		Day 8	
			Blastocysts/oocyte (%)	Blastocysts/cleaved (%)	Blastocysts/oocyte (%)	Blastocysts/cleaved (%)
0	5 (323)	66.6 ± 5.3	19.6 ± 1.7	30.3 ± 2.0	28.2 ± 2.9	43.5 ± 4.4
150	5 (464)	60.6 ± 5.3	10.7 ± 1.7**	17.6 ± 2.0**	17.2 ± 2.9*	28.2 ± 4.4*
300	5 (315)	47.4 ± 5.3*	4.9 ± 1.7***	10.2 ± 2.0**	8.6 ± 2.9**	18.8 ± 4.4**

Data are least-squares means ± SEM. Means that differ from 0 μM are indicated by superscripts: * P<0.05, ** P<0.01; *** P<0.001.
[a] number of replicates (total number of embryos evaluated per treatment)

CHAPTER 5
CAN PROGRAMMED CELL DEATH BE INDUCED IN POST-EJACULATORY BULL AND STALLION SPERMATOZOA?

Introduction

Apoptosis is a common characteristic of gametes undergoing spermatogenesis and is required to maintain homeostasis in the testes (Blanco-Rodriguez, 1998; Huckins, 1978; Allan et al., 1992; Sinha Hikim and Swerdloff, 1999). Ejaculates of healthy males contain two populations of sperm; a non-apoptotic fraction containing morphologically superior quality sperm and an apoptotic fraction associated with increased abnormal sperm morphology (Bull - Anzar et al., 2002; Boar - Peña et al., 2003; Man - Aziz et al., 2007).

Insults to the testis can lead to an increase in the proportion of tubular spermatocytes or ejaculated spermatozoa with characteristics of apoptosis and abnormal morphology. Among these insults are heat stress (Paul et al., 2008), scrotal heating (Sinha Hikim et al., 2003; Vera et al., 2004) and varicocele (Chen et al., 2004; Wu et al., 2008).

There are two major pathways for induction of apoptosis in mammalian cells. The intrinsic pathway involves the release of cytochrome c from the mitochondrial intermembrane space into the cytosol and sequential activation of caspase-9 and caspase-3 (Ravagnan et al., 2002; Riedl and Shi, 2004). Heat shock is one stress that activates the intrinsic pathway (Milleron and Bratton, 2007) as do agents such as carbonyl cyanide 3-chlorophenylhydrazone (CCCP) that depolarize mitochondria (Brad et al., 2007; Chaudhari et al., 2007). The extrinsic pathway is activated by ligands such as tumor necrosis factor-α that bind to receptors that activate caspase-8 and downstream caspases (Jin and El-Deiry, 2005). Given the profound morphological and molecular changes in male germ cells during spermatogenesis, it is possible that the signaling or effector pathways for induction of apoptosis become dysfunctional in the mature ejaculated sperm. Whether ejaculated spermatozoa can be induced to undergo apoptosis is not clear.

Cellular changes characteristic of apoptosis have been reported in ejaculated spermatozoa subjected to cryopreservation for the bull (Martin et al., 2004), stallion (Ortega-Ferrusola et al., 2008) and man (Weng et al, 2002; Paasch et al, 2004ab). While hydrogen peroxide has been reported to activate caspase-3 and -9 in man (Bejarano et al., 2007).

Induction of apoptosis after ejaculation by exposure of sperm to a stressful environment could compromise fertilization rate and developmental competence of the resulting embryo. Several studies indicate reduced fertilizing ability of sperm populations having a high percentage of apoptotic sperm (Paul et al., 2008; Tomsu et al., 2002; Benchaib et al., 2003; Seli et al., 2004). Moreover, an embryo formed from fertilization by an apoptotic sperm could conceivably have reduced developmental potential if DNA damage was not repaired or the sperm centriole which contributes to first cleavage in the embryo (Sutovsky et al., 1996) was damaged.

For the current study, bull and stallion spermatozoa were used to test the hypothesis that apoptosis can be triggered in ejaculated sperm. The pro-apoptotic signals were aging of sperm at 38.5ºC, which leads to a decline in fertilizing ability of sperm (Monterrosso et al., 1995; Lechniak et al., 2003; Hendricks et al., 2008), heat shock, and treatment with CCCP. The temperatures used for heat shock, 40 and 41ºC, are characteristic of lactating dairy cows exposed to heat stress (Elvinger et al., 1992; deCastro e Paula et al., 2008). Heat shock at 41ºC can cause apoptosis in bovine oocytes and preimplantation embryos (Roth and Hansen, 2004; Paula-Lopes et al., 2002; Brad et al., 2007) and CCCP has been demonstrated to cause apoptosis in bovine embryos (Brad et al., 2007). A second objective was to examine the mechanism by which apoptosis is blocked in bull spermatozoa by evaluating stress-induced changes in mitochondrial depolarization and activation of the caspase cascade.

Materials and Methods

Chemicals and Reagents

The medium Sperm-TL was purchased from Caisson (Sugar City, ID, USA) and used to prepare three media. Sp-TALP was made as previously described (Parrish et al., 1986), Sp-TALP-PVP was made similarly to Sp-TALP except bovine serum albumin was replaced with 6.3 mg/mL polyvinylpyrrolidone (PVP) (Eastman Kodak, Rochester, NY) and Sp-TALP-MOPS was prepared like Sp-TALP except the HEPES was replaced with MOPS (10 mM) and 1 mM sodium pyruvate and 0.1 mg/mL gentamicin were also added. Percoll was from GE Healthcare (Uppsala, Sweden). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide (DMSO) to make a 100 mM stock solution. Aliquots were stored at –20°C until the day of use. The stock solution of CCCP was diluted to 100 μM CCCP in 0.1% (v/v) DMSO. An equivalent amount of DMSO was added to Sp-TALP for control media. Tissue Culture Medium-199 (TCM-199), Eagle's Minimum Essential Medium (MEM), MEM with D-valine, Ham's F12, Dulbecco's phosphate buffered saline (DPBS), and penicillin–streptomycin were purchased from Sigma-Aldrich (St Louis, MO, USA). Horse serum was obtained from Hyclone (Logan, UT, USA),

Frozen semen from bulls of various beef breeds was donated by Southeastern Semen Services (Wellborn, FL, USA) and frozen Holstein semen was purchased from Select Sires (Rocky Mount, VA, USA) and ABS Global (Deforest, WI, USA). Mini-Brahman semen (Liu et al., 1999) was obtained from USDA-ARS Subtropical Agricultural Research Station, Brooksville, FL courtesy of Chad Chase Jr. Bovine endometrial (BEND) cells were obtained from ATCC (Rockville, MD, USA).

The In Situ Cell Death Detection Kit (fluorescein or TMR red) was obtained from Roche Diagnostics Corporation (Indianapolis, IN). An 8% (w/v) paraformaldehyde stock solution was

from Electron Microscopy Sciences (Hatfield, PA, USA). RQ1 RNase-free DNase was from Promega (Madison, WI, USA). Hoechst 33342 was from Sigma-Aldrich. Rabbit polyclonal antibody recognizing synthetic peptide derived from the sequence of human caspase-9 and rabbit polyclonal antibody against recombinant human caspase-3 were from Stressgen Bioreagents (Ann Arbor, MI, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

The mitochondrial polarity probe JC-1 (Molecular Probes, Invitrogen Eugene, Oregon) was prepared as a 7.67 mM stock in DMSO at –20ºC and diluted in Sp-TALP-PVP to 4 μM on the day of use. Caspase-3 inhibitor, z-DEVD-fmk, was from R & D Systems (Minneapolis, MN) and diluted with DMSO to make a 20 mM stock solution. All other reagents were purchased from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA, USA) unless otherwise stated.

Sperm Preparation

Extended bull semen was thawed, subjected to Percoll gradient purification to obtain motile spermatozoa (Parrish et al., 1986) and diluted in Sp-TALP medium to 10 to 20 x 10^6 spermatozoa/mL for experiments. Semen was collected from two fertile stallions using an artificial vagina, and the gel portion was removed with a mesh filter. The semen was diluted 1:2 with Sp-TALP, centrifuged (300 × g; 10 min) and resuspended in 2 mL Sp-TALP. One milliliter was layered over a 45% - 90% Percoll gradient and centrifuged (300 × g; 10 min). The sperm pellet was collected, resuspended in 5 mL HEPES-TALP, washed by centrifugation (300 × g; 10 min), and sperm pellet resuspended in 2 mL Sp-TALP and then adjusted to 20 × 10^6 sperm/mL.

BEND Cells

BEND cells, a bovine endometrial cell line (Staggs et al., 1998) , were grown in a 1:1 (v:v) mixture of Ham's F12 and the D-valine modification of Eagle's MEM supplemented with 200 U/L insulin, 10% (v/v) heat inactivated fetal bovine serum, 10% horse serum, 200 U/mL

penicillin and 2 mg/mL streptomycin. At confluence, cells were trypsinized, mixed with an equal amount of culture medium, centrifuged for 5 min at 110 g, resuspended in culture medium and split into two equal aliquots. At approximately 80% confluence, medium was removed and replaced with fresh medium and used for experiments.

Evaluation of TUNEL Labeling

DNA fragmentation was determined by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) procedure, whereby free 3'OH ends of DNA are labeled with TMR red- or fluorescein-conjugated dUTP by the enzyme terminal deoxynucleotidyl transferase.

Three experiments were performed. In the first, bull sperm were incubated at 20 x 10^6 spermatozoa/mL in Sp-TALP at 38.5ºC, 40ºC, 41ºC or 38.5ºC in Sp-TALP containing 100 µM CCCP in air for 4 h using a water bath. Additionally an aliquot of sperm was used as a non-incubated spermatozoa control. All treatments contained a similar concentration of DMSO [0.1% (v/v)]. At the end of the incubation period, sperm were fixed and subjected to the TUNEL labeling procedure. The experiment was replicated 4 to 7 times using a separate Holstein bull for each replicate. The second experiment was performed similarly except that incubation was for 24 h and sperm were incubated with and without 100 µM z-DEVD-fmk. All treatments contained the same amount of DMSO [0.1% (v/v)] with the exception of the non-incubated controls. The experiment was replicated 3 times using a separate Holstein bull for each replicate. The third experiment was performed using freshly ejaculated and Percoll-purified equine sperm. Sperm were incubated at 38.5ºC with or without 100 µM CCCP or at 41ºC in air for 4 h using a water bath. The experiment was replicated twice using a separate stallion for each replicate.

After incubation as described above, sperm were washed once by centrifugation (600 x g, 10 min) in 1mL PBS-PVP [10 mM KPO4, pH 7.4, containing 0.9% (w/v) NaCl (PBS) and 1 mg/mL PVP] and fixed. In some cases, sperm were fixed in 4% (w/v) paraformaldehyde in PBS for 15 min on ice, washed, and stored in PBS-PVP at 4ºC for up to 2 to 3 wk before assay. Slides were prepared using 20-50 µL of fixed sperm on the day of assay. Alternatively, sperm smears were prepared immediately following treatment, air dried and sperm fixed by covering slides with 4% (w/v) paraformaldehyde in PBS for 15 min on ice. Slides were washed gently three times by flooding with PBS-PVP, air dried and stored in the dark at 4ºC for up to 2 wk until assayed.

The TUNEL assay was initiated by permeabilizing fixed sperm with 0.5 or 0.1% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate in PBS for 30 min at room temperature. Samples were then incubated in 50-100 µL of TUNEL reaction mixture (containing TMR-red conjugated dUTP or fluorescein-conjugated dUTP and terminal deoxynucleotidyl transferase) for 1 h at 37ºC in the dark. Sperm were washed in PBS-PVP and incubated with 50 µL of 1 mg/mL Hoechst 33342 in PBS-PVP for 30 min at room temperature. Slides were washed three to four times with PBS-PVP, and cover slips mounted using 5 µL mounting medium containing ProLong® Gold antifade reagent (Invitrogen, Molecular Probes). Each TUNEL procedure contained sperm treated with RQ1 RNase-free DNase (50 U/mL) at 37ºC for 1 h as positive controls and sperm incubated with the TUNEL reagent in the absence of terminal deoxynucleotidyl transferase as a negative control. TUNEL labeling was observed using a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Inc., Göttingen, Germany). Images were acquired using AxioVision software and an AxioCam MRm digital camera (Zeiss).

Evaluation of Mitochondrial Membrane Potential

Percoll-purified sperm were diluted to 10×10^6 sperm/mL in pre-equilibrated Sp-TALP-MOPS maintained in an incubator at 38.5ºC and 5% (v/v) CO_2 or 42ºC and 7% (v/v) CO_2. The CO_2 concentration was varied with temperature to account for temperature-associated changes in gas solubility and to maintain an equal pH at both temperatures. Sperm were incubated at 38.5ºC and 5% CO_2 or 42ºC and 7% CO_2 for 4 h. A third aliquot of sperm was used as a killed control by freeze-thawing two to three times. Following incubation, mitochondrial polarity was measured by determining fluorescence emission of the polarity-dependent dye, JC-1. A total of 1×10^6 spermatozoa were incubated in triplicate with 2 μM JC-1 in a volume of 200 μL and Sp-TLP-MOPS medium at 37ºC in air for 15 min. Incubations were carried out in 96-well plates (black, tissue-culture treated, Corning, Acton, MA). Red (excitation, 550 nm; emission, 600 nm) and green fluorescence (excitation, 485 nm; emission, 535 nm) was measured using a BioTek FLx 800 fluorometer (BioTek, Winooski VT) and the ratio of red : green used to determine mitochondrial membrane potential. The experiment was replicated 4 times with a separate Angus, Brahman and Holstein bull in each replicate.

Experiment 2 was conducted similarly to Experiment 1 except that sperm from individual bulls of 4 breeds were used in each replicate: Angus, Brahman, Holstein and Mini-Brahman. The experiment was replicated a total of 6 times with 22 bulls, so that 3-4 bulls per breed were examined.

Western Blotting for Caspase-9 and Caspase-3

Sperm cells were subjected to the following treatments: control – no incubation, incubation at 38.5, 40 and 41ºC in Sp-TALP for 4 h and incubation at 38.5ºC in Sp-TALP containing 100 μM CCCP for 4 h. In addition, BEND cells were used to verify that the caspase-9 and -3 antibodies cross-reacted with bovine proteins. Cells were incubated at 37ºC in medium

with and without 100 μM CCCP for 24 h. Following treatments, sperm aliquots containing 10^6 cells were washed once by centrifugation in cold DPBS (1,800 × g for 10 min), resuspended in 15 μL DPBS and stored at -80ºC until analysis. Following thawing, cells were solubilized by boiling for 5 min in an equal volume of lysis buffer [125 mM Tris-HC1 buffer pH 6.8, containing 10% (w/v) sodium dodecyl sulfate (SDS), 20% (w/v) sucrose, and 5% (v/v) 2-mercaptoethanol]. Proteins in lysed sperm (10^6 cell equivalents per lane) and lysed BEND cells (10^5 cell equivalents per lane) were separated under reducing conditions using one-dimensional, discontinuous SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with 4–15% (w/v) gradient polyacrylamide gels and Tris-HCl buffer, pH 6.8. Proteins were transferred electrophoretically to Hybond ECL 0.2 mm nitrocellulose membranes. Membranes were washed for 10 min in TTBS [10 mM Tris pH 7.6, 0.9% (w/v) NaCl, and 0.3% (v/v) Tween-20] and blocked for 2 h or overnight in blocking buffer [TTBS containing 1% (w/v) gelatin]. Membranes were incubated for 2 h or overnight at room temperature with a rabbit polyclonal antibody recognizing caspase-9 (2 μg/mL in blocking buffer) or caspase-3 (0.5 μg/mL in blocking buffer), washed, and then incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution in blocking buffer). After additional washing, blots were developed using the ECL Plus Western blotting detection reagents as per manufacturer recommendations (GE Healthcare).

Statistical Analysis

Data were subjected to least-squares analysis of variance using the GLM and MIXED procedures of the Statistical Analysis System (SAS for Windows, Release 9.0, SAS Institute, Inc., Cary, NC). The p-diff procedure with Tukey adjustment was used as a means separation test. Data on percent TUNEL labeling were analyzed without transformation and again after arcsin transformation to correct for any non-normality associated with percentage data. Bull (i.e.

replicate) was considered random and other main effects were considered fixed. Data are reported as least-squares means ± SEM from the analysis of the untransformed data while probability values are derived from analyses of transformed data. Data on mitochondrial membrane potential were analyzed without transformation.

Results

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL)

Bull Sperm

In the first experiment, the TUNEL assay was used to assess differences in DNA damage among bull sperm aged for 4 h in Sp-TALP at 38.5ºC and under heat shock conditions of 40 and 41ºC. Results are summarized in Table 5-1. When compared to non-incubated controls, percent of sperm positive for TUNEL labeling was not increased by aging at 38.5, 40 or 41ºC. Similarly the mitochondrial depolarizing agent, CCCP, did not increase the percent of cells positive for TUNEL.

A second experiment was conducted where sperm were incubated for 24 h instead of 4 h. In addition, some sperm were treated with z-DEVD-fmk to block group II caspases. Sperm incubated for 24 h had a higher percentage that were positive for TUNEL as compared to control sperm that were not incubated ($P<0.01$), but there was no difference in the degree of TUNEL labeling between sperm incubated at 38.5, 40 and 41ºC (Table 5-2). In addition, the increase in TUNEL labeling caused by incubation for 24 h was not blocked by z-DEVD-fmk.

Equine Sperm

To investigate if TUNEL responses in stallion sperm were similar to those seen for bull sperm, equine sperm were evaluated for TUNEL labeling following aging for 4 h at 38.5ºC, 38.5ºC with 100 μM CCCP, and 41ºC (Figure 5-1). Two stallions were examined. For stallion 1, the proportion of sperm positive for the TUNEL reaction ranged from 5.7 to 9.2% and was not

affected by treatment. For stallion 2, it was noted that a large fraction of sperm (39.5%) were without tails. Among those with tails, the percent of cells positive for TUNEL was less than 3% and was unaffected by treatment (2.7%, 1.5% and 0.7% for sperm aged at 38.5ºC, with CCCP and at 41ºC respectively). However, there was a high incidence of TUNEL labeling among sperm without tails at all temperatures (94 to 97%).

Evaluation of Mitochondrial Membrane Potential

Results are given in Figure 5-2. Overall, there was no breed effect or interactions with breed and data are pooled across this classification. There was no difference in mitochondrial membrane potential between sperm incubated at 38.5 vs 42ºC in either experiment ($P>0.05$). In contrast, the ratio of red to green fluorescence was lower for sperm killed by freeze-thawing when compared to sperm incubated at 38.5 and 42ºC ($P<0.001$).

Western Blotting

Procaspase-9 (~50-54 kDa) and -3 (~36 kDa) were observed by Western blotting in bovine endometrial cells (BEND cells, Figure 5-3). There was also a band of ~30 kDa detected with the antibody to caspase-3. The manufacturer states that this protein is not caspase-3 because the band appears in cells lacking caspase-3. After addition of 100 µM CCCP, cleaved fragments of procaspase-9 (~30 kDa) and procaspase-3 (~20 kDa and 18 kDa) were observed; these represent the active caspases (Figure 5-3).

In bovine spermatozoa, procaspase-9 was evident in frozen-thawed sperm (control) and in sperm aged at 38.5, 40 and 41ºC. However, aging and exposure to 100 µM CCCP did not lead to the appearance of caspase-9. Moreover, neither procaspase-3 nor caspase-3 was observed (Figure 5-3).

Discussion

Short term aging of bull or equine sperm had no effect on DNA integrity as determined by the TUNEL assay. This was true even when spermatozoa were incubated at temperatures of 40ºC and 41ºC that are characteristic of heat-stressed cows (Elvinger et al., 1992; de Castro e Paula et al., 2008). Long-term aging for 24 h caused DNA damage to bovine spermatozoa but this was a caspase-independent phenomenon. The failure of aging to induce caspase-dependent apoptosis is due, at least in part, to failure of activation of procaspase-9 and a lack of procaspase-3. These latter results are similar to those of Martin et al. (2007) where procaspase-9 was present in bull sperm but procaspase-3 and procaspase-8 were absent.

One reason why procapase-9 was not activated by aging was that there was no change in mitochondrial potential caused by aging or heat shock. Other aspects of the activation pathway for procaspase-9 must be dysfunctional also since artificial depolarization of mitochondria with CCCP did not result in cleavage of procaspase-9. Cryopreservation, in contrast, has been reported to activate caspase-9 in bull sperm (Martin et al., 2007).

The lack of a functioning intrinsic pathway for activation of apoptosis may be a species-specific phenomenon. Cryopreservation has been reported to activate caspase-3 and -9 in man (Paasch et al., 2004ab; Bejarano et al., 2008) and activated caspase-3 has been found in ejaculated sperm in stallion (Ortega-Ferrusola et al., 2008), man (Kotwicka et al., 2008), and boar (Choi et al., 2008).

There was an increase in TUNEL labeling caused by aging of sperm for 24 h. However, this phenonomen was independent of the intrinsic activation pathway because the group II caspase inhibitor z-DEVD-fmk did not reduce the proportion of sperm cells that were positive for TUNEL. Mitochondria can induce DNA damage in a caspase-independent manner that involves apoptosis inducing factor (AIF) and endonuclease G (EndoG; van Gurp et al., 2003).

Sperm cells are particularly susceptible to oxidative stress because of their high content of polyunsaturated fatty acids in the plasma membrane (Parks et al., 1987; Aitken and Fishel, 1994). Perhaps, incubation for 24 h causes oxidative damage leading to mitochondrial membrane damage and the subsequent release of AIF and EndoG into the cytosol. Other nucleases also exist within the sperm (Sotolongo et al., 2005; Shaman et al., 2006) and these could also be activated by long-term incubation.

The fact that bull and stallion sperm are resistant to the induction of apoptosis after ejaculation by aging does not mean that apoptotic sperm are unlikely to be present in the ejaculate. Insults to the testis can increase the incidence of apoptosis of male germ cells (Paul et al., 2008; Sinha Hikim et al., 2003; Vera et al., 2004; Chen et al., 2004; Wu et al., 2008). The high incidence of apoptosis in the tailless sperm of one stallion in the present study probably represents the elimination of defective sperm by induction of apoptosis during spermatogenesis. While it is improbable that tailless sperm participate in fertilization, it is conceivable that other types of sperm with apoptotic changes in the nucleus could. Perhaps embryos formed from apoptotic sperm do not develop normally. Sperm with severe DNA damage decondense when injected into mouse oocytes but paternal DNA is not replicated during the first round of DNA synthesis and becomes digested (Yamauchi et al., 2007).

One hypothesis of the current study was that induction of apoptosis in ejaculated sperm by elevated temperatures, such as occur in the reproductive tract of heat-stressed females, could result in formation of defective embryos and be one source of the reduced fertility of heat-stressed females (Hansen, 2007). However, the failure of heat shock to induce apoptosis does not support this hypothesis.

Table 5-1. Effect of aging for 4 h at temperatures characteristic of normothermia (38.5°C) or heat stress (40 and 41°C) and of mitochondrial depolarization with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on the percent of frozen-thawed, ejaculated bull spermatozoa positive for the TUNEL reaction

Treatment	*n* [a]	Percent of spermatozoa positive for TUNEL
Control (no incubation)	4	1.2 ± 0.7
38.5°C, 4 h	7	1.6 ± 0.6
40°C, 4 h	7	2.7 ± 0.5
41°C, 4 h	4	1.5 ± 0.8
100 μM CCCP	7	1.8 ± 0.5
DMSO (control for CCCP)	7	2.1 ± 0.5

[a] Number of bulls examined per treatment. Data are least-squares means ± SEM. There was no effect of treatment.

Table 5-2. Effect of aging for 24 h at temperatures characteristic of normothermia (38.5°C) or heat stress (40 and 41°C) on the percent of frozen-thawed, ejaculated bull spermatozoa positive for the TUNEL reaction as affected by the group II caspase inhibitor, z-DEVD-fmk

		Percent of spermatozoa positive for TUNEL	
Incubation temperature	*n*[a]	- inhibitor	+ inhibitor
Control	3	1.0 ± 1.4	…
38.5°C	3	4.0 ± 1.4	4.2 ± 1.4
40°C	3	6.2 ± 1.4	4.4 ± 1.4
41°C	3	7.0 ± 1.4	8.5 ± 1.4

[a] Number of bulls examined per treatment. Data represents least-squares means ± SEM. The percent of cells positive for TUNEL was greater for cells incubated for 24 h than for control cells ($P<0.01$). When analyzed as a 2 x 3 factorial design (i.e., excluding the control), there was no effect of incubation temperature, inhibitor or temperature x inhibitor ($P>0.05$).

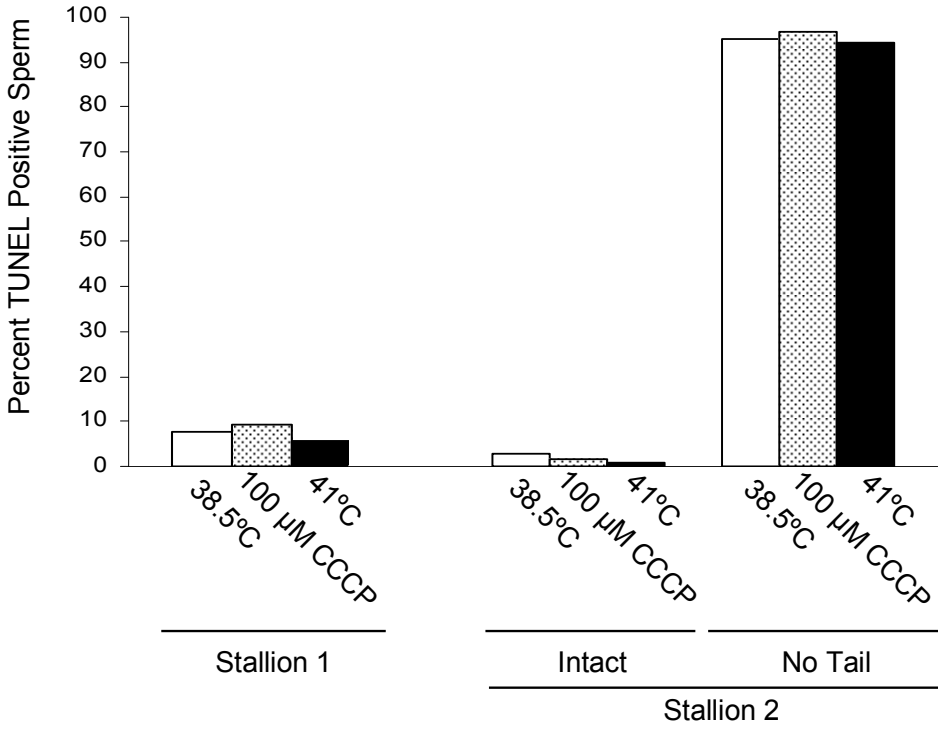

Figure 5-1. Effect of aging for 4 h at temperatures characteristic of normothermia (38.5°C) or heat stress (41°C) and of mitochondrial depolarization with carbonyl cyanide 3-chlorophenylhydrazone (CCCP) on the percent of freshly-ejaculated equine spermatozoa positive for the TUNEL reaction. Data on TUNEL labeling for stallion 2 were determined separately for sperm with and without tails because ≈ 39.5% of sperm were without tails. Few sperm (<1%) for stallion 1 were tailless.

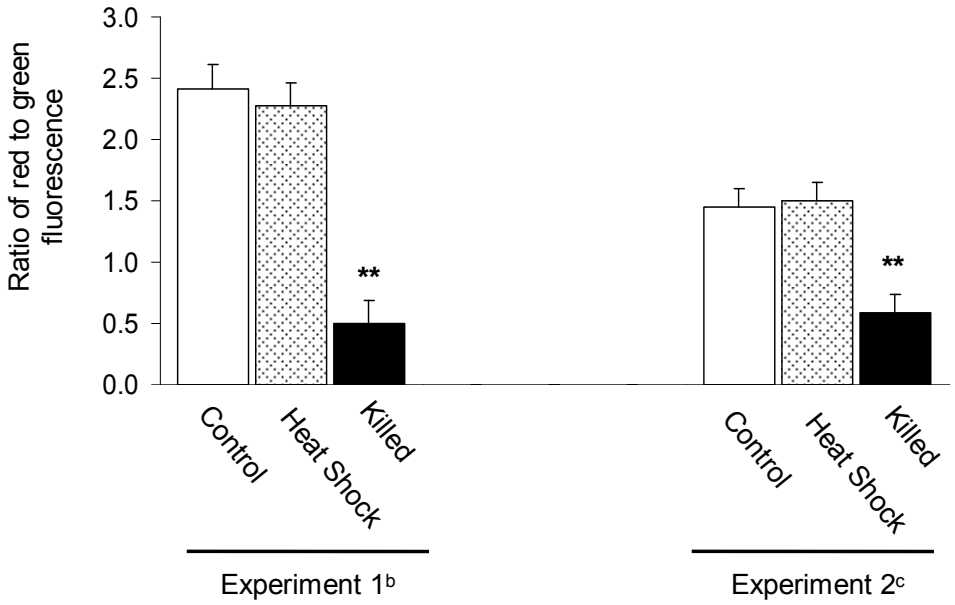

Figure 5-2. Effect of aging for 4 h at temperatures characteristic of normothermia (38.5°C) and heat stress (42°C) on mitochondrial membrane potential of frozen-thawed bull spermatozoa as measured by the cationic fluoroprobe JC-1. Data are least-squares means ± SEM of data from 16 bulls (Experiment 1) or 22 bulls (Experiment 2). There was no effect of temperature on mitochondrial membrane potential in either experiment ($P>0.05$) but killing of sperm reduced potential in both experiments ($P<0.01$;**).

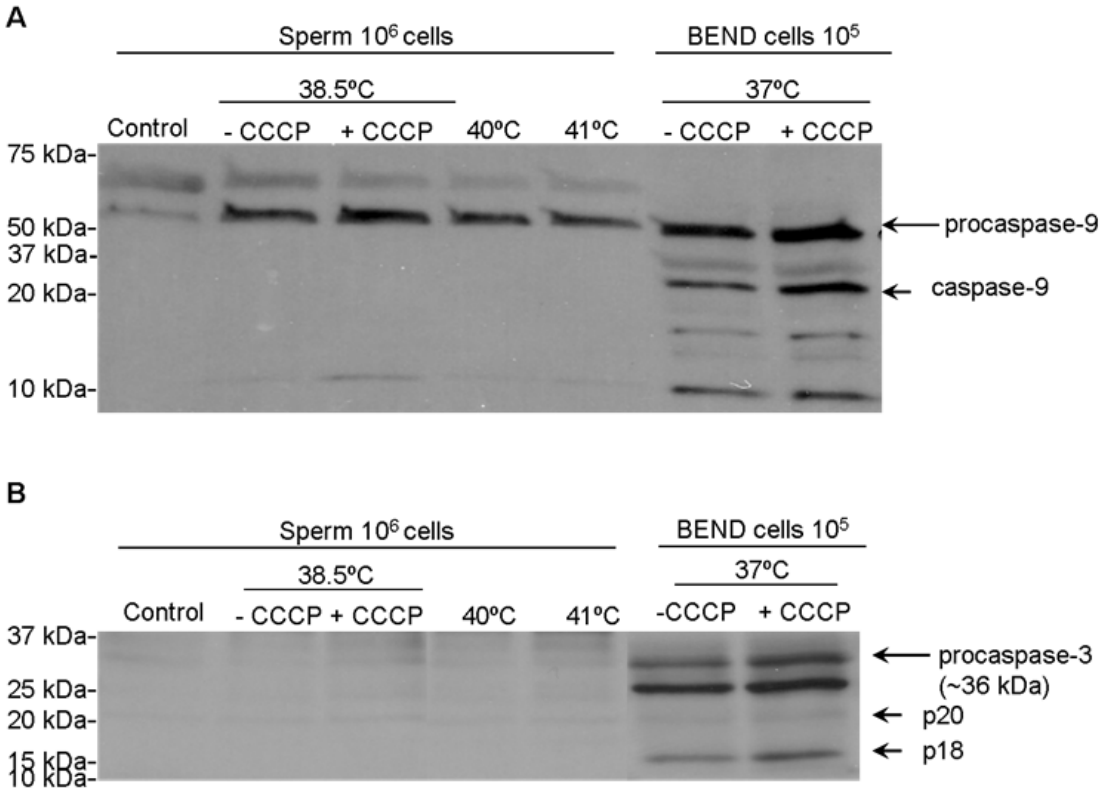

Figure 5-3. Representative western blots for the detection of caspase-9 (A) and caspase-3 (B). Bovine sperm: Control – a non-incubated sperm control; -CCCP – sperm incubated at 38.5ºC for 4 h without CCCP; +CCCP – sperm incubated at 38.5ºC for 4 h in 100 µM CCCP; 40ºC – sperm incubated at 40ºC for 4 h and 41ºC – sperm incubated at 41ºC for 4 h; BEND cells: -CCCP – cells incubated at 37ºC for 4 h without CCCP; +CCCP – cells incubated at 37ºC for 4 h in 100 µM CCCP. Note that the 30 kDa protein in the BEND cell lanes (panel B) is not caspase-3.

CHAPTER 6
GENERAL DISUSSION

The ability of the preimplantation embryo to complete its developmental program is determined in part by its genetic inheritance and non-genetic acquisition from the sperm and oocyte. The role of the oocyte in determination of embryo competence has been well established (Sirard et al., 2006). The thesis of this dissertation is that the sperm cell, too, contributes components to the embryo that affect its developmental competence and that damage to the sperm after ejaculation can result in the formation of an embryo with reduced competence for development.

Sperm may be exposed to post-ejaculatory stress while in the female reproductive tract following natural mating and/or AI or under conditions such as sperm processing for AI and IVF. Sperm must traverse the female reproductive tract to reach the site of fertilization. In cattle, sperm may remain in the female reproductive tract for up to 18 h or more prior to ovulation (Hawk, 1987) and up to 36 h prior to ovulation in swine (Hawk, 1987). Furthermore, intercourse in humans is not usually associated with ovulation, and sperm can remain in the reproductive tract for several days in women (Suarez and Pacey, 2006). Hence the time from deposition of sperm into the female reproductive tract and fertilization may be quite long with ample time for sperm to be exposed to stressors in the female reproductive tract.

Sperm within the female reproductive tract are foreign bodies and as such generate a robust inflammatory response that exposes them to immune cells. Within minutes of insemination, there is an influx of neutrophils into the uterus which peaks between 1 and 12 h after insemination (Schuberth et al., 2008). In the pig, seminal plasma induces the expression of pro-inflammatory cytokines in the uterus within 34 h of insemination (O'Leary et al., 2004).

Under normal physiological conditions it would be expected that the secretions of the female reproductive system would protect, maintain and/or enhance sperm function. For example, spermatozoa spend a large portion of time in the oviduct in sperm reservoirs waiting for ovulation to occur, and it has been demonstrated in vitro that the oviductal secretions may play a role in protecting sperm DNA structure (Robert et al., 2008), and in maintaining sperm survival and function (Pollard et al., 1991; Zhu et al., 1994; Quintero et al., 2005). Pathological conditions which lead to a change in the components of oviductal secretions may lead to loss of this protective effect, such as changes in pH (Rizvi et al., 2008).

Under both physiological and pathological conditions oxidative stress due to elevated concentrations of ROS has been associated with change in the reproductive and peritoneal microenvironments that can have negative effect on spermatozoa and hence on fertilization and early embryonic development (Agarwal et al., 2003). Surprisingly, in a study to examine the presence of ROS in the follicular fluid of women undergoing IVF, Attaran et al. (2000) found that women who became pregnant had higher levels of ROS than those who did not. However under pathological conditions where nitric oxide is generated in excessive amount over prolonged periods, such as urogenital tract infection in males and females it is possible that reduced fertility in these individuals is due to NO-induced sperm toxicity (Rosselli et al., 1995). Furthermore, it has been suggested that pathological changes due to oxidative stress and autoimmunity in infertile women could lead to changes in protein function and hence inhibit post-ejaculatory sperm functions such as sperm capacitation and oocyte fertilization, in addition to embryo implantation (Iborra et al., 2005).

Post-ejaculatory sperm processing for AI and IVF may entail staining sperm with Hoechst followed by flow cytometry sorting to produce sex-sorted semen and/or cryopreservation for

long term storage (Levinson et al., 1995; Johnson, 2000; Garner, 2008). Removal of seminal plasma with its associated anti-oxidant during sperm processing results in a pro-oxidant state, while repetitive washing and centrifugation increases the production of ROS and impairs sperm function (Aitken and Clarkson, 1988). Furthermore, sex-sorted sperm have lower viability and fertility than their unsorted counterparts (Maxwell et al., 1998; de Graaf et al., 2008). This reduction in viability and fertility may be associated to exposure to stressors, prior to, during and after passage through the flow cytometer and may include staining with Hoechst 33342, exposure to UV light, high temperatures during incubation, pressure changes, physical stress and shear forces as sperm are being sorted (Seidel and Garner, 2002; de Graaf et al., 2008).

The process of cryopreservation can lead to degenerative changes that include shortened lifespan (Gillian and Maxwell, 1999; Sankai et al., 1994; Rodríguez-Martínez et al., 2008), decreased motility (Gandini et al., 2006; Jin et al., 2008; Rodríguez-Martínez et al., 2008), a higher degree of membrane damage or membrane alterations (Hammerstedt et al., 1990; De Leeuw et al., 1990; Gillian et al., 1997; Gillian and Maxwell, 1999; Pegg, 2002; Nishizono et al., 2004), increased incidence of acrosome reacted sperm (Gillian et al., 1997; Gillian and Maxwell, 1999; Gillian et al., 1999), increase in chromatin abnormalities and DNA fragmentation (Horse: Baumber et al., 2003; Ram: Peris et al., 2004, 2007; Human: Gandini et al., 2006; Boar: Fraser and Strzezek, 2007; Mouse: Yildiz et al., 2007, 2008) and decreased fertility following intrauterine or cervical insemination (Dogs: Gill et al., 1970. Ewes: Jabbour and Evans, 1991; Maxwell et al., 1993; Gillian et al., 1997; Gómez et al., 1997; Donovan et al., 2004; Nizański, 2006; Mouse: Nishizono et al., 2004) and after intracytoplasmic sperm injection (ICSI; Gómez et al., 1997).

Most of the stresses that were applied to sperm did not affect the competence of the resulting embryos to become blastocysts. These stresses include aging *in vitro*, heat shock, and exposure to low doses of X-irradiation (Chapters 2 and 3). Failure of these stresses to affect the resultant embryo probably reflects the resistance of sperm DNA to damage as well as the limited contributions of other components of the sperm to the embryo. The highly compact nature of spermatozoal DNA due to the preponderance of protamines (Poccia, 1986), its organization into toroids (Ward and Coffey 1991; Ward 1993) and further disulphide-cross-linkage between protamines may protect spermatozoal DNA from damage due to mild stress. Perhaps, a greater magnitude of stress than applied here would compromise embryonic survival. Cozzi et al. (2001) found that exposure of epididymal mouse sperm to heat shock prior to ICSI resulted in embryos that had a lower ability to develop to the blastocyst stage compared to controls. More intense radiation than given here caused DNA damage in bovine ejaculated spermatozoa as measured by TUNEL labeling and embryos formed from X-irradiated spermatozoa had a lower ability to develop to the blastocyst stage compared to controls (Fatehi et al., 2006).

Tert-butyl hydroperoxide and menadione did reduce the competence of the resulting embryos to become blastocysts (measured as the proportion of cleaved embryos that developed to the blastocyst stage; Chapter 4). One of the possible mechanisms by which sperm damage affected embryo competence may be by delaying fertilization. A delay in fertilization leads to fertilization of an aged oocyte and this may account for the reduction in embryo competence seen in Chapter 4. Fertilization of aged oocytes results in asynchrony in pronuclear formation (Goud et al., 1999) and pronuclear asynchrony has been linked to developmental arrest in human zygotes (Zenezes et al., 1985; Schmiady and Kentenich, 1993). Another possibility for the reduction in the embryo's competence may have been due to the inheritance of damaged

macromolecules – DNA, centriole, membranes and/or RNAs. Pro-oxidants have been shown to cause oxidative damage to sperm plasma membrane, mitochondria, cytosol and DNA (Silva et al., 2007; Peris et al., 2007). Paternal DNA damage causes failure of spermatozoal pronuclear decondensation, reduction in embryo developmental potential and implantation rates following transfer in humans (Tomsu et al 2002; Seli et al., 2004) and induces developmental block after first cleavage in bovine embryos (Khalifa et al., 2007). The oocyte has a limited capacity to repair paternal DNA damage (Generoso et al., 1979; Fatehi et al., 2006) and thus some embryos will continue to develop.

The mammalian zygote, with exception to some rodents inherits the proximal centriole (which double and together forms the centrosome becoming the organizing center of the mitotic spindle) from sperm. Damage to the paternally inherited centriole may result in failure of pronuclear apposition and syngamy, in addition to the possibility of aberrant segregation of chromosomes following first cleavage, which has been linked to embryos with reduced competence for development (Chatzimeletiou et al., 2005).

Finally, the role of paternal RNAs in embryo development is unknown. In the event that these are essential to preimplantation embryo development, damage to mRNA, such as oxidation of bases will lead to failure in ribosomal translation and reduced protein expression (Shan et al., 2007). If vital to embryo survival and continued development, damage in this manner will certainly reduce the embryo's ability to develop.

Apoptosis is the typical route utilized to eliminate damaged sperm cells within the testes, both under physiological conditions, during normal spermatogenesis (Blanco-Rodriguez, 1998) or under conditions such as testicular hyperthermia (Hikim et al., 2003; Vera et al., 2004; 2005). There is also evidence in the literature that ejaculated sperm may have the capacity to undergo

cellular changes characteristic of apoptosis in response to stress (cryopreservation - Martin et al., 2004; Ortega-Ferrusola et al 2008: Weng et al., 2002; hydrogen peroxide - Bejarano et al., 2007). However, the results presented in Chapter 5 make it clear that the ejaculated bull spermatozoa are not capable of caspase-mediated apoptosis (Figure 6-1). This is because mitochondrial membrane depolarization due to aging, heat shock and CCCP (a known depolarizing agent) does not occur in ejaculated frozen-thawed sperm. Secondly, activation of procaspase 9 is blocked and this may be due to the resistance of mid-piece mitochondria to depolarize in response to the stresses used. Thirdly, procaspase 3 was not found in ejaculated bovine sperm; hence caspase-activated DNase activation is not likely. The fact that apoptosis is not possible in ejaculated spermatozoa increases the probability that a damaged spermatozoon fertilizes the oocyte.

Apoptosis is a mechanism utilized by most cell types to initiate removal of damaged cells, without damaging the surrounding tissue or cells. What biological function could the block to apoptosis in ejaculated spermatozoa serve? Perhaps the signaling mechanisms employed during apoptosis are utilized during sperm capacitation. One of the early events in the mitochondrial/intrinsic pathway of apoptosis is the release of cytochrome c into the cytosol (Figure 6-1). In ejaculated boar sperm, cytochrome c is up regulated during capacitation without activation of caspase-3 and apoptosis (Choi et al., 2008). This early apoptotic event can be detected using annexin V (Vermes et al., 1995). Incubation of human ejaculated sperm with A23187 – a calcium ionophore induces capacitated sperm to undergo the acrosome reaction and results in plasma membrane scrambling as measured by flow cytometric analysis of annexin V binding (Martin et al., 2005).

Furthermore, the externalization of PS due to A23187 is not associated with other key hallmarks of apoptosis in human sperm (attenuation of mitochondrial membrane potential,

caspase activation, increased plasma membrane permeability or increased DNA fragmentation; Martin et al., 2005). Activation of the apoptotic pathway while in the process of capacitation would be detrimental to sperm survival and blockade of the apoptosis pathway ensures fertilization by a competent spermatozoa.

It is possible that there were changes in embryo competence caused by stress of sperm that were not evident in the simple measure of embryo competence used in this study (blastocyst development). Perhaps, transfer of embryos to recipients would have revealed errors in embryonic or fetal development later in gestation. Burfening and Ulberg (1968) demonstrated that *in vitro* heat shock of ejaculated sperm decreased embryo survival as determined by the number of implantation sites noted on day 9 or 12 post insemination per number of cleaved ova 30 h post coitus in rabbits. Moreover, post-ejaculatory sperm damage *in utero* can result in the formation of an embryo with reduced competence for development (Howarth et al., 1965).

It was not until the end of this research that agents causing sperm damage that carried over into the embryo were identified. As a result, it was not possible to perform experiments to examine the mechanism for actions of *tert*-butyl hydroperoxide and mendaione.

Figure 6-2 illustrates the possible outcomes of post-ejaculatory oxidative stress on fertilization and subsequent embryo development. Post-ejaculatory oxidative damage could cause free radical lipid peroxidation (Chen et al., 1997) thereby destabilizing sperm membranes including those of mitochondria located in the mid-piece. Damage to sperm mitochondria can lead to a reduction in mitochondrial transmembrane potential and reduce the ATP content of sperm (Armstrong et al., 1999). The disturbance to the mitochondria and depletion in ATP can lead to a reduction in sperm motility. Fertilization failure occurs when the sperm fail to reach the oocyte or when sperm fail to bind to and penetrate the zona pellucida of the oocyte (Oehninger et

al., 1995), both of which may be due to either a reduction in sperm motility and/or damage to sperm plasma membrane.

Fertilization delay may also result from a reduction in sperm motility, as sperm take longer to reach the oocyte; at the same time the oocyte continues to age and fertilization of an aged oocyte can lead to pronuclear asynchrony and developmental arrest (Agung et al., 2006). However, in some cases the asynchrony in pronuclear development may not be severe enough to disrupt further development and the embryo develops normally.

Post-ejaculatory oxidative stress can lead to damage to the macromolecules inherited by the embryo. These marcomolecules include the paternal DNA and associated nuclear matrix, centriole and RNAs (especially mRNAs). Depending on the extent of paternal DNA damage, the damage may either be overlooked or repaired. However due to the limited ability of the oocyte to repair damaged DNA (Generoso et al., 1979; Brandriff and Pedersen, 1981), damage to the paternal genome may persist in the developing embryo. DNA damage leading to errors in the cell cycle, gene expression and/or metabolism may trigger cell cycle arrest at anytime during embryo development leading to apoptosis and embryo death. However, in the event that DNA damage does not compromise the embryo, development will continue unperturbed.

Oxidative damage to the paternally inherited centriole can lead to failure of pronuclear apposition and syngamy. Furthermore, centriolar damage may prevent duplication of the centriole and lead to aberrant chromosomal segregation prior to first cleavage. Failure of blastomeres to inherit the normal complement of chromosomes can lead to activation of cell cycle check points and cell cycle arrest, triggering apoptosis and embryo death.

There is currently a lack of knowledge on the role of paternal RNAs in early embryo development but there is one study that has identified unique paternal mRNA in the embryo

(Ostermeier et al., 2004). However, in the event that paternally inherited RNAs are essential for normal embryo development, oxidative damage mRNAs may lead to reduction in essential proteins (Shan et al., 2007) leading to developmental arrest, most likely prior to the maternal-zygotic transition.

Future research evaluating the effect of post-ejaculatory sperm damage on embryo competence should focus on the timing of fertilization and developmental arrest as a means of identifying which paternally inherited sperm components negatively affects embryo competence. Post-ejaculatory oxidative stress could affect fertilizing ability through sperm plasma membrane damage to either prohibit or delay fertilization (Figure 6-2). A time course study evaluating the timing of fertilization and the proportion of oocytes with sperm incorporated into the ooplasm would answer the question of delayed fertilization and concomitantly the possibility of fertilization of aged oocytes. In addition, defining the stage at which developmental arrest occurs could identify which paternally inherited sperm components negatively affect embryo competence. For example, failure of paternal pronuclei formation could point to DNA and nuclear matrix oxidative damage, while arrest at the 8- to 16- cell stage could point to inheritance of paternal damaged DNA which the oocyte was unable to repair. Examination of the zygote prior to syngamy may lead to evidence of inheritance of a damaged centriole by examining aster formation or in the 2-cell embryo examination of chromosome numbers may point to defective chromosome segregation associated with inheritance of a defective centriole.

A more difficult proposal is to examine the effect of inheritance of oxidative damaged mRNA, due to the current lack of knowledge on the role paternal mRNA play in the embryo and how these affect embryo competence. Gene expression profiles of 2, 8 and 16-cell embryo produced from sperm exposed to oxidative stress could give some insights into markers

associated with a damaged embryo. These markers may be used later to make selection of embryos that are more competent to complete its developmental program.

The fertility of lactating dairy cows has declined over the last 40 to 50 years (Lucy, 2001; Lopez-Gatius, 2003; Dobson et al., 2007). Some of this decline in fertility has been attributed to increased milk production and associated intensive management practices leading to disease such as mastitis, lameness, retained fetal membranes and endometritis (Dobson et al., 2007). Endocrine disrupters including environmental contaminants of either natural or synthetic origin, have been implicated as potential causes of reduced fertility in animals and humans (Brevini et al., 2005). Mouse embryos produced from fertilization of oocytes with spermatozoa from male mice fed the plant growth regulator, 2-chloroethyltrimethyl ammonium chloride, have reduced embryo survival (Torner et al., 1999) despite no alteration in spermatogenesis. The results of this dissertation clearly indicate that post-ejaculatory sperm damage can reduce the resultant embryo's competence for preimplantation development. Thus, the sperm is an important determinant in determining embryonic survival and should be considered when identifying causes of embryonic failure in dairy cows and other species.

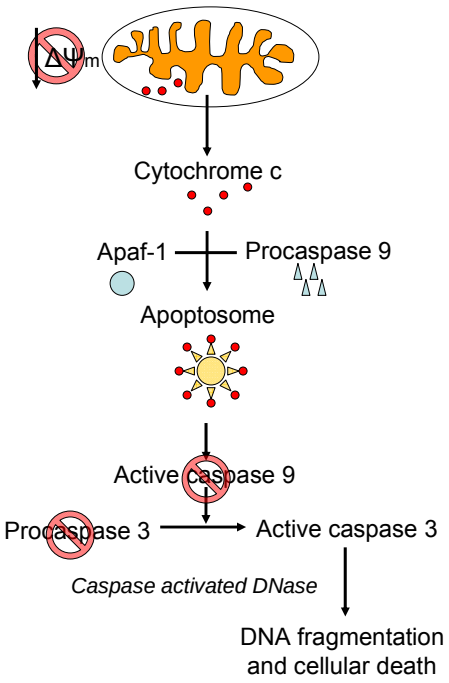

Figure 6-1. Model describing points in the mitochondrial/intrinsic pathway for apoptosis that are blocked in bovine ejaculated spermatozoa. Induction of apoptosis by post-ejaculatory stress triggers depolarization of the mitochondria (reduction in $\Delta\Psi_m$) within the mid-piece and release of various pro-apoptotic factors. Of these factors, Cytochrome c, APAF-1 and procaspase-9 form the apoptosome, resulting in the cleavage of procaspase-9 to its active form and subsequent activation of caspase 3. In addition to activation of other executioner caspases, caspase 3 cleaves caspase-activated DNase and DNA fragmentation and cell death occurs. This pathway is blocked at three points in the ejaculated spermatozoa. First, depolarization due to aging, heat shock and CCCP (a known depolarizing agent) does not occur in sperm. Activation of procaspase 9 is blocked, probably as a result of the resistance of mid-piece mitochondria to depolarization. Thirdly, procaspase 3 is absent in bovine ejaculated sperm so caspase-activated DNase activation via this enzyme is not possible.

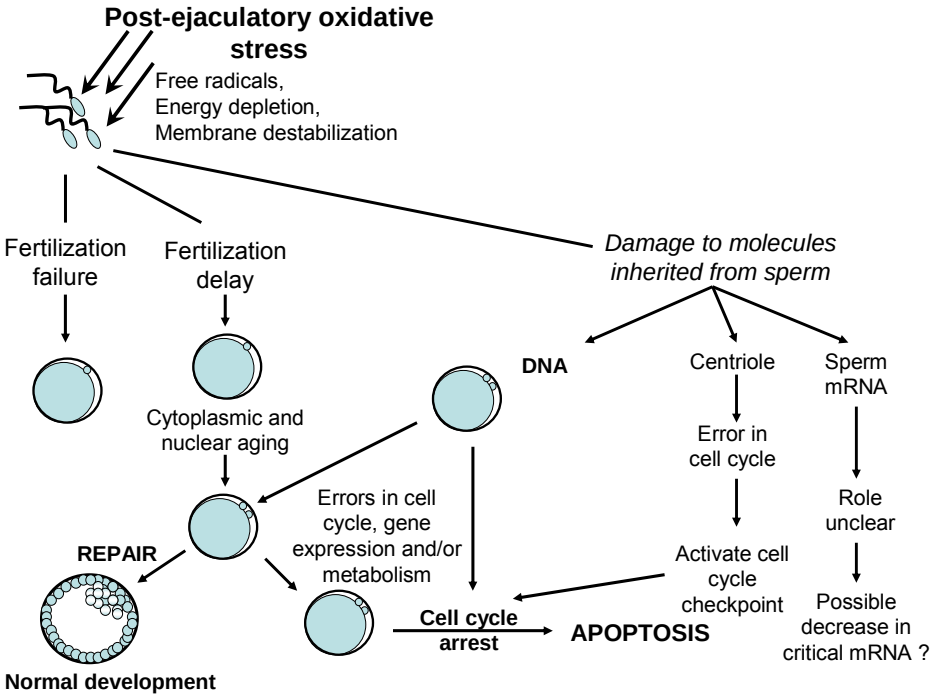

Figure 6-2. Possible scenario for the effect of post-ejaculatory oxidative stress on fertilization and subsequent embryo development

LIST OF REFERENCES

Adenot, P.G., Szöllösi, M.S., Geze, M., Renard, J.P., Debey, P., 1991. Dynamics of paternal chromatin changes in live one-cell mouse embryo after natural fertilization. Mol. Reprod. Dev. 28, 23-34.

Agarwal, A., Saleh, R.A., Bedaiwy, M.A., 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil. Steril. 79, 829-843.

Agarwal, A., Gupta, S., Sharma, R., 2005. Oxidative stress and its implications in female infertility - a clinician's perspective. Reprod. Biomed. Online. 11, 641-650.

Agarwal, A., Prabakaran, S.A., 2005. Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. Indian J. Exp. Biol. 43, 963-974.

Agung, B., Otoi, T., Wongsrikeao, P., Taniguchi, M., Shimizu, R., Watari, H., Nagai, T., 2006. Effect of maturation culture period of oocytes on the sex ratio of *in vitro* fertilized bovine embryos. J. Reprod. Dev. 52, 123-127.

Ahmadi, A., Ng, S.C., 1999a. Fertilizing ability of DNA-damaged spermatozoa. J. Exp. Zool. 284, 696-704.

Ahmadi, A., Ng, S.C., 1999b. Developmental capacity of damaged spermatozoa. Hum. Reprod. 14, 2279-2285.

Aitken, R.J., Liu, J., Best, F.S., Richardson, D.W., 1983. An analysis of the direct effects of gossypol on human spermatozoa. Int. J. Androl. 6, 157-167.

Aitken, R.J., Clarkson, J.S., 1988. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. J. Androl. 9, 367-376.

Aitken, R.J., Clarkson, J.S., Fishel, S., 1989. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol. Reprod. 41, 183-197.

Aitken, R.J., Fisher, H., 1994. Reactive oxygen species generation and human spermatozoa, the balance of benefit and risk. Bioessays. 16, 259-266.

Allan, D.J., Harmon, B.V., Roberts, S.A., 1992. Spermatogonial apoptosis has three morphologically recognizable phases and shows no circadian rhythm during normal spermatogenesis in the rat. Cell. Prolif. 25, 241-250.

Alvarez, J.G., Storey, B.T., 1995. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. Mol. Reprod. Dev. 42, 334-346.

Amanai, M., Brahmajosyula, M., Perry, A.C., 2006. A restricted role for sperm-borne microRNAs in mammalian fertilization. Biol. Reprod. 75, 877-884.

Anderson, D., 2005. Male-mediated developmental toxicity. Toxicol. Appl. Pharmacol. 207, 506-513. (supp)

Anzar, M., He, L., Buhr, M.M., Kroetsch, T.G., Pauls, K.P., 2002. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol, Reprod. 66, 354-360.

Aoki, V.W., Wilcox, A.L., Thorp, C., Hamilton, B.D., Carrell, D.T., 2004. Improved *in vitro* fertilization embryo quality and pregnancy rates with intracytoplasmic sperm injection of sperm from fresh testicular biopsy samples vs. frozen biopsy samples. Fertil. Steril. 82, 1532-1535.

Armstrong, J.S., Rajasekaran, M., Chamulitrat, W., Gatti, P., Hellstrom, W.J., Sikka, S.C., 1999. Characterization of reactive oxygen species induced effects on human spermatozoa movement and energy metabolism. Free Radic. Biol. Med. 26, 869-880

Ashwood-Smith, M.J., Edwards, R.G., 1996. DNA repair by oocytes. Mol. Hum. Reprod. 2, 46-51.

Attaran, M., Pasqualotto, E., Falcone, T., Goldberg, J.M., Miller, K.F., Agarwal, A., Sharma, R.K., 2000. The effect of follicular fluid reactive oxygen species on the outcome of in vitro fertilization. Int. J. Fertil. Womens Med. 45, 314-320.

Aziz, N., Said, T., Paasch, U., Agarwal, A., 2007. The relationship between human sperm apoptosis, morphology and the sperm deformity index. Hum. Reprod. 22, 1413-1419.

Bakos, H.W., Thompson, J.G., Feil, D., Lane, M., 2007. Sperm DNA damage is associated with assisted reproductive technology pregnancy. Int. J. Adrolo. 30, 1-9.

Balakrishnan, K., Wierda, W.G., Keating, M.J., Gandhi, V., 2008. Gossypol, a BH3 mimetic, induces apoptosis in chronic lymphocytic leukemia cells. Blood. 112, 1971-1980.

Ball, B.A., Vo, A.T., Baumber, J., 2001. Generation of reactive oxygen species by equine spermatozoa. Am. J. Vet. Res. 62, 508-515.

Barros, C.M., Pegorer, M.F., Vasconcelos, J.L., Eberhardt, B.G., Monteiro, F.M., 2006. Importance of sperm genotype (indicus versus taurus) for fertility and embryonic development at elevated temperatures. Theriogenology. 65, 210-218.

Baumann, C.G., Morris, D.G., Sreenan, J.M., Leese, H.J., 2007. The quiet embryo hypothesis, molecular characteristics favoring viability. Mol. Reprod. Dev. 74, 1345-1353.

Baumber, J., Ball, B.A., Linfor, J.J., Meyers, S.A., 2003. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. J. Androl. 24, 621-628.

Bedford, J.M., Moore, H.D., Franklin, L.E., 1979. Significance of the equatorial segment of the acrosome of the spermatozoon in eutherian mammals. Exp. Cell. Res. 119, 119-126.

Bejarano, I., Lozano, G.M., Ortiz, A., García, J.F., Paredes, S.D., Rodríguez, A.B., Pariente, J.A., 2007. Caspase-3 activation in human spermatozoa in response to hydrogen peroxide and progesterone. Fertil. Steril. (in press).

Benchaib, M., Braun, V., Lornage, J., Hadj, S., Salle, B., Lejeune, H., Guérin, J.F., 2003. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. Hum. Reprod. 18, 1023-1028.

Bilodeau, J.F., Chatterjee, S., Sirard, M.A., Gagnon, C., 2000. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol. Reprod. Dev. 55, 282-288.

Blanco-Rodriguez J., 1998. A matter of death and life, the significance of germ cell death during spermatogenesis. Int. J. Androl. 21, 236-248.

Block, J., Chase, C.C. Jr., Hansen, P.J., 2002. Inheritance of resistance of bovine preimplantation embryos to heat shock, relative importance of the maternal versus paternal contribution. Mol. Reprod. Dev. 63, 32-37.

Block, J., Drost, M., Monson, R.L., Rutledge, J.J., Rivera, R.M., Paula-Lopes, F.F., Ocon, O.M., Krininger, C.E. 3rd, Liu, J., Hansen, P.J., 2003. Use of insulin-like growth factor-I during embryo culture and treatment of recipients with gonadotropin-releasing hormone to increase pregnancy rates following the transfer of *in vitro*-produced embryos to heat-stressed, lactating cows. J. Anim. Sci. 81, 1590-1602.

Block, J., Hansen, P.J., 2007. Interaction between season and culture with insulin-like growth factor-1 on survival of *in vitro* produced embryos following transfer to lactating dairy cows. Theriogenology. 67, 1518-1529.

Blondin, P., Coenen, K., Sirard, M.A., 1997. The impact of reactive oxygen species on bovine sperm fertilizing ability and oocyte maturation. J. Androl. 18, 454-460.

Brad, A.M., Hendricks, K.E., Hansen, P.J., 2007. The block to apoptosis in bovine two-cell embryos involves inhibition of caspase-9 activation and caspase-mediated DNA damage. Reproduction. 134, 789-797.

Brandriff, B., Pedersen, R.A., 1981. Repair of the ultraviolet-irradiated male genome in fertilized mouse eggs. Science. 211, 1431-1433.

Breitbart, H., Rubinstein, S., Nass-Arden, L., 1984. Effect of gossypol-acetic acid on calcium transport and ATPase activity in plasma membranes from ram and bull spermatozoa. Int. J. Androl. 7, 439-447.

Brevini, T.A., Cillo, F., Antonini, S., Gandolfi, F., 2005. Effects of endocrine disrupters on the oocytes and embryos of farm animals. Reprod. Domest. Anim. 40, 291-299.

Brewer, L., Corzett, M., Lau, E.Y., Balhorn, R., 2003. Dynamics of protamine 1 binding to single DNA molecules. J. Biol. Chem. 278, 42403-42408.

Brinkworth, M.H., 2000. Paternal transmission of genetic damage, findings in animals and humans. Int. J. Androl. 23, 123-135.

Brocas, C., Rivera, R.M., Paula-Lopes, F.F., McDowell, L.R., Calhoun, M.C., Staples, C.R., Wilkinson, N.S., Boning, A.J., Chenoweth, P.J., Hansen, P.J., 1997. Deleterious actions of gossypol on bovine spermatozoa, oocytes, and embryos. Biol. Reprod. 57, 901-907.

Burfening, P.J., Ulberg, L.C., 1968. Embryonic survival subsequent to culture of rabbit spermatozoa at 38° and 40°C. J. Reprod. Fertil. 15, 87-92.

Cadet, J., Douki, T., Gasparutto, D., Ravanat, J.L., 2003. Oxidative damage to DNA, formation, measurement and biochemical features. Mutat. Res. 531, 5-23.

Cadet, J., Bellon, S., Douki, T., Frelon, S., Gasparutto, D., Muller, E., Pouget, J.P., Ravanat, J.L., Romieu, A., Sauvaigo, S., 2004. Radiation-induced DNA damage, formation, measurement, and biochemical features. J. Environ. Pathol. Toxicol. Oncol. 23, 33-43.

Chatterjee, S., Gagnon, C., 2001. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol. Reprod. Dev. 59, 451-458.

Chatzimeletiou, K., Morrison, E.E., Prapas, N., Prapas, Y., Handyside, A.H., 2005. Spindle abnormalities in normally developing and arrested human preimplantation embryos *in vitro* identified by confocal laser scanning microscopy. Hum. Reprod. 20, 672-682.

Chaudhari, A.A., Seol, J.W., Kim, SJ., Lee, Y.J., Kang, H.S., Kim, I.S., Kim, N.S., Park, S.Y., 2007. Reactive oxygen species regulate Bax translocation and mitochondrial transmembrane potential, a possible mechanism for enhanced TRAIL-induced apoptosis by CCCP. Oncol. Rep. 18, 71-76.

Chen, C.S., Chao, H.T., Pan, R.L., Wei, Y.H., 1997. Hydroxyl radical-induced decline in motility and increase in lipid peroxidation and DNA modification in human sperm. Biochem. Mol. Biol. Int. 43, 291-303.

Chen, C.H., Lee, S.S., Chen, D.C., Chien, H.H., Chen, I.C., Chu, Y.N., Liu, J.Y., Chen, W.H., Wu, G.J., 2004. Apoptosis and kinematics of ejaculated spermatozoa in patients with varicocele. J. Androl. 25, 348-353.

Chernos, J.E., Martin, R.H., 1989. A cytogenetic investigation of the effects of cryopreservation on human sperm. Am. J. Hum. Genet. 45, 766-777.

Choi, Y.H., Love, C.C., Love, L.B., Varner, D.D., Brinsko, S., Hinrichs, K., 2002. Developmental competence *in vivo* and *in vitro* of *in vitro*-matured equine oocytes fertilized by intracytoplasmic sperm injection with fresh or frozen-thawed spermatozoa. Reproduction. 123, 455-465.

Choi, Y.J., Uhm, S.J., Song, S.J., Song, H., Park, J.K., Kim, T., Park, C., Kim, J.H., 2008. Cytochrome c upregulation during capacitation and spontaneous acrosome reaction determines the fate of pig sperm cells, linking proteome analysis. J. Reprod. Dev. 54, 68-83.

Christova, Y., James, P.S., Jones, R., 2004. Lipid diffusion in sperm plasma membranes exposed to peroxidative injury from oxygen free radicals. Mol. Reprod. Dev. 68, 365-372.

Chung, H.S., Park, S.R., Choi, E.K., Park, H.J., Griffin, R.J., Song, C.W., Park, H., 2003. Role of sphingomyelin-MAPKs pathway in heat-induced apoptosis. Exp. Mol. Med. 35, 181-188.

Clarke, H.J., 1992. Nuclear and chromatin composition of mammalian gametes and early embryos. Biochem. Cell. Biol. 70, 856-866.

Comizzoli, P., Marquant-Le Guienne, B., Heyman, Y., Renard, J.P., 2000. Onset of the first S-phase is determined by a paternal effect during the G1-phase in bovine zygotes. Biol. Reprod. 62, 1677-1684.

Cordelli, E., Fresegna, A.M., Leter, G., Eleuteri, P., Spanò, M., Villani, P., 2003. Evaluation of DNA damage in different stages of mouse spermatogenesis after testicular X irradiation. Radiat. Res. 160, 443-451.

Cordier, S., 2008. Evidence for a role of paternal exposures in developmental toxicity. Basic Clin. Pharmacol. Toxicol. 102, 176-181.

Coutinho, E.M., 2002. Gossypol, a contraceptive for men. Contraception. 65, 259-263.

Cozzi, J., Monier-Gavelle, F., Lievre, N., Bomsel, M., Wolf, J.P., 2001. Mouse offspring after microinjection of heated spermatozoa. Biol. Reprod. 65, 1518-1521.

de Castro e Paula, L.A., Andrzejewski, J., Julian, D., Spicer, L.J., Hansen, P.J., 2008. Oxygen and steroid concentrations in preovulatory follicles of lactating dairy cows exposed to acute heat stress. Theriogenology. 69, 805-813.

De Croo, I., Van der Elst, J., Everaert, K., De Sutter, P., Dhont, M., 1998. Fertilization, pregnancy and embryo implantation rates after ICSI with fresh or frozen-thawed testicular spermatozoa. Hum. Reprod. 13, 1893-1897.

de Graaf, S.P., Beilby, K.H., Underwood, S.L., Evans, G., Maxwell, W.M., 2008. Sperm sexing in sheep and cattle: The exception and the rule. Theriogenology. (in press).

de Lamirande, E., Jiang, H., Zini, A., Kodama, H., Gagnon, C., 1997. Reactive oxygen species and sperm physiology. Rev. Reprod. 2, 48-54.

de Lamirande, E., Jiang, H., Zini, A., Kodama, H., Gagnon, C., 1997. Reactive oxygen species and sperm physiology. Rev. Reprod. 2, 48-54.

de Lamirande, E., O'Flaherty, C., 2008. Sperm activation, role of reactive oxygen species and kinases. Biochem. Biophys. Acta. 1784, 106-115.

De Leeuw, F.E., Chen, H.C., Colenbrander, B., Verkleij, A.J., 1990. Cold-induced ultrastructural changes in bull and boar sperm plasma membranes. Cryobiology. 27, 171-183.

Dinnyés, A., Lonergan, P., Fair, T., Boland, M.P., Yang, X., 1999. Timing of the first cleavage post-insemination affects cryosurvival of *in vitro*-produced bovine blastocysts. Mol. Reprod. Dev. 53, 318-324.

Dobrzyńska, M.M., Czajka, U., 2005. Male-mediated developmental toxicity in mice after 8 weeks' exposure to low doses of X-rays. Int. J. Radiat. Biol. 81, 793-799.

Dobrzyńska, M.M., 2007. Assessment of DNA damage in multiple organs from mice exposed to X-rays or acrylamide or a combination of both using the comet assay. In Vivo. 21, 657-662.

Dobson, H., Smith, R., Royal, M., Knight, C.H., Sheldon, I., 2007. The high-producing dairy cow and its reproductive performance. Reprod. Domest. Anim. 42, 17-23. (suppl 2).

Dodou, K., Anderson, R.J., Lough, W.J., Small, D.A., Shelley, M.D., Groundwater, P.W., 2005. Synthesis of gossypol atropisomers and derivatives and evaluation of their anti-proliferative and anti-oxidant activity. Bioorg. Med. Chem. 13, 4228-4237.

Donovan, A., Hanrahan, J.P., Kummen, E., Duffy, P., Boland, M.P., 2004. Fertility in the ewe following cervical insemination with fresh or frozen-thawed semen at a natural or synchronised oestrus. Anim. Reprod. Sci. 84, 359-368.

Eberhardt, B.G., Satrapa, R.A., Capinzaiki, R.L., Barros, C.M., 2005. Influence of the breed of bull and the breed of cow on the resistance of bovine embryos to heat shock *in vitro*, at early stages of development. Acta. Sci. Vet. 33, 362. (abstract).

Eid, L.N., Lorton, S.P., Parrish, J.J., 1994. Paternal influence on S-phase in the first cell cycle of the bovine embryo. Biol. Reprod. 51, 1232-1237.

Elvinger, F., Natzke, R.P., Hansen, P.J., 1992. Interactions of heat stress and bovine somatotropin affecting physiology and immunology of lactating cows. J. Dairy Sci. 75, 449-462.

Esen, M., Schreiner, B., Jendrossek, V., Lang, F., Fassbender, K., Grassme, H., Gulbins, E., 2001. Mechanisms of Staphylococcus aureus induced apoptosis of human endothelial cells. Apoptosis. 6, 431-439.

Fair, T., Gutierrez-Adan, A., Murphy, M., Rizos, D., Martin, F., Boland, M.P., Lonergan, P., 2004. Search for the bovine homolog of the murine ped gene and characterization of its messenger RNA expression during bovine preimplantation development. Biol. Reprod. 70, 488-494.

Fatehi, A.N., Bevers, M.M., Schoevers, E., Roelen, B.A., Colenbrander, B., Gadella, B.M., 2006. DNA damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages. J. Androl. 27, 176-188.

Franco, M., Block, J., Jousan, F.D., de Castro e Paula, L.A., Brad, A.M., Franco, J.M., Grisel, F., Monson, R.L., Rutledge, J.J., Hansen, P.J., 2006. Effect of transfer of one or two *in vitro*-produced embryos and post-transfer administration of gonadotropin releasing hormone on pregnancy rates of heat-stressed dairy cattle. Theriogenology. 66, 224-233.

Fraser, L., Strzezek, J., 2007. Is there a relationship between the chromatin status and DNA fragmentation of boar spermatozoa following freezing-thawing? Theriogenology. 68, 248-257.

Friedberg, E.C., 2003. DNA damage and repair. Nature. 421, 463-440.

Ford, W.C., 2004. Regulation of sperm function by reactive oxygen species. Hum. Reprod. Update. 10, 387-399.

Fumarola, C., Guidotti, G.G., 2004. Stress-induced apoptosis: toward a symmetry with receptor-mediated cell death. Apoptosis. 9, 77-82.

Fujimoto, S., Yoshida, N., Fukui, T., Amanai, M., Isobe, T., Itagaki, C., Izumi, T., Perry, A.C., 2004. Mammalian phospholipase Czeta induces oocyte activation from the sperm perinuclear matrix. Dev. Biol. 274, 370-383.

Gadea, J., Sellés, E., Marco, M.A., Coy, P., Matás, C., Romar, R., Ruiz, S., 2004. Decrease in glutathione content in boar sperm after cryopreservation: Effect of the addition of reduced glutathione to the freezing and thawing extenders. Theriogenology. 62, 690-701.

Gandini, L., Lombardo, F., Lenzi, A., Spanò, M., Dondero, F., 2006. Cryopreservation and sperm DNA integrity. Cell Tissue Bank. 7, 91-98.

Garner, D.L., 2008. Hoechst 33342: The dye that enabled differentiation of living X-and Y-chromosome bearing mammalian sperm. Theriogenology. (in press).

Generoso, W.M., Cain, K.T., Krishna, M., Huff, S.W., 1979. Genetic lesions induced by chemicals in spermatozoa and spermatids of mice are repaired in the egg. Proc. Natl. Acad. Sci. U S A. 76, 435-437.

Gill, H.P., Kaufman, C.F., Foote, R.H., Kirk, R.W., 1970. Artificial insemination of beagle bitches with freshly collected, liquid-stored, and frozen-stored semen. Am. J. Vet. Res. 3, 1807-1813.

Gillan, L., Evans, G., Maxwell, W.M., 1997. Capacitation status and fertility of fresh and frozen-thawed ram spermatozoa. Reprod. Fertil. Dev. 9, 481-487.

Gillan, L., Maxwell, W.M., 1999. The functional integrity and fate of cryopreserved ram spermatozoa in the female tract. J. Reprod. Fertil. 54, 271-283. (suppl)

Gillan, L., Skovgold, K., Watson, P.F., Evans, G., Maxwell, W.M., 1999. Fate and functional integrity of fresh and frozen-thawed ram spermatozoa following intrauterine insemination. Reprod. Fertil. Dev. 11, 309-315.

Gineitis, A.A., Zalenskaya, I.A., Yau, P.M., Bradbury, E.M., Zalensky, A.O., 2000. Human sperm telomere-binding complex involves histone H2B and secures telomere membrane attachment. J. Cell Biol. 151, 1591-1598.

Giovanetti, A., Deshpande, T., Basso, E., 2008. Persistence of genetic damage in mice exposed to low dose of X rays. Int. J. Radiat. Biol. 84, 227-235.

Gómez, M.C., Catt, J.W., Gillan, L., Evans, G., Maxwell, W.M., 1997. Effect of culture, incubation and acrosome reaction of fresh and frozen-thawed ram spermatozoa for *in vitro* fertilization and intracytoplasmic sperm injection. Reprod. Fertil. Dev. 9, 665-673.

Goodman, M.F., 2002. Error-prone repair DNA polymerases in prokaryotes and eukaryotes. Annu. Rev. Biochem. 71, 17-50.

Goud, P., Goud, A., Van Oostveldt, P., Van der Elst, J., Dhont, M., 1999. Fertilization abnormalities and pronucleus size asynchrony after intracytoplasmic sperm injection are related to oocyte postmaturity. Fertil. Steril. 72, 245-252.

Grasa, P., Coward, K., Young, C., Parrington, J., 2008. The pattern of localization of the putative oocyte activation factor, phospholipase C {zeta}, in uncapacitated, capacitated, and ionophore-treated human spermatozoa. Hum. Reprod. (in press).

Grassme, H., Gulbins, E., Brenner, B., Ferlinz, K., Sandhoff, K., Harzer, K., Lang, F., Meyer, T.F., 1997. Acidic sphingomyelinase mediates entry of N. gonorrhoeae into nonphagocytic cells. Cell. 91, 605-615.

Griffin, C.S., Tease, C., Fisher, G., 1990. The effect of low-dose X-irradiation on numerical and structural chromosome anomaly induction in mouse immature oocytes. Mutat. Res. 231, 137-142.

Griveau, J.F., Renard, P., Le Lannou, D., 1994. An *in vitro* promoting role for hydrogen peroxide in human sperm capacitation. Int. J. Androl. 17, 300-307.

Guthrie, H.D., Welch, G.R., 2006. Determination of intracellular reactive oxygen species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. J. Anim. Sci. 84, 2089-2100.

Guthrie, H.D., Welch, G.R., 2007. Use of fluorescence-activated flow cytometry to determine membrane lipid peroxidation during hypothermic liquid storage and freeze-thawing of viable boar sperm loaded with 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid. J. Anim. Sci. 85, 1402-1411.

Guthrie, H.D., Welch. G.R., Long, J.A., 2008. Mitochondrial function and reactive oxygen species action in relation to boar motility. Theriogenology. (in press).

Gyllensten, U., Wharton, D., Josefsson, A., Wilson, A.C., 1991. Paternal inheritance of mitochondrial DNA in mice. Nature. 352, 255-257.

Haines, G.A., Hendry, J.H., Daniel, C.P., Morris, I.D., 2001. Increased levels of comet-detected spermatozoa DNA damage following *in vivo* isotopic- or X-irradiation of spermatogonia. Mutat. Res. 495, 21-32.

Haines, G.A., Hendry, J.H., Daniel, C.P., Morris, I.D., 2002. Germ cell and dose-dependent DNA damage measured by the comet assay in murine spermatozoaa after testicular X-irradiation. Biol. Reprod. 67, 854-861.

Hansen, P.J., 2007. Exploitation of genetic and physiological determinants of embryonic resistance to elevated temperature to improve embryonic survival in dairy cattle during heat stress. Theriogenology. 68, S242-S249. (suppl).

Hammerstedt, R.H., Graham, J.K., Nolan, J.P., 1990. Cryopreservation of mammalian sperm, what we ask them to survive. J. Androl. 11, 73-88.

Hasler, J.F., Cardey, E., Stokes, J.E., Bredbacka, P., 2002. Nonelectrophoretic PCR-sexing of bovine embryos in a commercial environment. Theriogenology. 58, 1457-1469.

Haspel, H.C., Corin, R.E., Sonenberg, M., 1985. Effect of gossypol on erythrocyte membrane function, specific inhibition of inorganic anion exchange and interaction with band 3. J. Pharmacol. Exp. Ther. 234, 575-583.

Hawk, H.W., 1987. Transport and fate of spermatozoa after insemination of cattle. J. Dairy Sci. 70, 1487-1503.

Hendricks, K.E.M., Martins, L., Hansen, P.J., 2008. Consequences for the bovine embryo of being derived from a spermatozoan subjected to post-ejaculatory aging and heat shock, development to the blastocyst stage and sex ratio. J. Reprod. Dev. (in press).

Hernández-Cerón, J., Jousan, F.D., Soto, P., Hansen, P.J., 2005. Timing of inhibitory actions of gossypol on cultured bovine embryos. J. Dairy Sci. 88, 922-928.

Hikim, A.P., Lue, Y., Yamamoto, C.M., Vera, Y., Rodriguez, S., Yen, P.H., Soeng, K., Wang, C., Swerdloff, R.S., 2003. Key apoptotic pathways for heat-induced programmed germ cell death in the testis. Endocrinology. 144, 3167-3175.

Hillery, F.L., Parrish, J.J., First, N.L., 1990. Bull specific effect on fertilization and embryo development *in vitro*. Theriogenology. 33, 249. (abstract)

Høst, E., Lindenberg, S., Smidt-Jensen, S., 2000a. The role of DNA strand breaks in human spermatozoa used for IVF and ICSI. Acta. Obstet. Gynecol. Scand. 79, 559-563.

Høst, E., Lindenberg, S., Smidt-Jensen, S., 2000b. DNA strand breaks in human spermatozoa, correlation with fertilization *in vitro* in oligozoospermic men and in men with unexplained infertility. Acta. Obstet. Gynecol. Scand. 79, 189-193.

Howarth, B. Jr., Alliston, C.W., Ulberg, L.C., 1965. Importance of uterine environment on rabbit sperm prior to fertilization. J. Anim. Sci. 24, 1027-1032.

Hsu, P.C., Hsu, C.C., Guo, Y.L., 1999. Hydrogen peroxide induces premature acrosome reaction in rat sperm and reduces their penetration of the zona pellucida. Toxicology. 139, 93-101.

Huckins, C., 1978. The morphology and kinetics of spermatogonial degeneration in normal adult rats, an analysis using a simplified classification of the germinal epithelium. Anat. Rec. 190, 905-926.

Hud, N.V., Allen, M.J., Downing, K.H., Lee, J., Balhorn, R., 1993. Identification of the elemental packing unit of DNA in mammalian sperm cells by atomic force microscopy. Biochem. Biophys. Res. Commun. 193, 1347-1354.

Huxley, T.H., Sully, J., 1888. Evolution. 1. The Evolution of the Individual. In: Thomas S. Baynes (Ed.), Encyclopedia Britannica, A Dictionary of Arts, Sciences, and General Literature, Volume VIII. pp. 747.

Iborra, A., Palacio, J.R., Martinez, P., 2005. Oxidative stress and autoimmune response in the infertile woman. Chem. Immunol. Allergy. 88, 150-162.

Iwata, H., Shiono, H., Kon, Y., Matsubara, K., Kimura, K., Kuwayama, T., Monji, Y., 2008. Effects of modification of *in vitro* fertilization techniques on the sex ratio of the resultant bovine embryos. Anim. Reprod. Sci. 105, 234-244.

Jabbour, H.N., Evans, G., 1991. Fertility of superovulated ewes following intrauterine or oviducal insemination with fresh or frozen-thawed semen. Reprod. Fertil. Dev. 3, 1-7.

Jin, Z., El-Deiry, W.S., 2005. Overview of cell death signaling pathways. Cancer Biol. Ther. 4, 139-163.

Jin, B., Yamasaki, C., Yamada, N., Seki, S., Valdez, D.M. Jr., Kasai, M., Edashige, K., 2008. The mechanism by which mouse spermatozoa are injured during freezing. J. Reprod. Dev. 54, 265-269.

Johnson, L.A., 2000. Sexing mammalian sperm for production of offspring: the state-of-the-art. Anim. Reprod. Sci. 60-61, 93-107.

Kaji, K., Kudo, A., 2004. The mechanism of sperm-oocyte fusion in mammals. Reproduction. 127, 423-29.

Kamiguchi, Y., Tateno, H., Mikamo, K., 1990. Types of structural chromosome aberrations and their incidences in human spermatozoa X-irradiated *in vitro*. Mutat. Res. 228, 133-140.

Kanwar, U., Batla, A., Sanyal, S., Minocha, R., Majumdar, S., Ranga, A., 1989. Gossypol inhibition of Ca++ uptake and Ca++-ATPase in human ejaculated spermatozoal plasma membrane vesicles. Contraception. 39, 431-445.

Kanwar, U., Kaur, R., Chadha, S., Sanyal, S., 1990. Gossypol-induced inhibition of glucose uptake in human ejaculated spermatozoa may be mediated by lipid peroxidation. Contraception. 42, 573-587.

Kaur, P., Kaur, G., Bansal, M.P., 2006. Tertiary-butyl hydroperoxide induced oxidative stress and male reproductive activity in mice, role of transcription factor NF-kappaB and testicular antioxidant enzymes. Reprod. Toxicol. 22, 479-484.

Kemal Duru, N., Morshedi, M., Oehninger, S., 2000. Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. Fertil. Steril. 74, 1200-1207.

Kennedy, W.P., Van der Ven, H.H., Straus, J.W., Bhattacharyya, A.K., Waller, D.P., Zaneveld, L.J., Polakoski, K.L., 1983. Gossypol inhibition of acrosin and proacrosin, and oocyte penetration by human spermatozoa. Biol. Reprod. 29, 999-1009.

Khalifa, T.A., Rekkas, C.A., Lymberopoulos, A.G., Sioga, A., Dimitriadis, I., Papanikolaou, T., 2007. Factors affecting chromatin stability of bovine spermatozoa. Anim. Reprod. Sci. 104, 143-163.

Khatib, H., Monson, R.L., Schutzkus, V., Kohl, D.M., Rosa, G.J., Rutledge, J.J., 2008a. Mutations in the STAT5A gene are associated with embryonic survival and milk composition in cattle. J. Dairy Sci. 91, 784-793.

Khatib, H., Maltecca, C., Monson, R.L., Schutzkus, V., Wang, X., Rutledge, J.J., 2008b. The fibroblast growth factor 2 gene is associated with embryonic mortality in cattle. J. Anim. Sci. 86, 2063-2067.

Kim, S.T., Moley, K., 2008. Paternal effect on embryo quality in diabetic mice is related to poor sperm quality and associated with decreased GLUT expression. Reproduction. 136, 313-322.

Kimura, K., Spate, L.D., Green, M.P., Roberts, R.M., 2005. Effects of D-glucose concentration, D-fructose, and inhibitors of enzymes of the pentose phosphate pathway on the development and sex ratio of bovine blastocysts. Mol. Reprod. Dev. 72, 201-207.

King, W.A., Yadar, B.R., Xu, K. P., Picard, L., Sirard, M.A., Supplizi, A.V., Betteridge, K. J., 1991. The sex ratio of bovine embryos produced *in vitro* and *in vivo*. Theriogenology. 36, 779-788.

Knott, J.G., Kurokawa, M., Fissore, R.A., Schultz, R.M., Williams, C.J., 2005. Transgenic RNA interference reveals role for mouse sperm phospholipase Czeta in triggering Ca^{2+} oscillations during fertilization. Biol. Reprod. 72, 992-996.

Kochhar, H.S., Kochhar, K.P., Basrur, P.K., King, W.A., 2003. Influence of the duration of gamete interaction on cleavage, growth rate and sex distribution of *in vitro* produced bovine embryos. Anim. Reprod. Sci. 77, 33-49.

Korsmeyer, S.J., Wei, M.C., Saito, M., Weiler, S., Oh, K.J., Schlesinger, P.H., 2000. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. Cell Death Differ. 7, 1166-1173.

Kotwicka, M., Filipiak, K., Jedrzejczak, P., Warchol, J.B., 2008. Caspase-3 activation and phosphatidylserine membrane translocation in human spermatozoa, is there a relationship? Reprod. Biomed. 16, 657-63.

Kouchi, Z., Fukami, K., Shikano, T., Oda, S., Nakamura, Y., Takenawa, T., Miyazaki, S., 2004. Recombinant phospholipase Czeta has high Ca^{2+} sensitivity and induces Ca^{2+} oscillations in mouse eggs. J. Biol. Chem. 279, 10408-10412.

Krzyzosiak, J., McMillan, G., Molan, P., Vishwanath, R., 2000. Protein tyrosine phosphorylation during prolonged *in vitro* incubation of ejaculated bovine spermatozoa is regulated by the oxidative state of the medium. Biol. Reprod. 62, 1615-1623.

Krzyzosiak, J., Molan, P., McGowan, L., Vishwanath, R., 2001. Effect of sperm number and oxygenation state of the storage media on *in vitro* fertility of bovine sperm stored at ambient temperature. Theriogenology. 55, 1401-1415.

Kuroda, K., Fukushima, M., Harayama, H., 2007. Premature capacitation of frozen-thawed spermatozoa from subfertile Japanese black cattle. J. Reprod. Dev. 53, 1079-1086.

Kurokawa, M., Sato, K.I., Wu, H., He, C., Malcuit, C., Black, S.J., Fukami, K., Fissore, R.A., 2005. Functional, biochemical, and chromatographic characterization of the complete $[Ca^{2+}]_i$ oscillation-inducing activity of porcine sperm. Dev. Biol. 285, 376-392.

Kovacic, P., 2003. Mechanism of drug and toxic actions of gossypol, focus on reactive oxygen species and electron transfer. Curr. Med. Chem. 10, 2711-2718.

Kvist, L., Martens, J., Nazarenko, A.A., Orell, M., 2003. Paternal leakage of mitochondrial DNA in the great tit (*Parus major*). Mol. Biol. Evol. 20, 243-247.

Lalancette, C., Miller, D., Li, Y., Krawetz ,S.A., 2008. Paternal contributions, new functional insights for spermatozoal RNA. J. Cell. Biochem. 104, 1570-1579.

Lechniak, D., Strabel, T., Bousquet, D., King, A.W., 2003. Sperm pre-incubation prior to insemination affects the sex ratio of bovine embryos produced *in vitro*. Reprod. Domest. Anim. 38, 224-227.

Lee, C.H., Cho, Y.H., 1999. Aspects of mammalian spermatogenesis, electrophoretical analysis of protamines in mammalian species. Mol. Cells. 9, 556-559.

Levinson, G., Keyvanfar, K., Wu, J.C., Fugger, E.F., Fields, R.A., Harton, G.L., Palmer, F.T., Sisson, M.E., Starr, K.M., Dennison-Lagos, L., Calvo, L., Sherins, R.J., Bick, D., Schulman, J.D., Black, S.H., 1995. DNA-based X-enriched sperm separation as an adjunct to preimplantation genetic testing for the prevention of X-linked disease. Hum. Reprod. 10, 979-982.

Lewis, S.E., Aitken, R.J., 2005. DNA damage to spermatozoa has impacts on fertilization and pregnancy. Cell. Tissue Res. 322, 33-41.

Liang, L., Deng, L., Mendonca, M.S., Chen, Y., Zheng, B., Stambrook, P.J., Shao, C., Tischfield, J.A., 2007. X-rays induce distinct patterns of somatic mutation in fetal versus adult hematopoietic cells. DNA Repair (Amst). 6, 1380-1385.

Liang, X.W., Zhu, J.Q., Miao, Y.L., Liu, J.H., Wei, L., Lu, S.S., Hou, Y., Schatten, H., Lu, K.H., Sun, Q.Y., 2008. Loss of methylation imprint of Snrpn in postovulatory aging mouse oocyte. Biochem. Biophys. Res. Commun. 371, 16-21.

Li, H., Zhu, H., Xu, CJ., Yuan, J., 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell. 94, 491-501.

Linde-Forsberg, C., Forsberg, M., 1989. Fertility in dogs in relation to semen quality and the time and site of insemination with fresh and frozen semen. J. Reprod. Fertil. 39, 299-310. (suppl).

Lindsey, A.C., Schenk, J.L., Graham, J.K., Bruemmer, J.E., Squires, E.L., 2002. Hysteroscopic insemination of low numbers of flow sorted fresh and frozen/thawed stallion spermatozoa. Equine Vet. J. 34, 121-127.

Liu, J., Boyd, C.K., Kobayashi, Y., Chase, C.C. Jr., Hammond, A.C., Olson, T.A., Elsasser, T.H., Lucy, M.C., 1999. A novel phenotype for Lardon dwarfism in miniature Bos indicus cattle suggests that the expression of growth hormone receptor 1A in liver is required for normal growth. Domest. Anim. Endocrinol. 17, 421-437.

Lonergan, P., Kommisrud, E., Hafne, A.L., 1994. Comparison of two semen extenders in terms of *in vitro* development of bovine embryos following IVF. Acta. Vet. Scand. 35, 321-327.

Lonergan, P., Khatir, H., Piumi, F., Rieger, D., Humblot, P., Boland, M.P., 1999. Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. J. Reprod. Fertil. 117, 159-167.

López-Gatius, F., 2003. Is fertility declining in dairy cattle? A retrospective study in northeastern Spain. Theriogenology. 60, 89-99.

Lord, B.I., 1999. Transgenerational susceptibility to leukaemia induction resulting from preconception, paternal irradiation. Int. J. Radiat. Biol. 75, 801-810.

Lovelock, J.E., Polge, C., 1954. The immobilization of spermatozoa by freezing and thawing and the protective action of glycerol. Biochem. J. 58, 618-622.

Lucy, M.C., 2001. Reproductive loss in high-producing dairy cattle: where will it end? J. Dairy Sci. 84, 1277-1293.

Manandhar, G., Schatten, H., Sutovsky, P., 2005. Centrosome reduction during gametogenesis and its significance. Biol. Reprod. 72, 2-13.

Mann, S.L., Patton, W.C., King, A., Chan, P.J., 2002. Comparative genomic hybridization analysis of sperm DNA apoptosis after exposure to heat shock. J. Assist. Reprod. Genet. 19, 195-200.

Marti, E., Marti, J.I., Muiño-Blanco, T., Cebrián-Pérez, J.A., 2008. Effect of the cryopreservation process on the activity and immunolocalization of antioxidant enzymes in ram spermatozoa. J. Androl. 29, 459-467

Martin, R.H., Chernos, J.E., Rademaker, A.W., 1991. Effect of cryopreservation on the frequency of chromosomal abnormalities and sex ratio in human sperm. Mol. Reprod. Dev. 30, 159-163.

Martin, G., Sabido, O., Durand, P., Levy, R., 2004. Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol. Reprod. 71, 28-37.

Martin, G., Sabido, O., Durand, P., Levy, R., 2005. Phosphatidylserine externalization in human sperm induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction. Hum. Reprod. 20, 3459-3468.

Martin, G., Cagnon, N., Sabido, O., Sion, B., Grizard, G., Durand, P., Levy, R., 2007. Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. Hum. Reprod. 22, 380-388.

Matsuda, Y., Yamada, T., Tobari, I., 1985a. Studies on chromosome aberrations in the eggs of mice fertilized *in vitro* after irradiation. I. Chromosome aberrations induced in sperm after X-irradiation. Mutat. Res. 148, 113-117.

Matsuda, Y., Tobari, I., Yamada, T., 1985b. Studies on chromosome aberrations in the eggs of mice fertilized *in vitro* after irradiation. II. Chromosome aberrations induced in mature oocytes and fertilized eggs at the pronuclear stage following X-irradiation. Mutat. Res. 151, 275-280.

Matsuda, Y., Seki, N., Utsugi-Takeuchi, T., Tobari, I., 1989. X-ray- and mitomycin C (MMC)-induced chromosome aberrations in spermiogenic germ cells and the repair capacity of mouse eggs for the X-ray and MMC damage. Mutat. Res. 211, 65-75.

Maxwell, W.M., Evans, G., Rhodes, S.L., Hillard, M.A., Bindon, B.M., 1993. Fertility of superovulated ewes after intrauterine or oviducal insemination with low numbers of fresh or frozen-thawed spermatozoa. Reprod. Fertil. Dev. 5, 57-63.

Maxwell, W.M.C., Long, C.R., Johnson, L.A., Dobrinsky, J.R., Welch, G.R., 1998. The relationship between membrane status and fertility of boar spermatozoa after flow cytometric sorting in the presence or absence of seminal plasma. Reprod. Fertil. Dev. 10, 433-440.

McElhinny, A.S., Warner, C.M., 2000. Cross-linking of Qa-2 protein, the Ped gene product, increases the cleavage rate of C57BL/6 preimplantation mouse embryos. Mol. Hum. Reprod. 6, 517-522.

McKelvey-Martin, V.J., Melia, N., Walsh, I.K., Johnston, S.R., Hughes, C.M., Lewis, S.E., Thompson, W., 1997. Two potential clinical applications of the alkaline single-cell gel electrophoresis assay, (1). Human bladder washings and transitional cell carcinoma of the bladder, and (2). Human sperm and male infertility. Mutat. Res. 375, 93-104.

Ménézo, Y.J., 2006. Paternal and maternal factors in preimplantation embryogenesis, interaction with the biochemical environment. Reprod. Biomed. Online. 12, 616-621

Meseguer, M., Martínez-Conejero, J.A., O'Connor, J.E., Pellicer, A., Remohí, J., Garrido, N., 2008. The significance of sperm DNA oxidation in embryo development and reproductive outcome in an oocyte donation program: a new model to study a male infertility prognostic factor. Fertil. Steril. 89, 1191-1199.

Miller, D., Ostermeier, G.C., 2006. Towards a better understanding of RNA carriage by ejaculate spermatozoa. Hum. Reprod. Update. 12, 757-767.

Milleron, R.S., Bratton, S.B., 2007. 'Heated' debates in apoptosis. Cell. Mol. Life Sci. 64, 2329-2333.

Molls, M., Streffer, C., 1984. The influence of G2 progression on X-ray sensitivity of two-cell mouse embryos. Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 46, 355-365.

Monks, T.J., Hanzlik, R.P., Cohen, G.M., Ross, D., Graham, D.G., 1992. Quinone chemistry and toxicity. Toxicol. Appl. Pharmacol. 112, 2-16.

Monterroso, V.H., Drury, K.C., Ealy, A.D., Edwards, J.L., Hansen, P.J., 1995. Effect of heat shock on function of frozen/thawed bull spermatozoa. Theriogenology. 44, 947-961.

Moore, G.D., Salazar, A., Cárdenas, H., Croxatto, H.B., 1988. Inhibition of sperm migration, fertilization and implantation in rats by precoital intrauterine administration of gossypol or nonoxynol-9. Contraception. 37, 85-98.

Morris, I.D., Ilott, S., Dixon, L., Brison, D.R., 2002. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. Hum. Reprod. 17, 990-998.

Morris, L.H., Randall, A.E., King, W.A., Johnson, W.H., Buckrell, B.C., 2003. The contribution of the male to ovine embryogenesis in an *in vitro* embryo production system. Anim. Reprod. Sci. 75, 9-26.

Muriel, L., Garrido, N., Fernández, J.L., Remohí, J., Pellicer, A., de los Santos, M.J., Meseguer, M., 2006. Value of the sperm deoxyribonucleic acid fragmentation level, as measured by the sperm chromatin dispersion test, in the outcome of *in vitro* fertilization and intracytoplasmic sperm injection. Fertil. Steril. 85, 371-383.

Navara, C.S., First, N.L., Schatten, G., 1994. Microtubule organization in the cow during fertilization, polyspermy, parthenogenesis, and nuclear transfer, the role of the sperm aster. Dev. Biol. 162, 29-40.

Navara, C.S., Simerly, C., Zoran, S., Schatten, G., 1995. The sperm centrosome during fertilization in mammals, implications for fertility and reproduction. Reprod. Fertil. Dev. 7, 747-754.

Navara, C.S., First, N.L., Schatten, G., 1996. Phenotypic variations among paternal centrosomes expressed within the zygote as disparate microtubule lengths and sperm aster organization, correlations between centrosome activity and developmental success. Proc. Natl. Acad. Sci. U S A. 93, 5384-5388.

Nedambale, T.L., Dinnyes, A., Yang, X., Tian, X.C., 2004. Bovine blastocyst development *in vitro*, timing, sex, and viability following vitrification. Biol. Reprod. 71, 1671-1676.

Neild, D.M., Brouwers, J.F., Colenbrander, B., Agüero, A., Gadella, B.M., 2005. Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. Mol. Reprod. Dev. 72, 230-238.

Nishizono, H., Shioda, M., Takeo, T., Irie, T., Nakagata, N., 2004. Decrease of fertilizing ability of mouse spermatozoa after freezing and thawing is related to cellular injury. Biol. Reprod. 71, 973-978.

Niwa, O., Kominami, R., 2001. Untargeted mutation of the maternally derived mouse hypervariable minisatellite allele in F1 mice born to irradiated spermatozoa. Proc. Natl. Acad. Sci. USA. 98, 1705-1710.

Nizański, W., 2006. Intravaginal insemination of bitches with fresh and frozen-thawed semen with addition of prostatic fluid, use of an infusion pipette and the Osiris catheter. Theriogenology. 66, 470-483.

Oehninger, S., Blackmore, P., Mahony, M., Hodgen, G., 1995. Effects of hydrogen peroxide on human spermatozoa. J. Assist. Reprod. Genet. 12, 41-47.

O'Flaherty, C.M., Beorlegui, N.B., Beconi, M.T., 1999. Reactive oxygen species requirements for bovine sperm capacitation and acrosome reaction. Theriogenology. 52, 289-301.

O'Flaherty, C., Beorlegui, N., Beconi, M.T., 2003. Participation of superoxide anion in the capacitation of cryopreserved bovine sperm. Int. J. Androl. 26, 109-114.

O'Leary, S., Jasper, M.J., Warnes, G.M., Armstrong, D.T., Robertson, S.A., 2004. Seminal plasma regulates endometrial cytokine expression, leukocyte recruitment and embryo development in the pig. Reproduction. 128, 237-247.

Okada, S., Suzumori, K., Yagami, Y., 1995. Cytogenetic effects of cryopreservation on human sperm, assessment using an improved method for analyzing human sperm chromosomes. J. Obstet. Gynaecol. 21, 641-647.

Ortega-Ferrusola, C., Sotillo-Galán, Y., Varela-Fernández, E., Gallardo-Bolaños, J.M., Muriel, A., González-Fernández, L., Tapia, J.A., Peña, F.J., 2008. Detection of "apoptosis-like" changes during the cryopreservation process in equine sperm. J. Androl. 29, 213-221.

Ostermeier, G.C., Dix, D.J., Miller, D., Khatri, P., Krawetz, S.A., 2002. Spermatozoal RNA profiles of normal fertile men. Lancet. 360, 772-777.

Ostermeier, G.C., Miller, D., Huntriss, J.D., Diamond, M.P., Krawetz, S.A., 2004. Reproductive biology, delivering spermatozoan RNA to the oocyte. Nature. 429, 154.

Paasch, U., Grunewald, S., Agarwal, A., Glander, H.J., 2004a. Activation patterns in human spermatozoa. Fertil. Steril. 81, 802-809.

Paasch, U., Sharma, R.K., Gupta, A.K., Grunewald, S., Mascha, E.J., Thomas, A.J. Jr., Glander, H.J., Agarwal, A., 2004b. Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. Biol. Reprod. 71, 1828-1837.

Palermo, G.D., Colombero, L.T., Rosenwaks, Z., 1997. The human sperm centrosome is responsible for normal syngamy and early embryonic development. Rev. Reprod. 2, 19-27.

Pampfer, S., Streffer, C., Müller, W.U., 1989. Micronucleus formation in 2-cell embryos after *in vitro* X-irradiation of mouse spermatozoa. Mutat. Res. 210, 191-196.

Park, J.H., Lee, J.H., Choi, K.M., Joung, S.Y., Kim, J.Y., Chung, G.M., Jin, D.I., Im, K.S., 2001. Rapid sexing of preimplantation bovine embryo using consecutive and multiplex polymerase chain reaction (PCR) with biopsied single blastomere. Theriogenology. 55, 1843-1853.

Parks, J.E., Arion, J.W., Foote, R.H., 1987. Lipids of plasma membrane and outer acrosomal membrane from bovine spermatozoa. Biol. Reprod. 37, 1249-1258.

Parrish, J.J., Susko-Parrish, J.L., Critser, E.S., Eyestone, W.H., First, N.L., 1986. Bovine *in vitro* fertilization with frozen-thawed semen. Theriogenology. 25, 591-600.

Paul, C., Murray, A.A., Spears, N., Saunders, P.T., 2008. A single, mild, transient scrotal heat stress causes DNA damage, subfertility and impairs formation of blastocysts in mice. Reproduction. 136, 73-84.

Paula-Lopes, F.F., Hansen, P.J., 2002. Heat-shock induced apoptosis in preimplantation bovine embryos is a developmentally-regulated phenomenon. Biol. Reprod. 66, 1169-1177.

Pegg, D.E., 2002. The history and principles of cryopreservation. Semin. Reprod. Med. 20, 5-13.

Pegorer, M.F., Vasconcelos, J.L., Trinca, L.A., Hansen, P.J., Barros, C.M., 2007. Influence of sire and sire breed (Gyr versus Holstein) on establishment of pregnancy and embryonic loss in lactating Holstein cows during summer heat stress. Theriogenology. 67, 692-697

Peña, F.J., Johannisson, A., Wallgren, M., Rodríguez-Martínez, H., 2003. Assessment of fresh and frozen-thawed boar semen using an annexin-V assay, a new method of evaluating sperm membrane integrity. Theriogenology. 60, 677-89.

Peris, S.I., Morrier, A., Dufour, M., Bailey, J.L., 2004. Cryopreservation of ram semen facilitates sperm DNA damage, relationship between sperm andrological parameters and the sperm chromatin structure assay. J. Androl. 25, 224-233.

Peris, S.I., Bilodeau, J.F., Dufour, M., Bailey, J.L., 2007. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. Mol. Reprod. Dev. 74, 878-892.

Poccia, D., 1986. Remodeling of nucleoproteins during gametogenesis, fertilization, and early development. Int. Rev. Cytol. 105, 1-65.

Polge, C., Smith, A.U., Parkes, A.S., 1949. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature. 164, 666.

Pollard, J.W., Plante, C., King, W.A., Hansen, P.J., Betteridge, K.J., Suarez, S.S., 1991. Fertilizing capacity of bovine sperm may be maintained by binding of oviductal epithelial cells. Biol. Reprod. 44, 102-107.

Poulos, A., Sharp, P., Johnson, D., White, I., Fellenberg, A., 1986. The occurrence of polyenoic fatty acids with greater than 22 carbon atoms in mammalian spermatozoa. Biochem. J. 240, 891-895

Pukazhenthi, B., Comizzoli, P., Travis, A.J., Wildt, D.E., 2006. Applications of emerging technologies to the study and conservation of threatened and endangered species. Reprod. Fertil. Dev. 18, 77-90.

Quintero, I., Ghersevich, S., Caille, A.M., Munuce, M.J., Daniele, S.M., Morisoli, L., 2005. Effects of human oviductal in vitro secretion on spermatozoa and search of sperm-oviductal proteins interactions. Int. J. Androl. 28, 137-143.

Randel, R.D., Chase, C.C. Jr., Wyse, S.J., 1992. Effects of gossypol and cottonseed products on reproduction of mammals. J. Anim. Sci. 70, 1628-1638.

Rath, D., Niemann, H., 1997. In vitro fertilization of porcine oocytes with fresh and frozen-thawed ejaculated or frozen-thawed epididymal semen obtained from identical boars. Theriogenology. 47, 785-793.

Ravagnan, L., Roumier, T., Kroemer, G., 2002. Mitochondria, the killer organelles and their weapons. J. Cell. Physiol. 192, 131-137.

Riedl, S.J., Salvesen, G.S., 2007. The apoptosome, signalling platform of cell death. Nat. Rev. Mol. Cell. Biol. 8, 405-413.

Riedl, S.J., Shi, Y., 2004. Molecular mechanisms of caspase regulation during apoptosis. Nat. Rev. Mol. Cell. Biol. 5, 897-907.

Rivlin, J., Mendel, J., Rubinstein, S., Etkovitz, N., Breitbart, H., 2004. Role of hydrogen peroxide in sperm capacitation and acrosome reaction. Biol. Reprod. 70, 518-522.

Rizvi, A.A., Quraishi, M.I., Sarkar, V., Dubois, C., Biro, S., Mulhall, J., 2008. The effect of pH and viscosity on bovine spermatozoa motility under controlled conditions. Int. Urol. Nephrol. (in press).

Robert, C., Caille, A., Zumoffen, C., Cabada, M., Ghersevich, S., 2008. Effect of human oviductal in vitro secretion on human sperm DNA integrity. J. Assist. Reprod. Genet. 25, 263-270.

Rodriguez, P.C., O'Flaherty, C.M., Beconi, M.T., Beorlegui, N.B., 2005a. Nitric oxide induces acrosome reaction in cryopreserved bovine spermatozoa. Andrologia. 37, 166-172.

Rodriguez, P.C., O'Flaherty, C.M., Beconi, M.T., Beorlegui, N.B., 2005b. Nitric oxide-induced capacitation of cryopreserved bull spermatozoa and assessment of participating regulatory pathways. Anim. Reprod. Sci. 85, 231-242.

Rodríguez-Martínez, H., Saravia, F., Wallgren, M., Roca, J., Peña, F.J., 2008. Influence of seminal plasma on the kinematics of boar spermatozoa during freezing. Theriogenology. (in press).

Rosselli, M., Dubey, R.K., Imthurn, B., Macas, E., Keller, P.J., 1995. Effects of nitric oxide on human spermatozoa: evidence that nitric oxide decreases sperm motility and induces sperm toxicity. Hum. Reprod. 10, 1786-1790.

Roth, Z., Hansen, P.J., 2004. Involvement of apoptosis in disruption of oocyte competence by heat shock in cattle. Biol. Reprod. 71, 1898-1906.

Saacke, R.G., Nadir, S., Nebel, R.L., 1994. Relationship of semen quality to sperm transport, fertilization, and embryo quality in ruminants. Theriogenology. 41, 45-50.

Saacke, R.G., Dalton, J.C., Nadir, S., Nebel, R.L., Bame, J.H., 2000. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim. Reprod. Sci. 60-61, 663-677.

Sakkas, D., Urner, F., Bianchi, P.G., Bizzaro, D., Wagner, I., Jaquenoud, N., Manicardi, G., Campana, A., 1996. Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection. Hum. Reprod. 11, 837-843.

Sankai, T., Terao, K., Yanagimachi, R., Cho, F., Yoshikawa, Y., 1994. Cryopreservation of spermatozoa from cynomolgus monkeys (Macaca fascicularis). J. Reprod. Fertil. 101, 273-278.

Santana, P., Pena, L.A., Haimovitz-Friedman, A., Martin, S., Green, D., McLoughlin, M., Cordon-Cardo, C., Schuchman, E.H., Fuks, Z., Kolesnick, R., 1996. Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. Cell. 86, 189-199.

Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K., Lai, F.A., 2002. PLC zeta, a sperm-specific trigger of $Ca^{(2+)}$ oscillations in eggs and embryo development. Development. 129, 3533-3544.

Saunders, C.M., Swann, K., Lai, F.A., 2007. PLCzeta, a sperm-specific PLC and its potential role in fertilization. Biochem. Soc. Symp. 74, 23-36.

Schatten, G., 1994. The centrosome and its mode of inheritance, the reduction of the centrosome during gametogenesis and its restoration during fertilization. Dev. Biol. 165, 299-335

Schmiady, H., Kentenich, H., 1993. Cytological studies of human zygotes exhibiting developmental arrest. Hum. Reprod. 8, 744-751.

Schuberth, H.J., Taylor, U., Zerbe, H., Waberski, D., Hunter, R., Rath, D., 2008. Immunological responses to semen in the female genital tract. Theriogenology. 70, 1174-1181.

Schwartz, M., Vissing, J., 2002. Paternal inheritance of mitochondrial DNA. N. Engl. J. Med. 347, 576-580.

Seidel, G.E. Jr., Garner, D.L., 2002. Current status of sexing mammalian spermatozoa. Reproduction. 124, 733-743.

Seli, E., Gardner, D.K., Schoolcraft, W.B., Moffatt, O., Sakkas, D., 2004. Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after *in vitro* fertilization. Fertil. Steril. 82, 378-383.

Sengoku, K., Tamate, K., Yoshida, T., Takaoka, Y., Miyamoto, T., Ishikawa, M., 1998. Effects of low concentrations of nitric oxide on the zona pellucida binding ability of human spermatozoa. Fertil. Steril. 69, 522-527.

Shaman, J.A., Prisztoka, R., Ward, W.S., 2006. Topoisomerase IIB and an extracellular nuclease interact to digest sperm DNA in an apoptotic-like manner. Biol. Reprod. 75, 741-748.

Shaman, J.A., Yamauchi, Y., Ward, W.S., 2007. Function of the sperm nuclear matrix. Arch. Androl. 53, 135-140.

Shan, X., Chang, Y., Lin, C.L., 2007. Messenger RNA oxidation is an early event preceding cell death and causes reduced protein expression. FASEB J. 21, 2753-2764.

Sharma, K., Wang, R.X., Zhang, L.Y., Yin, D.L., Luo, X.Y., Solomon, J.C., Jiang, R.F., Markos, K., Davidson, W., Scott, D.W., Shi, Y.F., 2000. Death the Fas way: regulation and pathophysiology of CD95 and its ligand. Pharmacol. Ther. 88, 333-347.

Shi, D.S., Lu, K.H., Gordon, I., 1990. Effects of bulls on fertilization of bovine oocytes and their subsequent development *in vitro*. Theriogenology. 33, 324. (abstract)

Shi, D.S., Lu, K., Gordon, I., Polge, C., 1991. Variation in cleavage and embryonic development of bovine oocytes *in vitro* fertilized with different bull ejaculates. Theriogenology. 35, 271. (abstract)

Shi, Y.L., Bai, J.P., Wang, W.P., 2003. Ion-channels in human sperm membrane and contraceptive mechanisms of male antifertility compounds derived from Chinese traditional medicine. Acta. Pharmacol. Sin. 24, 22-30.

Shimura, T., Inoue, M., Taga, M., Shiraishi, K., Uematsu, N., Takei, N., Yuan, Z.M., Shinohara, T., Niwa, O., 2002. p53-dependent S-phase damage checkpoint and pronuclear cross talk in mouse zygotes with X-irradiated sperm. Mol. Cell. Biol. 22, 2220-2228.

Shitara, H., Hayashi, J.I., Takahama, S., Kaneda, H., Yonekawa, H., 1998. Maternal inheritance of mouse mtDNA in interspecific hybrids, segregation of the leaked paternal mtDNA followed by the prevention of subsequent paternal leakage. Genetics.148, 851-857.

Shitara, H., Kaneda, H., Sato, A., Inoue, K., Ogura, A., Yonekawa, H., Hayashi, J.I., 2000. Selective and continuous elimination of mitochondria microinjected into mouse eggs from spermatids, but not from liver cells, occurs throughout embryogenesis. Genetics.156, 1277-1284.

Silva, P.F., Gadella, B.M., Colenbrander, B., Roelen, B.A., 2007. Exposure of bovine sperm to pro-oxidants impairs the developmental competence of the embryo after the first cleavage. Theriogenology. 67, 609-619.

Sinha Hikim, A.P., Swerdloff, R.S., 1999. Hormonal and genetic control of germ cell apoptosis in the testis. Rev. Reprod. 4, 38-47.

Sinha Hikim, A.P., Lue, Y., Diaz-Romero, M., Yen, P.H., Wang, C., Swerdloff, R.S., 2003. Deciphering the pathways of germ cell apoptosis in the testis. J. Steroid Biochem. Mol. Biol. 85, 175-182.

Sirard, M.A., Richard, F., Blondin, P., Robert, C., 2006. Contribution of the oocyte to embryo quality. Theriogenology. 65, 126-136.

Songsasen, N., Betteridge, K.J., Leibo, S.P., 1997. Birth of live mice resulting from oocytes fertilized *in vitro* with cryopreserved spermatozoa. Biol. Reprod. 56, 143-152.

Soto, P., Natzke, R.P., Hansen, P.J., 2003. Identification of possible mediators of embryonic mortality caused by mastitis, actions of lipopolysaccharide, prostaglandin F2α, and the nitric oxide generator, sodium nitroprusside dihydrate, on oocyte maturation and embryonic development in cattle. Am. J. Reprod. Immunol. 50, 263-272.

Sotolongo, B., Huang, T.T., Isenberger, E., Ward, W.S., 2005. An endogenous nuclease in hamster, mouse, and human spermatozoa cleaves DNA into loop-sized fragments. J. Androl. 26, 272-280.

Staggs, K.L., Austin, K.J., Johnson, G.A., Teixeira, M.G., Talbott, C.T., Dooley, V.A., Hansen, T.R., 1998. Complex induction of bovine uterine proteins by interferon-τ. Biol. Reprod. 59, 293-297.

Streffer, C., 1993. Chromosomal damage in preimplantation mouse embryos and its development through the cell cycle. Mutat. Res. 299, 313-315.

Suarez, S., Pacey, A., 2006. Sperm transport in the female reproductive tract. Hum. Reprod. Updat. 12, 23-37.

Sun, Q.Y., 2003. Cellular and molecular mechanisms leading to cortical reaction and polyspermy block in mammalian eggs. Microsc. Res. Tech. 61, 342-348.

Sutovsky, P., Navara, C.S., Schatten, G., 1996. Fate of the sperm mitochondria, and the incorporation, conversion, and disassembly of the sperm tail structures during bovine fertilization. Biol. Reprod. 55, 1195-205.

Sutovsky, P., Oko, R., Hewitson, L., Schatten, G., 1997. The removal of the sperm perinuclear theca and its association with the bovine oocyte surface during fertilization. Dev. Biol. 188, 75-84.

Sutovsky, P., Moreno, R., Ramalho-Santos, J., Dominko, T., Simerly, C., Schatten, G., 1999. Ubiquitin tag for sperm mitochondria. Nature. 402, 371-372.

Sutovsky, P., Schatten, G., 2000. Paternal contributions to the mammalian zygote, fertilization after sperm-egg fusion. Int. Rev. Cytol. 195, 1-65.

Sutovsky, P., Moreno, R., Ramalho-Santos, J., Dominko, T., Simerly, C., Schatten, G., 2000. Ubiquitinated sperm mitochondria, selective proteolysis and the regulation of mitochondrial inheritance in mammalian embryos. Biol. Reprod. 63, 582-590.

Sutovsky, P., 2003a. Ubiquitin-dependent proteolysis in mammalian spermatogenesis, fertilization, and sperm quality control, killing three birds with one stone. Microsc. Res. Tech. 61, 88-102.

Sutovsky, P., Manandhar, G., Wu, A., Oko, R., 2003b. Interactions of sperm perinuclear theca with the oocyte, implications for oocyte activation, anti-polyspermy defense, and assisted reproduction. Microsc. Res. Tech. 61, 362-378.

Sutovsky, P., McCauley, T.C., Sutovsky, M., Day, B.N., 2003c. Early degradation of paternal mitochondria in domestic pig (*Sus scrofa*) is prevented by selective proteasomal inhibitors lactacystin and MG132. Biol. Reprod. 68, 1793-800.

Swann, K., Saunders, C.M., Rogers, N.T., Lai, F.A., 2006. PLCzeta (zeta), a sperm protein that triggers Ca2+ oscillations and egg activation in mammals. Semin. Cell. Dev. Biol. 17, 264-273.

Swanson, W.F., Magarey, G.M., Herrick, J.R., 2007. Sperm cryopreservation in endangered felids, developing linkage of in situ-ex situ populations. Soc. Reprod. Fertil. Suppl. 65, 417-432.

Tengowski, M.W., Wassler, M.J., Shur, B.D., Schatten, G., 2001. Subcellular localization of beta1,4-galactosyltransferase on bull sperm and its function during sperm-egg interactions. Mol. Reprod. Dev. 58, 236-244.

Tesarik, J., Mendoza, C., Greco, E., 2002. Paternal effects acting during the first cell cycle of human preimplantation development after ICSI. Hum. Reprod.17, 184-189.

Tomlinson, M.J., Moffatt, O., Manicardi, G.C., Bizzaro, D., Afnan, M., Sakkas, D., 2001. Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation, implications for assisted conception. Hum. Reprod. 16, 2160-2165.

Tomsu, M., Sharma, V., Miller, D., 2002. Embryo quality and IVF treatment outcomes may correlate with different sperm comet assay parameters. Hum. Reprod. 17, 1856-1862.

Torner, H., Blottner, S., Kuhla, S., Langhammer, M., Alm, H., Tuchscherer, A., 1999. Influence of chlorocholinechloride-treated wheat on selected *in vitro* fertility parameters in male mice. Reprod. Toxicol. 13, 399-404.

Tremellen, K., 2008. Oxidative stress and male infertility--a clinical perspective. Hum. Reprod. Update.14, 243-258.

Tso, W.W., Lee, C.S., 1928. Gossypol uncoupling of respiratory chain and oxidative phosphorylation in ejaculated boar spermatozoa. Contraception. 25, 649-655.

Ueno, H., Sahni, M.K., Segal, S.J., Koide, S.S., 1988. Interaction of gossypol with sperm macromolecules and enzymes. Contraception. 37, 333-341.

van Gurp, M., Festjens, N., van Loo, G., Saelens, X., Vandenabeele, P., 2003. Mitochondrial intermembrane proteins in cell death. Biochem. Biophys. Res. Commun. 304, 487-497.

Vera, Y., Diaz-Romero, M., Rodriguez, S., Lue, Y., Wang, C., Swerdloff, R.S., Sinha Hikim, A.P., 2004. Mitochondria-dependent pathway is involved in heat-induced male germ cell death, lessons from mutant mice. Biol. Reprod. 70, 1534-1540.

Vera, Y., Rodriguez, S., Castanares, M., Lue, Y., Atienza, V., Wang, C., Swerdloff, R.S., Sinha Hikim, A.P., 2005. Functional role of caspases in heat-induced testicular germ cell apoptosis. Biol. Reprod.72, 516-522.

Vermes, I., Haanen, C., Steffens-Nakken, H., Reutelingsperger, C., 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J. Immunol. Methods. 184, 39-51.

Virro, M.R., Larson-Cook, K.L., Evenson, D.P., 2004. Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in *in vitro* fertilization and intracytoplasmic sperm injection cycles. Fertil. Steril. 81, 1289-1895.

Vishwanath, R., Shannon, P., 1997. Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperature. Reprod. Fertil. Dev. 9, 321-331.

Walters, A.H., Eyestone, W.E., Saacke, R.G., Pearson, R.E., Gwazdauskas, F.C., 2004. Sperm morphology and preparation method affect bovine embryonic development. J. Androl. 25, 554-563.

Walters, A.H., Eyestone, W.E., Saacke, R.G., Pearson, R.E., Gwazdauskas, F.C., 2005a. Bovine embryo development after IVF with spermatozoa having abnormal morphology. Theriogenology. 63, 1925-1937.

Walters, A.H., Saacke, R.G., Pearson, R.E., Gwazdauskas, F.C., 2005b. The incidence of apoptosis after IVF with morphologically abnormal bovine spermatozoa. Theriogenology. 64, 1404-1421.

Walters, A.H., Saacke, R.G., Pearson, R.E., Gwazdauskas, F.C., 2006. Assessment of pronuclear formation following *in vitro* fertilization with bovine spermatozoa obtained after thermal insulation of the testis. Theriogenology. 65, 1016-1028.

Ward, J.F., 1988. DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation, and reparability. Prog. Nucleic Acid Res. Mol. Biol. 35, 95-125.

Ward, W.S., Coffey, D.S., 1991. DNA packaging and organization in mammalian spermatozoa, comparison with somatic cells. Biol. Reprod. 44, 569-574.

Ward, W.S., 1993. Deoxyribonucleic acid loop domain tertiary structure in mammalian spermatozoa. Biol. Reprod. 48, 1193-1201.

Ward, W.S., Kimura, Y., Yanagimachi, R., 1999. An intact sperm nuclear matrix may be necessary for the mouse paternal genome to participate in embryonic development. Biol. Reprod. 60, 702-706.

Ward, F., Rizos, D., Corridan, D., Quinn, K., Boland, M., Lonergan, P., 2001. Paternal influence on the time of first embryonic cleavage post insemination and the implications for subsequent bovine embryo development *in vitro* and fertility *in vivo*. Mol. Reprod. Dev. 60, 47-55.

Waring, P., Müllbacher, A., 1999. Cell death induced by the Fas/Fas ligand pathway and its role in pathology. Immunol. Cell Biol. 77, 312-317.

Warner, C.M., McElhinny, A.S., Wu, L., Cieluch, C., Ke, X., Cao, W., Tang, C., Exley, G.E., 1998. Role of the Ped gene and apoptosis genes in control of preimplantation development. J. Assist. Reprod. Genet. 15, 331-331.

Watson, P.F., Duncan, A.E., 1988. Effect of salt concentration and unfrozen water fraction on the viability of slowly frozen ram spermatozoa. Cryobiology. 25, 131-142.

Waterhouse, K.E., Haugan, T., Kommisrud, E., Tverdal, A., Flatberg, G., Farstad, W., Evenson, D.P., De Angelis, P.M., 2006. Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. Reprod. Fertil. Dev. 18, 781-788.

Weng, S.L., Taylor, S.L., Morshedi, M., Schuffner, A., Duran, E.H., Beebe, S., Oehninger, S., 2002. Caspase activity and apoptotic markers in ejaculated human sperm. Mol. Hum. Reprod. 8, 984-991.

Wildt, D.E., 2000. Genome resource banking for wildlife research, management, and conservation. ILAR J. 41, 228-234.

Wu, G.J., Chang, F.W., Lee, S.S., Cheng, Y.Y., Chen, C.H., Chen, I.C., 2008. Apoptosis-related phenotype of ejaculated spermatozoa in patients with varicocele. Fertil. Steril. (in press).

Yamauchi, Y., Shaman, J.A., Boaz, S.M., Ward, W.S., 2007. Paternal pronuclear DNA degradation is functionally linked to DNA replication in mouse oocytes. Biol. Reprod. 77, 407-415.

Yanagimachi, R., Noda, Y.D., 1970. Physiological changes in the postnuclear cap region of mammalian spermatozoa, a necessary preliminary to the membrane fusion between sperm and egg cells. J. Ultrastruct. Res. 31, 486-493.

Yildiz, C., Ottaviani, P., Law, N., Ayearst, R., Liu, L., McKerlie, C., 2007. Effects of cryopreservation on sperm quality, nuclear DNA integrity, *in vitro* fertilization, and *in vitro* embryo development in the mouse. Reproduction. 133, 585-595.

Yildiz, C., Fleming, C., Ottaviani, P., McKerlie, C., 2008. Fresh and frozen-thawed sperm quality, nuclear DNA integrity, invitro fertility, embryo development, and live-born offspring of N-ethyl-N-nitrosourea (ENU) mice. Cryobiology. (in press).

Yoneda, A., Kashima, M., Yoshida, S., Terada, K., Nakagawa, S., Sakamoto, A., Hayakawa, K., Suzuki, K., Ueda, J., Watanabe, T., 2006. Molecular cloning, testicular postnatal expression, and oocyte-activating potential of porcine phospholipase Czeta. Reproduction. 132, 393-401.

Yoon, S.Y., Fissore, R.A., 2007. Release of phospholipase Cζ and $[Ca^{2+}]_i$ oscillation-inducing activity during mammalian fertilization. Reproduction. 134, 695-704.

Young, C., Grasa, P., Coward, K., Davis, L.C., Parrington, J., 2008. Phospholipase C zeta undergoes dynamic changes in its pattern of localization in sperm during capacitation and the acrosome reaction. Fertil. Steril. (in press).

Yuan, Y.Y., Shi, Q.X., 2000. Inhibition of hamster sperm acrosomal enzyme by gossypol is closely associated with the decrease in fertilization capacity. Contraception. 62, 203-209.

Yuan, Y.Y., Shi, Q.X., Srivastava, P.N., 1995. Inhibition of rabbit sperm acrosomal enzymes by gossypol. Mol. Reprod. Dev. 40, 228-232.

Zalensky, A.O., Tomilin, N.V., Zalenskaya, I.A., Teplitz, R.L., Bradbury, E.M., 1997. Telomere-telomere interactions and candidate telomere binding protein(s) in mammalian sperm cells. Exp. Cell. Res. 232, 29-41.

Zamzami, N., Kroemer, G., 2001. The mitochondrion in apoptosis, how Pandora's box opens. Nat. Rev. Mol. Cell. Biol. 2, 67-71.

Zenzes, M.T., Belkien, L., Bordt, J., Kan, I., Schneider, H.P., Nieschlag, E,. 1985. Cytologic investigation of human *in vitro* fertilization failures. Fertil. Steril. 43, 883-891.

Zhang, Y., Mattjus, P., Schmid, P.C., Dong, Z., Zhong, S., Ma, W.Y., Brown, R.E., Bode, A.M., Schmid, H.H., 2001. Involvement of the acid sphingomyelinase pathway in uva-induced apoptosis. J. Biol. Chem. 276, 11775-11782.

Zhu, J., Barrat, C.L., Lippes, J., Pacey, A.A., Cooke, I.D., 1994. The sequential effects of human cervical mucus, oviductal fluid, and follicular fluid on sperm function. Fertil. Steril.61,1129-1135.

BIOGRAPHICAL SKETCH

Katherine Elizabeth May Hendricks was born in 1972, the first of two daughters, to Elsa May Binns and Lloyd Ivanhoe Binns, in St. Andrew, Jamaica. In 2002, she received her degree in veterinary medicine from the University of the West Indies, St. Augustine campus, located in the Republic of Trinidad and Tobago. In January 2003 she started her master's program in the College of Veterinary Medicine at the University of Florida under the supervision of Dr. Louis Archbald. Her master's research focused on reproductive strategies in the post partum cow with emphasis on anovulation and postpartum uterine health. In August 2004 Katherine was awarded a University of Florida Graduate Alumni Fellowship and began working on a Doctor of Philosophy degree in the Animal Molecular and Cell Biology Graduate Program under the supervision of Dr. Peter J. Hansen. After completion of her program, Katherine will continue her career in academia.