Attempts to promote the use of cryopreserved bovine semen: Effect of prostaglandin F2-alpha, sucrose and short-term dry ice storage

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D7

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DEDICATION

To

My children Rahma, Fatima, Hajara and Ahmad for being a source of inspiration to me

My wife Asma'u for your understanding, loyalty, support and prayers during my long absence from home

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LIST OF ABBREVIATIONS

µl	Microlitre
AI	Artificial insemination
AV	Artificial vagina
CASA	Computer-assisted semen analysis
cm	Centimetre
EE	Electro-ejaculation
GLM	General linear model
h	Hour
LDL	Low-density lipoprotein
LN_2	Liquid nitrogen
min	Minute
ml	Millilitre
mM	Millimolar
^oC	degrees Celsius
$PGF_{2\alpha}$/ $PGF_2\alpha$/ PGF2-alpha	Prostaglandin F2-alpha
RM	Transrectal massage
s	Second
TRIS/ Tris	Tris (hydroxymethyl) aminomethane
TV	Television
v/v	Volume by volume
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity
ZP	Zona pellucida

SUMMARY

This dissertation argues that improvement of conception to artificial insemination (AI) through approaches that enhance post-thaw quality of bull semen might sustain enthusiasm for this technology and present a greater chance of capturing superior genetics from proven AI sires; thus, promoting the use of cryopreserved bovine semen. The general objective of this work is to determine the efficacy of some strategies aimed at improving post-thaw motility of bull spermatozoa. The general objective was, therefore, divided into three specific objectives:

1. Investigate the effect of PGF2-alpha addition after thawing on viability of bovine spermatozoa. In that context, extenders with or without egg yolk were compared to establish extender most suitable for PGF2-alpha supplementation.

2. Determine the effect of incorporation of sucrose alone or in combination with glycerol into customized (Tris-egg yolk) and two commercial (Steridyl® and AndroMed®) extenders on post-thaw motility of bovine spermatozoa.

3. Assess the effect of temporary storage of bovine semen originally stored in liquid nitrogen on dry ice and of refreezing of thawed semen on spermatozoon motility.

The above-mentioned specific objectives were analysed in Chapters 2 to 4. Chapter 2 is entitled, "*In vitro* effect of type of extender and addition of prostaglandin F2-alpha post-thawing on the motility of bovine spermatozoa" and it addresses the first specific objective. Results of Experiment 1 show that percent total motility decreased significantly ($p<0.05$) as bull semen passed through different stages of cryopreservation from extension (65 ± 1%) via equilibration (60 ± 1%) to 15 min (49 ± 3%) and 7 days (46 ± 2%) of immersion in liquid nitrogen (LN_2). The

difference between storage times (15 min or 7 days) in LN_2 was not significant. Post-thaw motility in AndroMed® extender was significantly ($p<0.05$) higher (59 ± 2%) than in both Triladyl™ and Tris-egg yolk-glycerol extenders (both 53 ± 2%). No effect of individual bull was observed ($p>0.05$). In Experiment 2, results revealed that there was no significant ($p>0.05$) effect of PGF2-alpha concentration on spermatozoon motility. However, a slight but significant ($p<0.05$) effect of individual bull was recorded. In conclusion, frozen-thawed bull spermatozoa were capable of tolerating PGF2-alpha up to a concentration of 30% (v/v) in AndroMed® without adverse effect on total motility. Further studies should be attempted to test the effect of prostaglandin F2-alpha added to semen after thawing on success rates at insemination.

Chapter 3 is concerned with the second specific objective and it is captioned "Sugar supplementation in customized and commercial extenders: The use of sucrose solely and in combination with glycerol". Results show that relative to Tris-egg yolk containing 6.8% glycerol without sucrose (control), semen in same extender containing 150 mM sucrose and 3.4% glycerol had a relative motility of 68 (SE 3) % which decreased to 52 (SE 3) % in extender with 300 mM sucrose and devoid of glycerol. In semen diluted in Steridyl® with 150 mM or 300 mM sucrose, relative motility was significantly decreased to 67 (SE 3) % in the former and 31 (SE 4) % in the latter. In AndroMed® extender with 150 mM sucrose, motility had significantly decreased to 70 (SE 5) % and with 300 mM sucrose to 42 (SE 6) %. Tris-egg yolk extender containing 150 mM sucrose with 3.4% glycerol proved to be better than the same extender containing 300 mM sucrose without glycerol. Motility in Steridyl® and AndroMed® extenders containing 150 mM sucrose was better than that of the same extenders with 300 mM sucrose. However, motility in Tris-egg yolk extender containing 300 mM sucrose without glycerol was better than that of commercial extenders containing glycerol in combination with 300 mM

sucrose. Efforts were made to employ an extender devoid of glycerol but it was observed that a small amount of sucrose in combination with glycerol proved to be advantageous. Complete replacement of glycerol with sucrose in the customized Tris-egg yolk extender retained some motility though not as good as same extender with 150 mM sucrose and 3.4% glycerol. It, therefore, appears that sucrose could be used in semen extenders in order to reduce, partly or completely, the amount of glycerol added to such extenders; thus, ameliorating the toxicity of glycerol when used in high concentrations.

Chapter 4 deals with the last specific objective and is entitled "Temporary storage on dry ice of bovine semen originally stored in liquid nitrogen and the effect of refreezing". Results show that neither a 1h nor a 6h sojourn on dry ice affected spermatozoon motility regardless whether semen was thawed immediately or after being returned to LN_2 ($p>0.05$). Intensity of progressive motility was virtually unimpaired by the respective treatments. Post treatment motility rates were reduced by a factor of 10 as compared to semen not subjected to refreezing. No significant difference in percent post-thaw motility after refreezing was observed between semen samples that had been transiently stored on dry ice for 0, 1 or 6 hours ($p>0.05$). Semen that underwent refreezing on dry ice rather than in LN_2 vapour before being returned to LN_2 exhibited a significantly higher post-thaw motility rate ($p<0.05$). Although the proportion of motile spermatozoa was drastically reduced, the intensity of progressive forward motion was satisfactory. Temporary dry ice storage appears to have no adverse effect on percent motility of bovine spermatozoa; thus, its use as a viable option for transport of frozen semen should be explored provided *in vivo* fertility is proven.

Chapter 1

Introduction and literature review

Chapter 1 - Introduction and literature review

1.1. Preamble

It is no gainsaying that the most frequently utilized reproductive technologies in cattle worldwide are artificial insemination (AI) and embryo transfer (ET) (Dalton, 1999). According to Peel and Bauman (1987), semen selection and AI can result in milk production increases of approximately 100 kg/yr. The dairy industry is where technical advancement in AI and semen technology has been captured most successfully (Vishwanath and Shannon, 2000). Although AI offers the potential to choose sires that will supply proven genetics, few beef producers have embraced the technology (Jaeger, 2005). AI accounts for less than 5% of the replacement animals in the world beef cattle population (Vishwanath, 2003). This, however, does not imply that AI in the dairy cattle population is free from its own peculiar problems. First service conception rates, for example, rarely exceed 50% in lactating cows (Dalton, 1999). Although many personnel factors contribute to low conception rates, including heat detection accuracy, semen handling, site of semen deposition, and time of insemination, many producers have lost enthusiasm for AI (Chupin and Thibier, 1995). Furthermore, as herd sizes continue to increase without new employees being hired, many producers have erroneously chosen to save time and money in labour costs and use herd bulls (Dalton, 1999). Irrespective of the reasons advanced against AI, failure to use it results in loss of superior genetics. Therefore, if conception to AI could be improved through strategies that enhance post-thaw quality of semen, enthusiasm for this technology might be sustained and a greater chance of capturing superior genetics from proven AI sires could be achieved. The resultant effect, in this case, being a promotion of the use of cryopreserved semen.

1.2. Factors influencing semen production and quality

Thibier and Wagner (2002), in their classical work on world statistics for artificial insemination in cattle, concluded that the artificial insemination industry seems very active with 2.5 times more doses processed than utilized for insemination which implies that

production of frozen bull semen far outweighs its utilization for artificial insemination (AI) in cows. Therefore, as artificial insemination becomes increasingly utilized, opportunities for substantial increases in the production and use of frozen semen will emerge. To supply this demand, the AI industry must optimize the factors that affect sperm production and semen quality. Semen production, being a quantitative trait, is affected by genetic as well as non-genetic factors (Chauhan et al., 2010). Some of these factors are discussed below.

1.2.1. Season/ Environment

Semen production is affected by season (Ibrahim et al., 1983, Schwab et al., 1987, Graffer et al., 1988; Stalhåmmar et al., 1989; Chauhan et al., 2010). In the tropics, sperm production and semen quality decrease during the hot season (Igboeli and Rakha, 1971; Fields et al., 1979; Kumi-Diaka et al., 1981; Rekwot et al., 1987), however, seasonal variations cannot be attributed only to greater ambient temperature (Brito et al., 2002). Seasonal effects are not only the result of various factors such as temperature, humidity, length of day, feed composition but also management (Fuerst-Waltl et al., 2006). Consequently, significant effects of season on semen production were partly contradictory (Fuerst-Waltl et al., 2006). While Stalhåmmer et al. (1989) observed the highest sperm concentration and total number of spermatozoa during summer, Mathevon et al. (1998) found higher values during winter and spring. However, Brito et al. (2002) failed to detect any effect of season on semen production. Optimal ambient temperature for semen production was found to be approximately 15-20 ^{o}C (Taylor et al., 1985; Parkinson, 1987). It is believed that not only the temperature recorded on the day of semen collection affects sperm production, but also the temperature during the entire spermatogenesis period until 70 days before collection (Igna et al., 2010).

1.2.2. Age of bull

Age of bull affects ejaculate characteristics such as semen volume and sperm concentration (Mathevon et al., 1998; Fuerst-Waltl et al., 2006). Generally, semen production and quality were reported to increase with age of bull (Stalhåmmar et al., 1989; Mathevon et al., 1998; Brito et al., 2002). However, management and health effects may bias results for age effects (Fuerst-Waltl et al., 2006). Some older bulls may benefit from preferential treatment while a possible unfavourable state of health may decrease semen quantity and quality in others. A better knowledge of the influence of age of the bull at collection, season of collection, and frequency of collection on semen production will help the AI industry to adapt management of bulls to improve semen output (Mathevon et al., 1998).

1.2.3. Management

Varying the quality of feed may affect semen quality up to several weeks (Peter, 1991). Bull handler and semen collector are also essential for adequate semen production and quality by being responsible for proper sexual preparation (Fuerst-Waltl et al., 2006). Mathevon et al. (1998) observed a significant effect of the collection team on ejaculate volume and total number of spermatozoa while no significant effect was found on concentration and motility. Large effects of collection interval were reported by Everett and Bean (1982) and Mathevon et al. (1998). The same was recorded for frequency of collection. First ejaculates resulted in significantly higher ejaculate volumes, sperm concentration and total number of spermatozoa (Everett et al., 1978; Everett and Bean, 1982) while motility was less affected. Similarly, weekday may influence semen production as observed by Everett and Bean (1982) and Taylor et al. (1985). Shorter intervals between collections reduce the number of sperm produced per ejaculate (Everett and Bean, 1982; Schwab et al., 1987), but increase the amount of semen produced per unit of time (Mathevon et al., 1998).

1.2.4. Genetics

Semen quality, like other phenotypic expressions, no doubt consists of a genetic component and environmental component and a variety of interactions between the two (Foote, 1978). The genetic component is generally thought to be small because the heritability of fertility usually is low (Rollinson, 1955; Foote, 1970). Levels of exotic inheritance and breed components of crossbred bulls have very important consequences on semen production (Chauhan et al., 2010). *Bos indicus* breeds are better adapted to the tropics and are more resistant to heat stress than are *B. taurus* breeds developed in temperate climates because they usually have a smaller frame, greater skin surface to body size ratio, more sweat glands and lower thermogenesis (Turner, 1980). Godfrey et al. (1990) reported that in a temperate climate, *B. indicus* (but not *B. taurus*) bulls suffered from cold stress that was reflected in decreased sperm production and semen quality during the winter. In the tropics and semi-tropics, sperm production and semen quality decreased during the hot season only in *B. taurus* and crossbred bulls, but *B. indicus* bulls were not affected (Fields et al., 1979; Kumi-Diaka et al., 1981). However, some authors showed that in Africa, sperm production (ejaculate volume, sperm concentration and total sperm number) and percentage of normal sperm cells decreased during the hot season in *B. indicus* bulls (Igboeli and Rakha, 1971; Rekwot et al., 1987). Knowledge of heritability of semen volume, sperm concentration and total number of sperm per ejaculate indicates the importance of genetics in bull semen production (Mathevon et al., 1998). Repeatabilities of these traits indicate the possibility of predicting future semen production of bulls in relation to preselection of bulls for progeny testing based on semen production characteristics (Mathevon et al., 1998).

1.3. Factors affecting the survival of cryopreserved spermatozoa

The production potential of livestock can be increased by genetic improvement using one of the modern ways of breed improvement, e.g., artificial insemination (AI) (Andrabi,

2009). Moreover, the quality of frozen-thawed semen is one of the most influential factors affecting the likelihood of conception (Saacke, 1984) which is the ultimate confirmatory test of the successful outcome of artificial insemination. Cryopreservation is a non-physiological method that involves a high level of adaptation of biological cells to the osmotic and thermal shocks that occur both during the dilution, cooling-freezing and during the thawing procedures (Watson et al., 1992; Holt, 2000a, b). Cryopreservation of semen has long been seen as a means of benefitting the breeding of animals of agricultural importance, and has been recognized as contributing to the conservation of endangered species and to overcoming aspects of male infertility in humans (Watson, 2000). At each stage of the cryopreservation cycle, which includes the entire process of semen collection, dilution, equilibration and freezing, the spermatozoa may lose the ability to fertilize normally (Watson, 1995). It is therefore mandatory to optimize a cryopreservation cycle, which would cryopreserve the largest number of structurally and functionally normal spermatozoa (Sundararaman and Edwin, 2008). Hence, successful cryopreservation of livestock semen would aid in the provision of long-term storage of male gametes and the maintenance of genetic stock that could improve milk and meat production as well as their associated economic gains.

The first step in the creation of a cryopreserved semen bank is the use of an effective method of semen recovery or ejaculate collection (Marco-Jiménez et al., 2005; Jiménez-Rabadán et al., 2012). In addition to an effective method of semen collection, the survival of spermatozoa in frozen-thawed semen is affected by other factors, such as male, extenders, cryoprotectants, packaging and storage, freezing and thawing rates, as well as the quality of the semen used for freezing (Pace et al., 1981; Tuli et al., 1981; Abdelhakeam et al., 1991; Karabinus et al., 1991; Thomas et al., 1993; Park et al., 1995; Heitland et al., 1996; Eriksson and Rodriguez-Martinez, 2000; Watson, 2000; Aires et al., 2003; Sieme et al., 2004; Dorado et al., 2007; Muiño et al., 2008; Andrabi, 2009).

1.3.1. Semen collection

Ideally semen collection method employed should be repeatable, reliable, and, preferably, non-invasive but should not influence sperm characteristics or sperm function (Morrell et al., 1996). Semen can be collected from live animals by artificial vagina (AV) (Terrill, 1940; Schaffer et al., 1990; Deen et al., 2003; Sieme et al., 2004; Marco-Jiménez et al., 2005; Jiménez-Rabadán et al., 2012), electro-ejaculation (EE) (Terril, 1940; Asher et al., 1993; Marco-Jiménez et al., 2005; Palmer et al., 2005; Okano et al., 2006; Jiménez-Rabadán et al., 2012), collection from vagina after service or copulation (Terril, 1940; Morrell et al., 1996), and by transrectal massage (RM) (Schaffer et al., 1990; Palmer et al., 2005). Quinn et al. (1968) suggested that spermatozoa in semen collected with an AV were more resistant to cold shock than when ejaculated electrically. Marco-Jiménez et al. (2005) reported a higher number of stable and functional spermatozoa in frozen-thawed spermatozoa from semen collected by EE when compared with semen collected by AV. EE is known to alter semen characteristics (Quinn and White, 1966; Lightfoot, 1968), diminish semen quality (Brady and Gildow, 1939; Terrill, 1940; Mattner and Voglmayr, 1962) and is worse for predicting fertility (Hulet et al., 1964). Flaws in semen collection method have been shown when electrical stimulation was used (Dziuk et al., 1954). EE could affect the ejaculation reflex as a consequence of the aggressiveness of these techniques, stress management or insufficient stimulation in rectal mucosae (Dziuk et al., 1954). Palmer et al. (2004) demonstrated that semen samples obtained by RM had a lower percentage of motile and live sperm compared to samples obtained by EE; interpreted to be a function of lack of thermal control (chilling) in the collection vessel (Palmer et al., 2005). Sperm morphology was not affected by both EE and RM, but percent of motile sperm and live sperm were lower in semen samples collected by RM (Palmer et al., 2005). For domestic males, the AV procedure is the preferred method

of semen collection (Leboeuf et al., 2000), but this technique requires a previous training period (Wulster-Raddiffe et al., 2001).

1.3.2. Extenders

In normal, freshly ejaculated bull semen, the concentration of spermatozoa is high. Therefore, dilution (or extension) is necessary to provide a convenient inseminate volume that will contain enough cells to ensure maximum fertilization rates without wasting spermatozoa (Eljarah, 2007). Semen extenders (or diluents) were developed in order to provide an acceptable buffering capacity, osmolality, energy in the form of metabolizable substrates, and to minimize bacterial growth and to provide protection due to decreases in temperature (Phillips, 1939; Phillips and Lardy, 1940; Salisbury et al., 1941; Foote and Berndtson, 1976; Salisbury et al., 1978; Shannon, 1978; Vishwanath et al., 1996; Vishwanath and Shannon, 1997). The composition of the extender in which semen is diluted before freezing is one of the main factors that influence the success of cryopreservation (De Leeuw et al., 1993; Dhami et al., 1994; Woelders et al., 1997). Karabinus et al. (1991) demonstrated the existence of differential extender effects on post-thaw bull semen quality and indicated that altering extender composition or sequence of addition of extender components may improve post-thaw quality of cryopreserved sperm.

Aires et al. (2003) reported significantly lower post-thaw sperm motility when bull semen was extended in TRIS egg-yolk diluent than when extended in Soya lecithin diluent. Field trials revealed that non-return rates of Soya lecithin-extended semen showed significantly higher insemination success compared with non-return rates for TRIS egg-yolk extender (Aires et al., 2003). It was found that TRIS-based extender gave better *in vitro* performance in cryopreserved goat semen compared to milk-based extender, though these improvements were not noticed in fertility results (Dorado et al., 2007). Comparison of the resultant fertility showed inconsistent differences between the two extenders, which indicates

that either TRIS or milk extender can be used for goat semen cryopreservation for subsequent use in cervical insemination (Dorado et al., 2007).

When captive Japanese black bear spermatozoa were frozen in egg yolk-TRIS-citrate-glucose, egg yolk-TRIS-citrate-fructose, and egg yolk-TRIS-citrate-fructose-lactose-raffinose extenders, none of the parameters (i.e., % motility, % viability, % abnormal morphology and % intact acrosomes) examined after thawing were statistically different among the three (Okano et al., 2006). TRIS provided enhanced cryoprotection of bull spermatozoa than Bioxcell®, resulting in greater motility and integrity of sperm membranes, and when combined with 4 h equilibration, resulted in the greatest quality of frozen-thawed semen (Leite et al., 2010). For cryopreservation of electroejaculated bison semen, Triladyl® yielded slightly greater but statistically significant motilities immediately after thawing while TRIS-citric acid enhanced the ability of bison sperm to sustain motility and structural characteristics during post-thaw incubation to a greater extent (Hussain et al., 2011). Goat semen freezing with Biladyl® and AndroMed® resulted in a greater sperm quality after thawing in relation to milk-based extender (Jiménez-Rabadán et al., 2012). Bull semen extended in AndroMed® and Bioxcell® exhibited a superior progressive motility when compared to semen in Triladyl® (Janett et al., 2005). However, bull sperm viability was significantly better in semen extended in Triladyl® compared to semen in AndroMed® or Bioxcell® (Janett et al., 2005).

1.3.3. Cryoprotectants

The need for control of ice crystal formation during freezing is of prime importance (Mazur, 1980). Many compounds have been tested for their efficacy as sperm cryoprotectants (Jeyendran and Graham, 1980; Molinia et al., 1994a); but most semen preservation protocols still favour glycerol in the cryoprotective media (Holt, 2000b), following the example set by Polge et al. (1949). Glycerol reduces thermal stress and prevents fracture in the frozen

solutions by reducing the total ice volume expansion during water solidification (Gao et al., 1995). Furthermore, it acts through salt-buffering mechanism (Rasul, 2000), binds with metallic ions (Lohmann et al., 1964) and dehydrates the cell while preserving the extracellular media (Meryman, 1971). In certain instances other cryoprotectants are possibly better than glycerol; for example, dimethyl sulphoxide (DMSO) was preferred for elephant spermatozoa (Jones, 1973). Glycerol has been used almost universally as the cryoprotectant for stallion semen (Sieme et al., 2008), although it has been reported that other cryoprotectants such as DMSO, ethylene glycol, methyl formamide or dimethyl formamide may yield similar or superior results (Squires et al., 2004; Alvarenga et al., 2005). Mantovani et al. (2002) reported that ethylene glycol could substitute glycerol as cryoprotectant, if used at the same or lower concentration when stallion semen is diluted in skimmed milk extender. Bull spermatozoa exhibited higher percentages for total and progressive motilities when frozen in extender containing 3% glycerol compared to 3, 2 and 1% ethylene glycol or 3, 2 and 1% methanol (Awad, 2011). The choice of cryoprotectant seems to have been a matter of trial and error in nearly all investigations; this is partly because a complete and satisfactory explanation for the action of cryoprotectants does not exist (Holt, 2000).

Cryoprotectant compounds can be roughly classified into groups, with differing modes of action (Holt, 2000b). Glycerol, together with substances such as methanol, ethylene glycol, 1, 2-propanediol, butanediol, acetamide and DMSO, belong to a group which permeate into the cellular cytoplasm (penetrating or permeating cryoprotectants). Besides glycerol and other penetrating cryoprotectants, sugars such as sucrose, raffinose, trehalose and lactose, polymers such as polyvinyl pyrrolidone (PVP) and the amphipathic compounds glycine betaine, glutamine and proline have been identified as potentially cryoprotective (Lahnsteiner et al., 1996; Holt, 2000b; Sztein et al., 2001, Sànchez et al., 2011; Kumar et al., 2012). Raffinose has been used, with or without glycerol, for the preservation of mouse

spermatozoa; 11% lactose in combination with glycerol has been found useful in combination with pellet freezing methods, where it has been used for carnivore (e.g., ferret, Howard et al., 1991; Giant panda, Moore et al., 1984) as well as for ram and boar spermatozoa (Salamon and Lightfoot, 1969; Wilmut and Polge, 1977).

Supplementation of extenders with sugars, such as sucrose, trehalose or raffinose, tends to protect the sperm cells against freeze-damage (Jafaroghli et al., 2011). These sugars, as non-permeating cryoprotectants, are not able to diffuse across the plasma membrane, but create an osmotic pressure that induces cell dehydration before freezing, thus decreasing the extent of cell injury by intracellular ice formation (Molinia et al., 1994b; Liu et al., 1998). Experimentally, the amphipathic substances glycine betaine, glutamine and proline have only proved effective in the presence of glycerol and egg yolk when tested with ram and stallion spermatozoa (Koskinen et al., 1989; Sanchez-Partida et al., 1992). In addition to these various cryoprotective compounds, egg yolk is routinely included in cryopreservation protocols for semen from domestic animals and many exotic species (Holt, 2000b). Egg yolk is regarded as protecting against cold-shock, a lipid-phase transition effect (Drobnis et al., 1993). Given current needs for disease control and therefore the avoidance of biologically derived substances in cryoprotective media, there is a pressing requirement to find an egg yolk substitute (Holt, 2000b).

1.3.4. Packaging, storage, freezing and thawing rates

To maximally utilize the genetics of desired sires on a commercial basis, attempts are made to package a minimal number of spermatozoa per insemination unit without sacrificing fertility (Foote and Parks, 1993; Shannon and Vishwanath, 1995). The surface-to-volume ratio of the package in which semen is frozen has important implications for cooling, freezing and thawing rates of the semen (Maxwell et al., 1995). Semen packaging is also important for practical reasons since it determines both the means of identification of each dose of semen

and how it may be arranged for storage in the liquid nitrogen container (Maxwell et al., 1995).

Several methods are available for the packaging of spermatozoa for freezing in different species (Lemma, 2011). They include glass ampoules or vials, polypropylene, polyvinyl or plastic round or flat straws (usually 0.5 – 1.0 ml in volume), flat aluminum packets (10 – 15 ml); pellets (0.1 – 0.2 ml), and microtubes (Park et al., 1995; Heitland et al., 1996). Both ampoules and straws are traditionally frozen by suspension over liquid nitrogen, followed by plunging into liquid nitrogen at -196 ^{o}C. Although pellets have the advantage of allowing a rapid drop in temperature to be achieved, they are not suited for easy identification after freezing (Lemma, 2011). In addition, the reuse of the carbon dioxide block or metal plate carries the potential risk of cross-contamination with semen from the previous freezing batch. On the other hand, the use of vials or straws readily allows the accurate identification of samples and considerably reduces the risk of cross-contamination during cryopreservation. The geometric configuration of a straw as a unit for the cryopreservation of bovine spermatozoa provides flexibility in freezing and thawing procedures (Pace et al., 1981). Freezing bovine semen in plastic straws resulted in improved storage efficiency with recovery of more live spermatozoa and higher maintenance of the acrosome following freeze-thaw process when compared to 1.0 ml glass ampoules (Pickett and Berndtson, 1974; Senger et al., 1976).

Since different methods of storage of semen abound, the need to know whether means of storage has any bearing on the successful outcome of cryopreservation has been raised. This is confirmed by studies that compared spermatozoa stored in different packages (Park et al., 1995; Heitland et al., 1996). Results from these studies showed an effect on spermatozoa quality due to reduced motility and conception rate. The role of different extenders used, the interaction between extender, and means of packaging were also implicated. On the other

hand, Clulow et al. (2008) reported that stallion spermatozoa frozen at a low concentration (40 x 10^6 spermatozoa/ ml) in 0.25 ml straws gave no negative effect on sperm motility, morphology or acrosome integrity. Thomas et al. (1993) showed that Tris-Citrate extender either in pellets or 0.5 ml straws was the most efficacious combination for canine semen when extender x packaging interaction was examined.

When the effect of straws alone was analyzed, it was verified that the use of the 0.5 ml straw promoted better conservation of goat semen than the 0.25 ml straw in terms of progressive motility and acrosomal integrity after freeze-thawing procedures (Bezerra et al., 2012). Battista et al. (1988) found that Tris extender in 0.5 ml straws yielded better post-thaw motility in canine semen than unbuffered lactose extender in pellet form. They also found that lactose in pellet form was better than lactose in straws. El-Bahrawy (2010) reported significant decrease in post-thaw motility of dromedary spermatozoa in semen cryopreserved as pellets (20.8%) compared to straws (43.3%). Also, sperm survival rates were 68.7% and 33.1% for both French straws and pellets post-cryopreservation, respectively (El-Bahrawy, 2010).

Freezing and thawing rates have been shown to influence the post-thaw survival of a number of cells (Mazur, 1985). This also applies to spermatozoa from various species, although the range for optimum cooling rates (10 – 100 ^oC/ min) of semen of domestic animals is generally considered to be wide (Watson, 1990). The FlatPack gave better post-thaw motility of boar spermatozoa than the Maxi-straw, probably by allowing a quicker rate of thaw (Eriksson and Rodriguez-Martinez, 2000). Freezing and thawing rates, extenders, packages and boars affected post-thaw sperm survival with thawing rate having the greatest influence on post-thaw survival followed by boar and freezing rate (Eriksson and Rodriguez-Martinez, 2000). Also, neither freezing rate nor thawing rate had any effect on plasma membrane integrity of boar spermatozoa (Eriksson and Rodriguez-Martinez, 2000). Higher

post-thaw motility was obtained when bovine spermatozoa were frozen in straws placed horizontally as opposed to vertically (Rodriguez et al., 1975). The post-thaw motility of bovine spermatozoa frozen rapidly in straws and thawed at 55 to 90 ^{o}C exceeded that for ampoules from split-ejaculates frozen in 1.0 ml ampoules (Rodriguez et al., 1975). Thawing bovine semen by immersing 0.25 ml straws in 75 ^{o}C water was superior to thawing in iced water or palm thawing, but there was no difference between the latter two methods (Ennen et al., 1976).

Senger et al. (1976) reported a thaw rate x post-thaw treatment interaction for percent intact acrosomes and percent sperm motility in bovine semen. There was a significant ejaculate x thaw rate interaction for bull sperm motility and percentage intact acrosomes (Olar et al., 1977). Overall means for percentage of motile bull spermatozoa were greater following thaws above 35 ^{o}C when compared to 5 ^{o}C and 20 ^{o}C thaws (Olar et al., 1977). Research has shown that faster thawing rates result in greater survival of spermatozoa frozen in straws (Pace et al., 1981). Söderquist et al. (1997) compared thawing of ram semen frozen in mini straws at 70, 50, and 35 ^{o}C and found that the post-thaw sperm motility, as well as the percentage of spermatozoa were significantly higher in straws thawed at 70 ^{o}C compared to at 35 ^{o}C. Söderquist et al. (1999) observed that thawing of mini straws at 50 ^{o}C for 9 s, instead of 70 ^{o}C for 5 s, did not seem to further affect fertility or litter size. Paulenz et al. (2004) reported that AI in sheep using minitubes resulted in the highest overall lambing rates and was superior to mini straws independent of thawing procedure used.

1.4. Strategies for improving post-thaw semen quality

The application of frozen-thawed semen technology is currently increasing worldwide (Lemma, 2011). The duration of motility and other sperm characteristics during post-thawing incubation is an indication of usability of the semen (Bag et al., 2002). Several studies have focused on exploring different approaches to improve the post-thaw quality of semen from

domestic, wild and endangered animals. These approaches include sperm washing to remove seminal plasma (Garcia and Graham, 1987; Tibary et al., 1990; Graham, 1994; Tuli and Holtz, 1994; Aurich et al., 1996; Brinsco et al., 2000; Gil et al., 2000; Moore et al., 2005; Fraser et al., 2007; El-Bahrawy, 2010; Naing et al., 2011; Natali, 2011; Jiménez-Rabadán et al., 2012a); sperm selection to isolate live and motile spermatozoa from semen (Bangham and Hancock, 1955; Maki-Laurila and Graham, 1968, Arcidiacono et al., 1983; Lessley and Garner, 1983; White et al., 1984; Parrish and Foote, 1987; Anzar and Graham, 1995; Garcia-López et al., 1996; Morrel and Rodriguez-Martinez, 2011; Natali, 2011; Jiménez-Rabadán et al., 2012b); the use of zwitterion (ZI) extenders to provide good buffering (Tuli and Holtz, 1992; Molinia et al., 1994c; Rasul et al., 2000; Garde et al., 2003; Hussain et al., 2013); adding glycerol at lower temperature to reduce its toxic effect (Hussain et al., 2013); adding antioxidants to control oxidative stress (Zahariev et al., 2007; Anghel and Zamfirescu, 2010; Hu et al., 2010a; Reddy et al, 2010; Ansari, 2011; Kaeoket, 2011; Chhillar et al., 2012; Singh et al., 2012; Hussain et al., 2013); adding egg low-density lipoprotein (LDL) to protect against cold shock (Moussa et al., 2002; Amirat-Briand et al., 2010; Hu et al., 2010b; Tonieto et al., 2010); using cyclodextrins preloaded with cholesterol to replenish cholesterol in sperm plasma membranes (Mocé et al., 2010; Hussain et al., 2013); supplementing semen with prostaglandin $F_{2\alpha}$ to enhance intra-uterine sperm transport due to myometrial contractility at the time of insemination (Abbitt et al., 1977; Reddy et al., 1992; Willenburg et al., 2003; Jaeger, 2005; Epishina, 2009); addition of sugars to maintain osmotic pressure of diluents by inducing cell dehydration and less ice crystal into the spermatozoa (Chen et al., 1993; Woelders et al., 1997; Isachenko et al., 2008; Tonieto et al., 2010; Jafaroghli et al., 2011; Kumar et al., 2012; Singh et al., 2012) and the continuous and alternate storage of semen in liquid nitrogen and dry ice (Pickett et al., 1960). The last three approaches constitute the main components of this dissertation.

1.4.1. Statement of research problem

Spermatozoa are transported to the oviduct by a combination of sperm motility, uterine motility, and epithelial cilia activity (Troedson et al., 2005). Myometrial contractility is an essential component in the fertilization process because it is the mechanism by which spermatozoa are transported to the site of fertilization (Cheng et al., 2001). This implies that myometrial contractions have a significant role to play in the fertilization process, and prostaglandin F2-alpha has been shown to enhance uterine and/or oviduct motility in several species (Abbitt et al., 1977). An increase in *in vitro* myometrial contractility was recorded with prostaglandin $F_{2\alpha}$-supplemented boar semen when compared to extended semen or extender treatment alone after 72 h of incubation (Cheng et al., 2001). The addition of prostaglandin E2 and prostaglandin $F_{2\alpha}$ to diluted ram semen, comparable to the total amounts of prostaglandins in one ejaculate, increased the fertility of rams by more than 15% (Dimov and Georgiev, 1977).

Literature reports on supplementation of $PGF_{2\alpha}$ to bovine semen are scanty and even when available they present equivocal results. Abbitt et al. (1977) reported a statistically significant decrease in sperm motility with increase in concentration of $PGF_{2\alpha}$ when supplemented to bovine semen. Exogenous $PGF_{2\alpha}$ supplementation to bovine semen at 500 and 5000 pg/ml had no effect on post-thaw motility at 0 or 180 minutes post-thaw (Jaeger, 2005). However, semen supplementation with 125 and 250 µg prostaglandin $F_{2\alpha}$ caused a statistically significant increase in the motility of diluted bull semen stored at 4 ^{o}C after 24 h when compared with diluted prostaglandin $F_{2\alpha}$-free semen (Karahan, 2006). Of the three authors cited, only Jaeger (2005) was able to determine the endogenous levels of $PGF_{2\alpha}$ in whole semen, seminal plasma, and extended semen as a basis for the concentrations used for *in vitro* supplementation of $PGF_{2\alpha}$ to bovine semen. This could probably explain why levels of $PGF_{2\alpha}$ used in his study were not detrimental to post-thaw motility in any way by neither

increasing nor decreasing sperm motility. Therefore, works of the aforementioned authors suggest that exogenous supplementation of semen with prostaglandin $F_{2\alpha}$ could stimulate myometrial contractions and enhance sperm transport and fertility.

Studies have been carried out on the composition of cryoprotectant media in numerous species, by varying the cryoprotectant agent, adding sugars (monosaccharides or disaccharides), or changing the concentrations of the buffers used (Gramajo-Bühler et al., 2012). The composition of the cryoprotectant medium is a critical factor in the cryopreservation of sperm (Curry, 2000; Yoshida, 2000). Glycerol was the first cryoprotective agent used on sperm cells (Polge et al., 1949). However, numerous studies have been carried out since then to optimize glycerol concentration in the different freezing protocols (Gramajo.Bühler et al., 2012). Despite its benefits, glycerol can induce chemical and osmotic toxic effects on spermatozoon (Fiser and Fairfull, 1984; Fahy, 1986) and changes in the lipid packing structure of the membrane (Watson, 1995; Hay et al., 1997). These effects and changes could result in a lower fertility rate when AI is used (Bezerra et al., 2011), which indicates the need for alternative cryoprotectants.

In male dogs (Sanchez et al., 2011), goat bucks (Farshad and Akhonzadeh, 2008; Khalili et al., 2009), male humans (Hossain and Osuamkpe, 2007), rabbit bucks (Gramajor-Bühler et al., 2012), and the ram (Jafaroghli et al., 2011), sucrose has been used in comparison to glycerol for semen freezing. Sugars are known to maintain the osmotic pressure of diluents by inducing cell dehydration and less ice crystal formation into the spermatozoa (Leibo and Songsasen, 2002; Purdy, 2006). Moreover, sugars have the ability to form glass (vitrification) by depressing the membrane phase transition temperature of dry lipids. They also interact with phospholipid membranes at low hydration and thus cause stabilization of the membranes (Aisen et al., 2002; Naing et al., 2010). Furthermore, sugars are utilized by spermatozoa as an energy source during glycolysis and mitochondrial

oxidative phosphorylation to support sperm motility and movement (Naing et al., 2010; Ponglowhapan et al., 2004).

The use of sucrose as a cryoprotectant has found wide application in female gametes and embryos of different mammalian species including humans (Chen et al., 2001; Cervera and Garcia-Ximenez, 2003; Isachenko et al., 2004a; Silva and Berland, 2004). Due to deleterious osmotic effects of highly concentrated permeable cryoprotectants like glycerol, use of a nonpermeable cryoprotectant like sucrose cannot be directly extrapolated to male gametes (Sanchez et al., 2011). Isachenko et al. (2004a, b) have shown that excluding permeable cryoprotectants from cryopreservation solutions, increasing the cooling rate and using carbohydrates, proteins and other extracellular agents; to increase the viscosity of the surrounding medium of cells and prevent the formation of intra- and extracellular crystals, can reverse this situation by producing favourable results in human spermatozoa. Use of only sucrose as a nonpermeable cryoprotectant has been shown to provide a high recovery rate of motile cells and effectively protects the mitochondrial membrane (Isachenko et al., 2008) and the DNA integrity of human spermatozoa after warming (Isachenko et al., 2004b).

Bridging geographical barriers to breeding of animals could be achieved through semen transportation as a result of the use of suitable storage methods (Lemma, 2011). Dry ice and liquid nitrogen are the two main refrigerants that have dominated the history of bovine semen storage. Classical studies on bull semen storage in both refrigerants are found in the literature. Results of these studies show that factors such as type of extender, freezing method, duration of storage and fluctuations in storage temperature have some influence on sperm motility and fertility (MacPherson, 1954; MacPherson, 1955; Bratton et al., 1957; Pickett et al., 1960; Pickett et al., 1961 and Bean et al., 1963).

During the last three decades, several improvements in sperm cryobanking have occurred and storage in liquid nitrogen has become the standard (Anger et al., 2003). In

countries that cannot cope with the high investment and maintenance costs for liquid nitrogen installations needed in the production and storage of deep-frozen semen, transport of imported frozen bull semen on dry ice for immediate use could promote the utilization of cryopreserved semen by farmers. In some developing countries frozen semen is often thawed at the home of the technician or at the sub-centre and then sometimes carried to the farmer after thawing because of the inconvenience of using a liquid nitrogen container (Schuh, 1992). In this situation, transporting the frozen semen on dry ice in a styrofoam box could prove less cumbersome when compared to a liquid nitrogen tank. This also has the added advantage of preserving semen quality in comparison with transporting already thawed semen under the prevailing unreliable public transport system in these countries.

1.4.2. Objectives of the study

The studies in the present dissertation were conducted to determine: 1) the effect of prostaglandin F2-alpha (PGF2-alpha) addition after thawing on viability of bovine spermatozoa following comparison of extenders with or without egg yolk in order to establish the most suitable extender for PGF2-alpha supplementation, 2) effect of incorporation of sucrose alone or in combination with glycerol into customized (Tris-egg yolk) and two commercial (Steridyl® and AndroMed®) extenders on post-thaw motility of bovine spermatozoa, and 3) the effect of temporary storage of bovine semen originally stored in liquid nitrogen on dry ice and of refreezing of thawed semen on spermatozoon motility.

1.4.3. Structure of the dissertation

Chapter 2 is entitled, "*In vitro* effect of type of extender and addition of prostaglandin F2-alpha post-thawing on the motility of bovine spermatozoa" and it addresses the first specific objective. Chapter 3 is concerned with the second specific objective and it is captioned "Sugar supplementation in customized and commercial extenders: The use of sucrose solely and in combination with glycerol". Chapter 4 deals with the last specific

objective and is entitled "Temporary storage on dry ice of bovine semen originally stored in liquid nitrogen and the effect of refreezing". Chapter 5 is all about the overall conclusions from studies presented in this dissertation.

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Chapter 2

In vitro effect of type of extender and addition of prostaglandin F2-alpha post-thawing on the motility of bovine spermatozoa

Chapter 2 – In vitro effect of type of extender and addition of prostaglandin F2-alpha post-thawing on the motility of bovine spermatozoa

Abstract

The aim of the current study is to determine the effect of PGF2-alpha on viability of bovine spermatozoa in order to utilize its presumptive effect on myometrial contractility. Two experiments were carried out in this study. Experiment 1 was designed to compare extenders with or without egg yolk in order to determine the most suitable extender for PGF2-alpha supplementation. Two egg yolk-based extenders (Triladyl™ and Tris-egg yolk-glycerol) and a soybean lecithin-based extender (AndroMed®) were used. In Experiment 2, the effect of semen supplementation with prostaglandin F2-alpha (PGF2-alpha) at a concentration of 0, 10, 20, and 30 % (v/v) after thawing on percent motility of bull spermatozoa was evaluated immediately after incubation at 35°C and 1 h later. Percent total motility in Experiment 1 decreased significantly ($p<0.05$) as bull semen passed through different stages of cryopreservation from extension (65 ± 1%) via equilibration (60 ± 1%) to 15 min (49 ± 3%) and 7 days (46 ± 2%) of immersion in liquid nitrogen (LN_2). The difference between storage times (15 min or 7 days) in LN_2 was not significant. Post-thaw motility in AndroMed® extender was significantly ($p<0.05$) higher (59 ± 2%) than in both other extenders (both 53 ± 2%). There was no effect of individual bull ($p>0.05$). There was no significant ($p>0.05$) effect of PGF2-alpha concentration in Experiment 2. However, a slight but significant ($p<0.05$) effect of individual bull was recorded. AndroMed® appeared to be better in terms of preservation of bull sperm motility compared to the two egg yolk-based extenders. Frozen-thawed bull spermatozoa were capable of tolerating PGF2-alpha up to a concentration of 30% (v/v) in AndroMed® without adverse effect on total motility. Further studies should be attempted to test the effect of prostaglandin F2-alpha on success rates at insemination.

Chapter 2 – In vitro effect of type of extender and addition of prostaglandin F2-alpha post-thawing on the motility of bovine spermatozoa

2.1. Introduction

Recently, Gabriel et al. (2011) reported that intrauterine administration of PGF2-alpha following insemination appeared to lead to improved pregnancy rates. The present study was conducted with the intention to verify this result on a larger scale and improve the practicability of the operation by adding PGF2-alpha to the inseminate rather than having to administer it separately. As a prerequisite it was necessary to determine what concentration of PGF2-alpha spermatozoa will tolerate without impairment of viability. To that end four preliminary (unpublished) in vitro experiments preceded the current study. It was shown that a concentration of up to 20% PGF2-alpha does not affect spermatozoon motility even after 4 hours of incubation at 35°C (preliminary Experiments 1 and 2). Preliminary Experiment 3 confirmed this finding and showed that, if the PGF2-alpha concentration was increased to 40%, the proportion of motile spermatozoa was reduced from 65% to, on average, 50% within 1 hour of incubation and to 0% after 4 hours. At a concentration of 60% PGF2-alpha motility had ceased within 1 hour. As the intention was to add PGF2-alpha to extended semen prior to freezing, in preliminary Experiment 4, semen was diluted with Triladyl^{TM} extender supplemented with between 4 and 12% PGF2-alpha and cryopreserved. The result revealed that as long as the PGF2-alpha concentration did not exceed 6%, pre-freezing motility after 1 hour of incubation was unimpaired. At 8 and 10% it had decreased from 75 to 64% within 1 hour and at 12% to 10%. After cryopreservation, post-thaw motility had dropped to 27 and 15% at PGF2-alpha concentrations of 4 and 6% respectively, and to 0% at higher PGF2-alpha concentrations.

As a consequence of the preliminary experiments it was decided that PGF2-alpha should be added to semen after thawing rather than prior to freezing immediately before insemination. The aim of the current study, which is a follow-up to the above-mentioned preliminary experiments, was to determine the effect of PGF2-alpha addition after thawing

on viability of spermatozoa. In that context, extenders with and without egg yolk were compared to establish the extender most suitable for PGF2-alpha supplementation.

2.2. Materials and methods

2.2.1. Experiment 1: Comparison of three semen extenders

Freshly collected semen from three bulls of the Göttingen University AI station, routinely collected at weekly intervals, was used for this experiment. Of each ejaculate volume and initial motility (assessed subjectively at 400x using a closed circuit TV microscope equipped with a heating stage) were recorded and in 25 µl semen, diluted 200-fold with physiological saline, sperm density was determined photometrically. Two egg yolk containing extenders (Triladyl™ and Tris-egg yolk extender) and one extender devoid of egg yolk (AndroMed®) were tested. Triladyl™ extender was prepared by adding 60 ml double distilled water and 20 ml chicken egg yolk not older than 72 hours to 20 ml Triladyl™ concentrate (Minitüb, Tiefenbach, Germany), containing Tris, citric acid, sugar, buffers, glycerol, tylosin, gentamicin, spectinomycin and lincomycin. A customized Tris-egg yolk extender was made up by adding 20 ml chicken egg yolk, 1 g fructose (Serva Feinbiochemica, Heidelberg, Germany), 100,000 IU penicillin and 100 mg streptomycin (PAA Laboratories, Pasching, Austria) to 80 ml Tris buffer (3.786 g Tris [Merck, Darmstadt, Germany], 2.115 g citric acid monohydrate [Sigma-Aldrich Chemie, Steinheim, Germany] in 100 ml double distilled water, adjusted to pH 6.75 by addition of 10% citric acid solution). To this extender 6.8 ml glycerol (Merck, Darmstadt, Germany) was added. AndroMed® was made up by adding 80 ml double distilled water to 20 ml AndroMed® concentrate (Minitüb, Tiefenbach, Germany) containing phospholipids, Tris, citric acid, sugars, antioxidants, buffers, glycerol, tylosin, gentamicin, spectinomycin and lincomycin. This extender requires no addition of egg yolk. The three extenders were stored at -20°C until use.

Each of nine ejaculates was split into three equal portions of which each was diluted with one of three extenders (Triladyl™, Tris-egg yolk-glycerol and AndroMed®). The amount required to arrive at the desired sperm density of 100 million motile spermatozoa/ml of diluted semen depended on the original sperm density. Extended semen was aspirated into 0.25 ml straws with the aid of a filling and sealing equipment (IMV, L'Aigle, France). After 2 h at 4°C straws were placed 2 cm above the liquid nitrogen surface in a liquid nitrogen vat (Taylor-Wharton Harsco, Husum, Germany) and, after 10 min, immersed in liquid nitrogen. For thawing, straws were immersed in a water bath at 35°C for 1 min. Motility assessment was carried out after extension, after equilibration at 4°C and after 15 min and 7 days of storage in liquid nitrogen.

2.2.2. Experiment 2: Post-thaw supplementation of semen with PGF2-alpha

Freshly collected semen was diluted in AndroMed® extender and processed as described in Experiment 1. Semen was collected from the same three bulls used in Experiment 1. Three straws of frozen semen from each bull and PGF2-alpha concentration were thawed as described previously. For the control group (devoid of PGF2-alpha), the content of three straws of frozen semen from a bull was pooled in a 1.5 ml Eppendorf tube held at 35°C. For the 10, 20 and 30% PGF2-alpha containing samples the same procedure was followed. AndroMed® extender containing 0, 10, 20 and 30% v/v PGF2-alpha (Dinoprost-Trometamol; Dinolytic®, Pharmacia – Pfizer Group, Berlin, Germany; 5 mg/ml PGF2-alpha) was prepared and frozen in a similar fashion as described for semen. After 1 h, three straws from each of the PGF2-alpha concentrations were thawed in a water bath at 35°C for 1 min and added to frozen-thawed semen at a temperature of 35°C contained in 1.5 ml Eppendorf tubes. After gentle thorough mixing and one hour of incubation at 35°C, percent total motility was evaluated immediately and one hour later. The experiment was replicated three times.

2.2.3. Statistical analyses

Data from Experiment 1 was analyzed by a three-way analysis of variance (ANOVA) with extender and bull as between-subject variables and cryopreservation stage as within-subject variable. For Experiment 2 a three-way analysis of variance (ANOVA) was conducted with PGF2-alpha concentration and bull as between-subject variables and post-incubation time as within-subject variable. For both analyses the GLM repeated measures procedure of SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL, USA) was employed.

2.3. Results

The results of Experiment 1, summarized in Table 2.1, indicate that motility decreased significantly ($p<0.05$) as the semen passed through different stages of cryopreservation from extension (65 ± 1%) via equilibration (60 ± 1%) to 15 min (49 ± 3%) and 7 days (46 ± 2%) of immersion in liquid nitrogen (LN_2). The difference between storage times in LN_2 was not significant. Post-thaw motility in AndroMed® extender was significantly ($p<0.05$) higher (59 ± 2%) than in both other extenders (both 53 ± 2%). There was no effect of individual bull ($p>0.05$).

The results of Experiment 2, summarized in Table 2.2, indicate the absence of a significant effect of post-incubation time and concentration of PGF2-alpha up to 30% on the motility of bovine semen ($p>0.05$). There was, however, a slight but significant ($p<0.05$) bull effect.

Table 2.1 Percent total motility as affected by different stages of cryopreservation, extender and bull (Experiment 1). The experiment was conducted with 9 replications (= 9 ejaculates).

Variable	Motility (%) Mean	SE
Stage of Cryopreservation		
After Extension	65[a]	1
After Equilibration	60[b]	1
After in 15 min LN_2	49[cd]	3
After 7 days in LN_2	46[d]	2
Extender		
Triladyl™	53[b]	2
Tris-egg yolk-glycerol	53[b]	2
AndroMed®	59[a]	2
Bull		
1	55	2
2	58	2
3	52	2

[a,b,c,d] Different superscripts indicate difference at $p<0.05$.

Table 2.2 Percent total motility as affected by post-incubation time, PGF2-alpha concentration and bull (Experiment 2). The experiment was conducted with 3 replications (= 36 frozen semen straws per replicate).

	Motility (%)	
Variable	Mean	SE
Post-incubation time (h)		
0	72	1
1	68	3
PGF2-alpha concentration (%)		
0	76	3
10	74	3
20	67	3
30	64	3
Bull		
1	73^{ab}	3
2	75^{a}	3
3	64^{c}	3

^{a,b,c} Different superscripts indicate difference at $p<0.05$.

2.4. Discussion

The results indicate that, as bull spermatozoa passes through various stages of cryopreservation, total motility decreases with every step. Once in a frozen state, storage time (15 min or 7 days) does not seem to matter. The challenge for future research is, thus, to devise improved methods of cryopreservation, in particular during the temperature zone (-10° to -50°C) that sperm cells go through twice during a freeze-thaw procedure (Saacke, 1983). Extension and equilibration stages do not involve freezing and thawing which could explain the higher motilities recorded at these stages. According to Foote (2002), sperm survival in liquid nitrogen (LN_2) is virtually infinite; hence, the absence of significant differences between the two storage times employed in this study.

Since AndroMed® appeared to be superior to the other extenders used; it was the extender of choice in Experiment 2. AndroMed® contains soybean lecithin, known to be responsible for protecting the cells against damage from the freezing-thawing process, whereas the other extenders (Triladyl™ and Tris-egg yolk-glycerol) contain whole egg yolk. Concerning the use of AndroMed®, Müller-Schlösser et al. (2001) found no significant differences in post-thaw motility, inducibility of acrosome reaction with ZP proteins or progesterone, or *in vivo* fertility of bull spermatozoa cryopreserved with AndroMed® or a Tris-standard diluent. The presence of yolk globules in egg yolk-based extenders has been shown to interfere with microscopic evaluation of diluted semen (Vishwanath and Shannon, 2000). Aires et al. (2003) observed that Tris-egg yolk extender was more viscous than soybean lecithin-based extender and concluded that the difference in sperm motility between the two could result from differences in viscosity. The existence of an effect of individual bull on freezability has been amply documented (Gil et al., 2000; Thun et al., 2002; Ježková et al. 2008; Beran et al. 2012). The equilibration stage (Muiño et al., 2007), temperature,

freezing protocol, container, individual freezing and extender composition (Amirat et al., 2005) has been shown to affect spermatozoa viability.

The present study has confirmed that bull spermatozoa is capable of maintaining better motility when up to 30% PGF2-alpha was added to semen after thawing compared with addition prior to freezing in our preliminary experiments. Salamon and Maxwell (2000) observed that prostaglandins improved sperm motility and lambing when added to thawed semen, but not when used before freezing. According to Gabriel et al. (2011), PGF2-alpha could improve pregnancy rate by enhancement of spermatozoon motility through stimulation of myometrial contractility. Based on our findings, we tested a two-column straw technique (data not shown) in a small insemination trial in which extended semen and PGF2-alpha were frozen in the same straw but separated by an air space to prevent mixing. Results revealed that pregnancies could be achieved on the field using this technique. However, a large scale trial is necessary to determine the efficacy of the technique.

2.5. Conclusion

The current investigation concludes that AndroMed® appeared to be better in terms of preservation of motility of bovine spermatozoa compared to Triladyl™ and Tris-egg yolk-glycerol extenders; hence, its possible use for PGF2-alpha supplementation. Frozen-thawed bull spermatozoa were capable of tolerating PGF2-alpha up to a concentration of 30% (v/v) in AndroMed® without adverse effect on total motility. Further studies should be attempted to test the effect of prostaglandin F2-alpha on success rates at insemination.

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Chapter 3

Sugar supplementation in customized and commercial extenders: The use of sucrose solely and in combination with glycerol

Chapter 3 – Sugar supplementation in customized and commercial extenders: The use of sucrose solely and in combination with glycerol

Abstract

The present study was an attempt to establish a glycerol-free extender for bovine semen and was, therefore, designed to investigate the effect of the incorporation of sucrose alone or in combination with glycerol into customized (Tris-egg yolk) and two commercial (Steridyl® and AndroMed®) extenders on post-thaw motility. Three different concentrations (0, 300 and 600 mM) of sucrose were supplemented solely and in combination with glycerol in the customized extender and only in combination with glycerol in the commercial types. Ten ejaculates each were used for each of the three extenders in this experiment. Each of the ejaculates were divided into 3 equal portions and diluted with the respective extenders such that final concentrations in extended semen were 0 mM sucrose with 6.8% glycerol, 150 mM sucrose with 3.4% glycerol and 300 mM sucrose without glycerol for the customized extender and 0 mM sucrose with glycerol, 150 mM sucrose with glycerol and 300 mM sucrose with glycerol for both commercial extenders, respectively. The endpoint measurement was relative percent total motility, calculated as percent total motility relative to the control. In relation to semen diluted in Tris-egg yolk extender containing 6.8% glycerol (control), semen in same extender containing 150 mM sucrose and 3.4% glycerol had a relative motility of 68 (SE 3) % which decreased to 52 (SE 3) % in extender with 300 mM sucrose and devoid of glycerol. In semen diluted in Steridyl® with 150 mM or 300 mM sucrose, relative motility was significantly decreased to 67 (SE 3) % in the former and 31 (SE 4) % in the latter. In AndroMed® extender with 150 mM sucrose, motility had significantly decreased to 70 (SE 5) % and with 300 mM sucrose to 42 (SE 6) %. Efforts were made to employ an extender devoid of glycerol but it was observed that a small amount of sucrose in combination with glycerol proved to be advantageous. The present investigation, therefore, shows that sucrose could be used in semen extenders in order to reduce the amount of glycerol added to such extenders; thus, ameliorating the toxicity of glycerol when used in

high concentrations. However, the extent of sucrose incorporation needs further study in order to clarify its optimal concentration in extenders with or without glycerol. Our results were based on *in vitro* evaluations; therefore, further fertility trials are required.

3.1. Introduction

Ever since Polge discovered the cryoprotective effect of glycerol (Polge et al., 1949), most semen cryopreservation protocols favour that substance as cryoprotective agent. Together with methanol and ethylene glycol, glycerol belongs to a group of permeating cryoprotectants (Awad, 2011). However, when used at high concentrations glycerol is cytotoxic and, due to its exceptional penetrability, may be considered the cryoprotectant causing the greatest osmotic cell damage (Guthrie et al., 2002). Sugars such as glucose, fructose, raffinose, sucrose and trehalose have been included in semen-freezing extenders as energy substrates, osmotically active components and cryoprotective agents (Watson, 1979; Chen et al., 1993; Woelders et al., 1997; Becker-Silva et al., 2004a; Chaveiro et al., 2006; Naing et al., 2010; Jafaroghli et al., 2011; Kumar et al., 2012; Singh et al., 2012). Spermatozoa are known to be able to metabolize glucose, fructose and mannose (Mann, 1980) as well as arabinose (White, 1954). On the other hand, di- or trisaccharides such as lactose, sucrose and raffinose are virtually unable to permeate cell membranes but have cryoprotective properties (England, 1992). The protective effects of these sugars constitute cell dehydration, thus reducing intracellular ice crystal formation (Chen et al., 1993), prevention of liposome fusion and leakage of trapped solutes during dehydration and rehydration (Crowe et al., 1986). The cryoprotective impact of sugars on sperm cells is dependent on their molecular weight (Molinia et al., 1994), the type of buffer (Abdelhakeam et al., 1991) and storage temperature (Lapwood and Martin, 1966).

A study on goat semen conducted in this laboratory by Becker-Silva (2004) indicated that glycerol may, at least in part, be substituted by sucrose. This finding gave rise to the

current study which attempts to establish a low-glycerol or glycerol-free extender for the cryopreservation of bovine semen. In addition, the possibility of improving commercially available extenders (Steridyl® and AndroMed®) by adding a certain amount of sucrose was investigated.

3.2. Materials and methods

3.2.1. Semen collection and evaluation

Five ejaculates from each of two bulls of the Göttingen University AI station, routinely collected at weekly intervals, were used for each of three trials. Volume and initial spermatozoon motility, assessed subjectively at 400x using a closed circuit TV microscope equipped with a heating stage, were recorded and, in a sample diluted 200-fold with physiological saline, sperm density was determined photometrically. Trial 1 was conducted to examine the effect of a sucrose supplemented customized glycerol-free Tris-egg yolk extender on post-thaw spermatozoon motility. In Trials 2 and 3 the effect on post-thaw motility of various concentrations of sucrose in two commercial extenders, one based on egg yolk (Steridyl®) the other on soybean lecithin (AndroMed®), was determined.

3.2.2. Trial 1: Tris-egg yolk extender supplemented with glycerol and/or sucrose

Tris-egg yolk extender was made up by adding 20 ml chicken egg yolk, not older than 72 h, 1 g fructose (Serva Feinbiochemica, Heidelberg, Germany), 100,000 IU penicillin and 100 mg streptomycin (PAA Laboratories, Pasching, Austria) to 80 ml Tris buffer (3.786 g Tris [Merck, Darmstadt, Germany], 2.115 g citric acid monohydrate [Sigma-Aldrich Chemie, Steinheim, Germany] in 100 ml double distilled water, adjusted to pH 6.75 by addition of 10% citric acid solution). To this extender 6.8% glycerol (Merck, Darmstadt, Germany) (Extender 1), 6.8% glycerol and 300 mM sucrose (Sigma-Aldrich Chemie, Steinheim, Germany) (Extender 2) or 600 mM sucrose (Extender 3) was added. To facilitate sucrose

dissolution, extenders were swirled over a stream of mildly warm tap water. The three diluents were stored at -20°C until use.

Ten ejaculates (five from each of two bulls) were each divided into three equal portions that were diluted with Extenders 1, 2 and 3, respectively. The amount required to arrive at the desired sperm density of 100 million motile spermatozoa/ml diluted semen depended on the original sperm density. In the case of Extender 1, semen was diluted in three steps (1 part semen and 1 part extender; then a drop of extender, and finally, the rest of the extender). After extension, percent total motility was evaluated subjectively as described previously. Extended semen was aspirated into 0.25 ml straws with the aid of a straw filling and sealing equipment (IMV, L'Aigle, France). After 2 h at 4°C, straws were placed 2 cm above the surface of liquid nitrogen in a liquid nitrogen vat (Taylor-Wharton Harsco, Husum, Germany) and, after 10 min, immersed in liquid nitrogen. With Extenders 2 and 3, one half of the required volume of extender, devoid of supplements, was added in three steps (1 part semen and 1 part extender; then a drop of extender, and finally, the rest of the extender). After 2 h at 4°C the other half of the extender, containing the respective supplements, was added in three steps such that final concentrations were 3.4% glycerol and 150 mM sucrose in Extender 2 and 300 mM sucrose with no glycerol in Extender 3. The procedure was carried out in a cold room at 4°C. Further processing and storage for both extenders was conducted as previously described. Semen in Extender 1 (devoid of sucrose) was treated differently from semen in Extenders 2 and 3 (containing sucrose) in order to minimize dehydration damage to spermatozoa as a result of sucrose supplementation.

3.2.3. Trial 2: Steridyl® extender supplemented with sucrose

Of the commercially available Steridyl® concentrate (Minitüb, Tiefenbach, Germany), containing Tris, citric acid, sugar, buffers, glycerol, irradiated sterile egg yolk, tylosin, gentamicin, spectinomycin and lincomycin, 40 ml was diluted with 60 ml double distilled

water. To this extender either nothing (control) or the same amount of extender supplemented with 300 mM or 600 mM sucrose was added to give a final concentration of 150 mM or 300 mM sucrose after addition to semen. Ten ejaculates (five each from two bulls) were each divided into three equal portions that were diluted with these three extenders, processed and stored in the way described in Trial 1.

3.2.4. Trial 3: AndroMed® extender supplemented with sucrose

Of the commercially available AndroMed® concentrate (Minitüb, Tiefenbach, Germany), containing phospholipids, Tris, citric acid, sugars, antioxidants, buffers, glycerol, tylosin, gentamicin, spectinomycin and lincomycin, 20 ml was diluted with 80 ml double distilled water. To this extender either nothing (control) or the same amount of extender supplemented with 300 mM or 600 mM sucrose was added to give a final concentration of 150 mM or 300 mM sucrose after addition to semen. Ten ejaculates (five each from two bulls) were each divided into three equal portions that were diluted with these three extenders, processed and stored in the way described in Trial 1.

3.2.5. Thawing and rehydration

For thawing and rehydration of spermatozoa, four straws of the control group containing semen in an extender devoid of sucrose were placed in a water bath at 35°C for one min and pooled in a 12 ml test tube at 35°C. After 15 min, spermatozoon motility was assessed. Four straws each containing semen in extender with 150 and 300 mM sucrose, respectively, were thawed in water bath at 4°C for 1 min. Their content was transferred to 12 ml test tubes. After 80 s extender with no sucrose was added to rehydrate spermatozoa to near isosmolality in a stepwise manner. Briefly, initially 500 µl of the respective diluent (Tris-egg yolk, Steridyl® and AndroMed®) was added, followed, 2 min later by 500 µl, after another 4 min by 1000 µl, after another 5 min by 2000 µl and after another 6 min by 3000 µl

(according to Becker-Silva, 2004b). After these steps of rehydration semen was incubated in a water bath at 35°C and after 15 min motility was assessed.

3.2.6. Statistical analyses

The endpoint measurement was relative percent total motility, calculated as percent total motility relative to the control. The three trials were analyzed separately using two-way factorial ANOVA (SPSS 16.0 software for Windows, SPSS Inc., Chicago, IL, USA). Main effects of extender and bull and extender x bull interactive effects were included in the model. When F tests were significant, means were separated and compared using Tukey's procedure.

3.3. Results

The results of Trials 1, 2 and 3 are summarized in Table 3.1. In relation to semen diluted in Tris-egg yolk extender containing 6.8% glycerol (control), semen in extender containing 150 mM sucrose and 3.4% glycerol had a relative motility of 68 (SE 3) % which decreased to 52 (SE 3) % in semen diluted in extender with 300 mM sucrose and devoid of glycerol. In semen diluted in Steridyl® with 150 mM or 300 mM sucrose, relative motility was significantly decreased to 67 (SE 3) % and 31 (SE 4) %, respectively. In AndroMed® extender with 150 mM sucrose, motility had significantly decreased to 70 (SE 5) % and with 300 mM sucrose to 42 (SE 6) %.

Table 3.1 Relative motility of spermatozoa after thawing and rehydration of bull semen frozen in Tris-egg yolk extender in which sucrose was added in combination with glycerol or without glycerol and in Steridyl® and AndroMed® extenders supplemented with different concentrations of sucrose. Absolute motility of controls was 65.4% for Tris-egg yolk extender with 6.8% glycerol, 65.3% for Steridyl® without sucrose and 64.0% for AndroMed® without sucrose. Supplement levels in extenders are their final concentrations in diluted semen. Trials were conducted with 10 replications (= separate ejaculates).

Extender	Supplement	Relative motility (%)	
		Mean	SE
Tris-egg yolk (Trial 1)	6.8% glycerol (control)	100[a]	0
	3.4% glycerol + 150 mM sucrose	68[b]	3
	0% glycerol + 300 mM sucrose	52[c]	3
Steridyl® (Trial 2)	0 mM sucrose (control)	100[a]	0
	150 mM sucrose	67[b]	3
	300 mM sucrose	31[c]	4
AndroMed® (Trial 3)	0 mM sucrose (control)	100[a]	0
	150 mM sucrose	70[b]	5
	300 mM sucrose	42[c]	6

[a,b,c] Different superscripts indicate difference at $p<0.05$.

3.4. Discussion

The objective of the present investigation was to evaluate the effect of sucrose as a component of semen extenders, either alone or in combination with glycerol, on post-thaw motility of bovine spermatozoa. The cryoprotective benefits of glycerol, the most commonly used cryoprotectant in semen freezing, are attributed mostly to its water-binding properties (Morrier et al., 2002). When used at high concentrations, glycerol is cytotoxic and, due to its exceptional penetrability, causes osmotic cell damage (Guthrie et al., 2002). According to McLaughlin et al. (1992), the major cause of cellular damage is the rapid expansion of cell volume that occurs upon post-thaw dilution due to rapid inflow of water compared with the slower outflow of glycerol. The viscosity of cytoplasm in specific compartments or organelles of the spermatozoa is altered when glycerol enters the cell (Hammerstedt and Graham, 1992). We therefore explored the possibility of partially dehydrating the cells prior to freezing by exposing them to a hyperosmotic sucrose extender, thus by withdrawing intracellular water, prevent the formation of needle-shaped ice crystals known to inflict damage upon cell membranes and organelles and minimizing penetration of glycerol.

To fathom out what pre-freezing sucrose concentration is tolerated by goat spermatozoa, Farshad (1994) arrived at an optimum of 220 mM contained in a Tris diluent devoid of glycerol. According to Becker-Silva (2004b), there was little difference in percent motility and membrane integrity in goat spermatozoa cryopreserved in an extender containing 300 mM sucrose alone vs. one containing 300 mM sucrose and a small amount of glycerol.

In the present study Tris-egg yolk extender containing 150 mM sucrose with 3.4% glycerol proved to be better than the same extender containing 300 mM sucrose with no glycerol. Efforts were made to employ an extender devoid of glycerol but it was evident that a small amount of sucrose in combination with glycerol proved to be advantageous. According to Unal et al. (1978), post-thaw motility of bull spermatozoa was better in

extenders containing 120 and 180 mM lactose and 3% glycerol than in extenders devoid of lactose. Synergistic effects of sucrose and glycerol on cryopreservation of murine embryos have been reported by Biery et al. (1986). We, therefore, tested glycerol-containing commercial extenders (Steridyl® and AndroMed®) with sucrose. Relative motility in semen in Tris-egg yolk extender containing 150 mM sucrose dropped when semen was diluted in the same extender containing twice the sucrose concentration. This trend is similar to semen in commercial extenders with corresponding sucrose concentrations. However, the reduction in motility rating was more pronounced in commercial extenders containing 300 mM sucrose than in Tris-egg yolk extender with the same sucrose concentration and no glycerol. This proves that it is possible to completely replace glycerol with sucrose in the customized Tris-egg yolk extender and still retain some motility though not as good as in the extender with 150 mM sucrose and 3.4% glycerol. According to Chaveiro et al. (2006), incorporation of sucrose into Tris-based media leads to an increase in viscosity and turbidity. Higher viscosity and turbidity of Tris-based media containing sucrose could lead to lower percent motile sperm, VAP, VSL, and VCL as determined by CASA system (Chaveiro et al., 2006). This might have contributed to a lower rating concerning motility observed in our study in semen diluted with extenders containing 300 mM sucrose.

In the current study, extended semen was exposed to liquid nitrogen vapour within 10 min after addition of sucrose. Temperature and time spermatozoa remain in a dehydrated state affect hypertonic damage (Gao et al., 1993; Caiza de la Cueva et al., 1997a). We thawed the sucrose-containing semen in a cold room at 4°C and rehydrated by arbitrarily adding extender at the same temperature in a stepwise manner to near isosmolality according to Becker-Silva (2004b). This is in order to restore motility and regulate volume and/or osmolality excursions. Thawing and rehydration at 4°C was reported to restore better sperm motility in sucrose extender compared to thawing and rehydration at 38°C (Becker-Silva,

2004b). According to Caiza de la Cueva et al. (1997b), the temperature of incubation is involved in modulation of the ability of spermatozoa to regulate their volume and osmolality.

3.5. Conclusion

In summary, the results of this study indicate that sucrose could be used in semen extenders in order to reduce the amount of glycerol added to such extenders; thus, ameliorating the toxicity of glycerol when used in high concentrations. However, the extent of sucrose incorporation needs further attention in order to clarify its optimal concentration when used alone as in glycerol-free extenders or in combination with glycerol. Our results were based on *in vitro* evaluations. We, therefore, recommend further fertility trials.

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Chapter 4

Temporary storage on dry ice of bovine semen originally stored in liquid nitrogen and the effect of refreezing

Chapter 4 – Temporary storage on dry ice of bovine semen originally stored in liquid nitrogen and the effect of refreezing

Abstract

The possibility of semen transportation across national and continental borders could provide a means of exchange of genetic material. The overall objective of this study was to determine the effect of temporary storage of bovine semen originally stored in liquid nitrogen (LN_2) on dry ice and of refreezing of thawed semen on spermatozoon motility. Two experiments were carried out as follows: Experiment 1 was concerned with temporary dry ice storage of semen originally stored in LN_2 and Experiment 2 investigated the effect of refreezing of thawed semen on spermatozoon motility. Neither a 1h nor a 6h sojourn on dry ice affected spermatozoon motility regardless whether semen was thawed immediately or after being returned to LN_2 ($p>0.05$). Intensity of progressive motility was virtually unimpaired by the respective treatments. Post treatment motility rates were reduced by a factor of 10 as compared to semen not subjected to refreezing. No significant difference in percent post-thaw motility after refreezing was observed between semen samples that had been transiently stored on dry ice for 0, 1 or 6 hours ($p>0.05$). Semen that underwent refreezing on dry ice rather than in LN_2 vapour before being returned to LN_2 exhibited a significantly higher post-thaw motility rate ($p<0.05$). Although the proportion of motile spermatozoa was drastically reduced, the intensity of progressive forward motion was satisfactory. The results of the present *in vitro* study indicated that temporary dry ice storage appears to have no adverse effect on percent motility of bovine spermatozoa; thus, its use as a viable option for transport of frozen semen should be explored provided *in vivo* fertility is proven.

Chapter 4 – Temporary storage on dry ice of bovine semen originally stored in liquid nitrogen and the effect of refreezing

4.1. Introduction

Geographical barriers to breeding animals have long been reduced because of possibilities of semen transportation (Lemma, 2011). This, in turn, depends on suitable ways of storage and shipping. Early freezing and storage was accomplished with dry ice-alcohol (Parish, 2000). A major change in storage occurred in the 1950s with the shift from dry ice storage at -79°C to liquid nitrogen (LN_2) at -196°C (Foote, 2002). The impetus for such transition could be linked to the need for frequent resupply of dry ice and the fact that sperm survival in liquid nitrogen (LN_2) is virtually infinite (Foote, 2002). The successful cryopreservation of spermatozoa and development of efficient liquid nitrogen containers provided a foundation upon which today's entire cryopreservation industry is built (Foote, 2002).

Transport of frozen semen on dry ice for immediate use could be a more practical approach in countries where facilities for liquid nitrogen storage are scarce or not available. For transporting frozen semen at -196°C, a dry shipper has to be used and returned to the consigner (Okamoto et al., 2001). Shipment of dry ice is far less problematic and requires merely a styrofoam box. This paper, therefore, deals with the effect of temporary storage of bovine semen originally stored in LN_2 on dry ice and of refreezing of thawed semen on spermatozoon motility.

4.2. Materials and methods

The present study was conducted on frozen semen from 5 fertility-proven Holstein-Friesian AI sires. The semen had been collected by artificial vagina and processed according to standard practice. It was diluted in Triladyl^{TM} extender, packaged in 0.25 ml straws, chilled at 4°C for 2h, frozen in LN_2 vapour 2 cm above the LN_2 surface for 10 min and stored in LN_2. At the end of each treatment, straws were thawed by immersing them in a water bath at 35°C for one min, pooling their content and assessing percent total motility and intensity of

motility by placing a drop of semen on a cover slip, inverting it on a glass slide (Shuttleworth, 2002) and examining it at 400x using a light microscope with phase contrast stage objective, stage warmer set to 35°C and a camera connecting the microscope to a monitor. Percent motile spermatozoa and intensity of motility (sperm vigour), scored on a scale of 0 to 5, was determined subjectively. The scores were: 0 – no sperm movement; 1 – slight tail undulation without forward motion; 2 – slow tail undulation with slow or stop and go forward motion; 3 – forward progression at a moderate speed; 4 – rapid forward progression, 5 – very rapid progression, cells being difficult to follow visually. All motility assessments were done by the same individual. Two experiments were conducted, each with 15 straws from each of five bulls.

4.2.1. Experiment 1: Temporary storage on dry ice

Of the 15 straws from each of five bulls, three were thawed immediately to examine spermatozoon motility. The remaining 12 straws were transferred, with precooled forceps, to grooves, 2 mm deep, on a block of dry ice. After 1 h three of these straws were thawed, another three were returned to LN_2 to be thawed at a later stage. The same procedure was followed with the remaining six straws after 6 h on dry ice.

4.2.2. Experiment 2: Effect of refreezing

Of the 15 straws from each of five bulls, three were thawed, refrozen by placing them in LN_2 vapour 2 cm above the LN_2 surface for 10 min and returning them to LN_2, to be thawed once again one hour later when spermatozoon motility was assessed. Another six straws were transferred from LN_2 to dry ice. After 1 h three of these were thawed, refrozen as described above and thawed again to assess motility. After 6 h the same procedure was followed with the other three. The remaining six straws were thawed, then transferred to dry ice and, after 1 h and 6 h, respectively, returned to LN_2 and, eventually, thawed to assess motility.

4.2.3. Statistical analyses

Motility data from the two experiments were analyzed separately by analyses of variance (ANOVA) in a randomized complete block design with 5 replications (bulls) as blocks in both experiments and five treatments each. Percent motility data in Experiment 1 was analyzed after arcsine transformation to near normality. The results are presented as means ± SE of untransformed data. Transformation was not applicable to percent motility data in Experiment 2 due to their conformity with normality. All analyses were carried out using the GLM univariate procedure of SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL, USA).

The data on intensity of motility (sperm vigour) was descriptively analyzed using the crosstab procedure of SPSS 16.0 software for Windows (SPSS Inc., Chicago, IL, USA). A stacked bar chart was used to describe the distribution of sperm vigour.

4.3. Results

As shown in Table 4.1, post-freezing spermatozoon motility in Experiment 1 was satisfactory. Neither a 1 h nor a 6 h sojourn on dry ice affected the percentage of motile spermatozoa, regardless whether semen was thawed immediately or after having been returned to LN_2 ($p>0.05$). Figure 4.1 indicates that the intensity of progressive motility was virtually unimpaired by the respective treatments. In Experiment 2 post-treatment motility rates were reduced by a factor of 10 as compared to semen not subjected to refreezing (Table 4.1). No significant difference in percent post-thaw motility after refreezing was observed between semen samples that had been transiently stored on dry ice for 0, 1 or 6 hours ($p>0.05$). Semen that underwent refreezing on dry ice rather than in LN_2 vapour before being returned to LN_2 exhibited a significantly higher post-thaw motility rate ($p<0.05$). Although the proportion of motile spermatozoa was drastically reduced, the intensity of progressive forward motion was satisfactory (Figure 4.2).

Table 4.1 Percent total motility of bovine spermatozoa in semen originally stored in LN_2 as affected by temporary dry ice storage (Experiment 1) and refreezing (Experiment 2) (5 replications per treatment per experiment)

		Motility	
Experiment	Treatment	Mean	SE
1	LN_2 - Thaw	68.60	3.22
	LN_2 - 1 h CO_2 - Thaw	68.80	1.46
	LN_2 - 6 h CO_2 - Thaw	68.40	3.14
	LN_2 - 1 h CO_2 - LN_2 - Thaw	69.00	1.79
	LN_2 - 6 h CO_2 - LN_2 - Thaw	68.00	1.10
2	LN_2 - Thaw - LN_2 - Thaw	7.20^{b}	2.27
	LN_2 - 1 h CO_2 - Thaw - LN_2 - Thaw	6.00^{ab}	1.92
	LN_2 - 6 h CO_2 - Thaw - LN_2 - Thaw	7.40^{b}	2.79
	LN_2 - Thaw - 1 h CO_2 - LN_2 - Thaw	13.00^{ab}	2.55
	LN_2 - Thaw - 6 h CO_2 - LN_2 - Thaw	17.00^{a}	4.47

^{a,b,c} Different superscripts indicate difference at $p<0.05$.

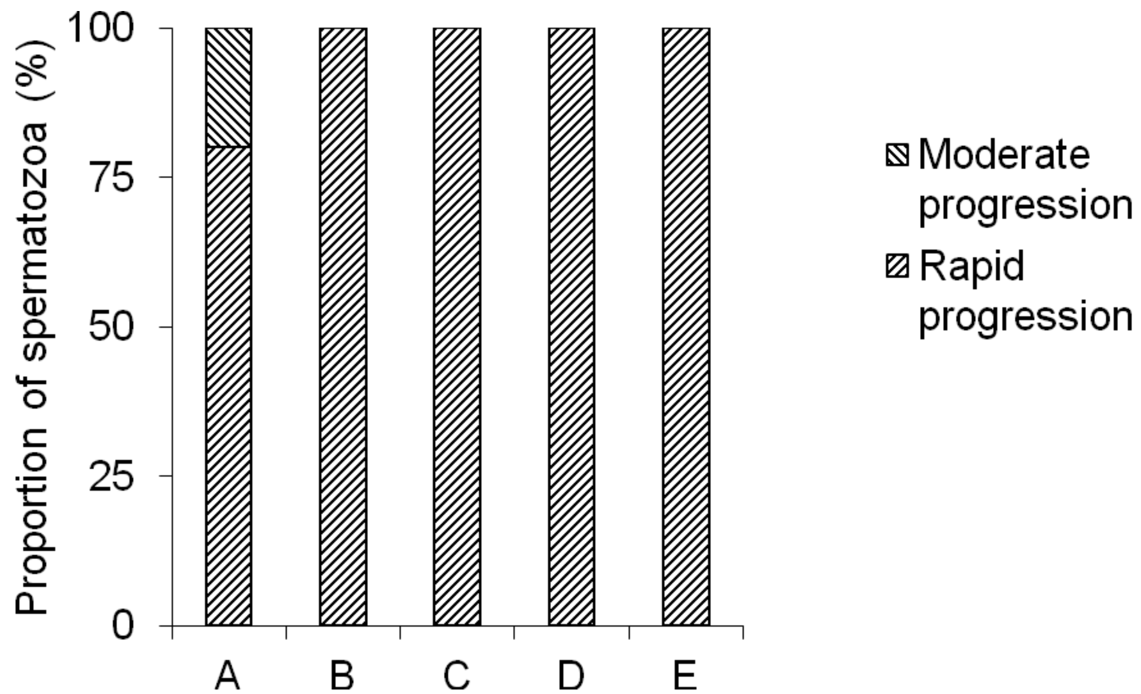

Figure 4.1 Intensity of post-thaw motility in cryopreserved semen thawed directly (A), after 1 h (B) or after 6 h on dry ice (C) prior to thawing or after 1 h (D) or 6 h on dry ice and return to liquid nitrogen (E) (Experiment 1).

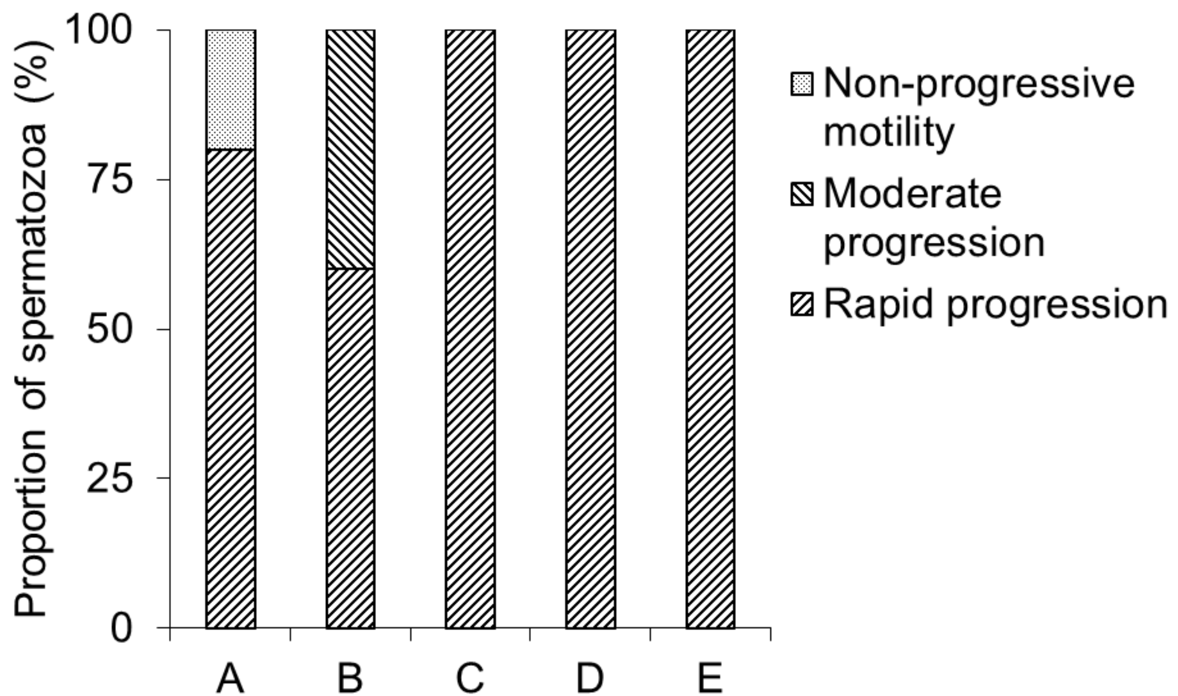

Figure 4.2 Intensity of post-thaw motility in cryopreserved semen thawed directly and refrozen in liquid nitrogen vapour (A), thawed and refrozen in liquid nitrogen vapour after 1 h (B) or 6 h (C) on dry ice or thawed and refrozen on dry ice for 1 h (D) or 6 h (E) before return to liquid nitrogen (Experiment 2).

4.4. Discussion

As the results show, spermatozoon motility in semen stored in LN_2 is not impaired by a one or six hour sojourn on dry ice, no matter whether thawing occurred directly after the period on dry ice or after return to LN_2. Okamoto et al. (2001) observed that mouse spermatozoa stored at -79°C for 4 months survived. Bean et al. (1963) found that motility of bovine spermatozoa stored at -92 and -196°C (liquid nitrogen) was approximately equal but superior to that of semen stored at -79°C (dry ice). Citing Larson and Graham (1950), Pickett et al. (1960) report that bull semen transferred from -196 to -79°C showed a decline in motility, but less so after seven- or ten- than after three days in LN_2. They conclude that an adaptation period of at least one week is required before transferring semen from LN_2 to dry ice. Also, semen stored in LN_2 6 weeks before it was transferred to dry ice exhibited greater motility than any paired samples stored in dry ice originally, irrespective of the nature of subsequent transfers (Pickett et al., 1960). The semen used in the present study was stored in LN_2 for a period of 7 to 18 years. Whether longer periods of keeping semen stored in LN_2 on dry ice will be tolerated and what the effect on fertilizability will be needs to be investigated before the practicability of maintaining or transporting cryopreserved semen on dry ice is proven.

Refreezing semen successfully could provide a means of rescuing genetics through salvaging of mistakenly thawed semen (James, 2004; Carwell et al., 2010); allowing judicious use of precious semen that is in limited supply (McCue et al., 2004) as in the case of sperm sorting with flow cytometry (Evans et al., 2004), and maximizing sperm utilization in the case of large volume or high concentration cryobanking (Arav et al., 2002). Our results showed that semen refrozen on dry ice compared to LN_2 vapour exhibited a significantly higher post-thaw motility rate. In that respect temporary storage on dry ice appeared to have been rather beneficial. Although the proportion of motile spermatozoa was drastically

reduced with refreezing, a satisfactory progressive forward motion was maintained. The possibility of using this subpopulation of sperm cells in assisted reproduction, such as IVF, cannot be overlooked. According to Muino et al. (2008), ejaculates with the highest subpopulations of rapid and progressive sperm were also resistant to cryopreservation and showed post-thaw sperm longevity.

Attempts to subject semen to further refreezing produced an appalling outcome (data not shown). Refreezing in the current study involved two thawing cycles. According to Saragusty et al. (2009), two freeze-thaw cycles are bound to put the sperm cells through stressful conditions resulting in the death or loss of biological function in a large proportion of cells. Rofeim et al. (2001) demonstrated that it is possible to preserve human sperm motility and viability for up to 7 cycles of the thaw-refreeze process depending on the method of refreezing. They suggested that multiple factors such as plasma membrane damage by free radicals and superoxide, osmotic changes, and intracellular and extracellular ice formation play a role in sperm integrity during thaw-refreeze process.

The subjective approach used in the determination of total sperm motility could be seen as a possible drawback of the current study, especially when objective methods like the computer-assisted sperm analysis (CASA) exist. This approach could be justified from the point-of-view of less cost of equipment and its practicality from the simple fact that a well-trained technician equipped with a light microscope could easily and reliably assess sperm motility, even under field conditions; however, results obtained may not be repeatable in all species and with all operators (Viveiros et al., 2010). According to Tuli et al. (1992), both subjective and computer-assisted (HTM-2000 motility analyzer) assessment of sperm motility in fresh and in frozen-thawed semen of bulls, boars and goat bucks yielded comparable results. They, however, posit that the use of motility analyzer renders consistently more accurate estimates when motility is vigorous as in fresh bull semen.

Similarly, Viveiros et al. (2010) reported no significant difference between subjective evaluation of post-thaw sperm motility using light microscope and objective evaluation using CASA in the streaked prochilod fish, *Prochilodus lineatus*.

4.5. Conclusion

It may be concluded that temporary dry ice storage has no adverse effect on percent motility of bovine spermatozoa; thus, it presents a viable option for transport of frozen semen for immediate use. Refreezing on dry ice with return to liquid nitrogen seems to maintain satisfactory progressive forward motion compared with refreezing in liquid nitrogen vapour with return to liquid nitrogen. However, refreezing irrespective of the method used yielded a drastic decline in percent motility when compared with semen samples temporarily stored on dry ice without refreezing. The *in vitro* nature of the current investigation necessitates further *in vivo* fertility studies.

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Chapter 5

Conclusions and suggestions for further study

Chapter 5 – Conclusions and suggestions for further study

5.1. Concluding remarks

Attempts have been made to supplement semen with hormones such as prostaglandin F2-alpha (PGF2-alpha) in order to improve post-thaw sperm motility and fertility post-insemination. Our preliminary unpublished experiments on supplementation of PGF2-alpha to bovine semen have shown that spermatozoon can tolerate addition of a concentration of up to 20% PGF2-alpha without freezing even after 4 hours of incubation at 35°C. When the PGF2-alpha concentration was increased to 40%, the proportion of motile spermatozoa was reduced from 65% to, on average, 50% within 1 hour of incubation and to 0% after 4 hours. At a concentration of 60% PGF2-alpha motility had ceased within 1 hour. When diluted semen was supplemented with PGF2-alpha in the concentration range of between 4 and 12%, results reveal that as long as the PGF2-alpha concentration did not exceed 6%, pre-freezing motility after 1 hour of incubation was unimpaired. At 8 and 10% PGF2-alpha concentration, it had decreased from 75 to 64% within 1 hour and at 12% to 10%. After freezing, post-thaw motility had dropped to 27 and 15% at PGF2-alpha concentrations of 4 and 6% respectively, and to 0% at higher PGF2-alpha concentrations. These preliminary experiments were the basis for the current study where it was proposed that addition of PGF2-alpha to semen after thawing could improve spermatozoon motility with more encouraging results than those obtained with the preliminary experiments. In the context of this proposition, extenders with and without egg yolk were compared to establish the extender most suitable for PGF2-alpha supplementation.

It can be deduced from results of the current work that, as bull spermatozoa passes through various stages of cryopreservation, total motility decreased in the order: extension > equilibration > frozen storage for 15 min or 7 days. This implies that total motility decreases with every step. However, storage time (15 min or 7 days) does not seem to matter once semen is in a frozen state. Cryopreservation in liquid nitrogen (-196°C) is an important

strategy for conserving semen quality. The outcome of the cryopreservation process depends on the protocol used in diluting semen with different extenders and additives as well as the use of different cooling, freezing and thawing rates. The influence of freezing medium composition (cryoprotectants and extenders) is of great importance for sperm survival during cryopreservation. Since AndroMed® appeared to be superior to the other extenders (Triladyl™ and Tris-egg yolk-glycerol) used in this study, it was made the extender of choice for PGF2-alpha supplementation. AndroMed® contains soybean lecithin, known to be responsible for protecting the cells against damage from the freezing-thawing process, whereas the other extenders contain whole egg yolk. There was no significant difference in preservation of bull sperm motility between Triladyl™ and Tris-egg yolk-glycerol. Bull spermatozoa in AndroMed®-extended semen supplemented with up to 30% (v/v) PGF2-alpha showed no change in total motility; implying that a concentration of up to 30% (v/v) PGF2-alpha is tolerated by bovine spermatozoa. Our study has, therefore, confirmed that bull spermatozoa is capable of maintaining better motility when up to 30% PGF2-alpha was added to semen after thawing compared with addition prior to freezing in our preliminary experiments.

Most semen cryopreservation protocols still use glycerol as the gold standard cryoprotectant. Despite this, sugars, especially the higher molecular weight ones (such as lactose, sucrose and raffinose) due to their low membrane permeability, have been used as cryoprotective agents. Equivocal reports are found in the literature regarding claims of effectiveness of these sugars as cryoprotectants. This could be due to the fact that cryoprotective ability of these sugars depends on the storage temperature, molecular weight and type of buffer; all of which usually vary in works that focus on this subject. In an attempt to establish a glycerol-free extender for bovine semen in the current study, the effect of the incorporation of sucrose alone or in combination with glycerol into customized (Tris-egg

yolk) and two commercial (Steridyl® and AndroMed®) extenders on post-thaw motility was investigated to initiate partial dehydration of semen prefreezing; thus, ameliorating the dehydrating effects of freezing on bull spermatozoa. The results of our study show that relative to Tris-egg yolk containing 6.8% glycerol without sucrose (control), semen in same extender containing 150 mM sucrose and 3.4% glycerol had a relative motility of 68 (SE 3) % which decreased to 52 (SE 3) % in extender with 300 mM sucrose and devoid of glycerol. This implies that Tris-egg yolk extender containing 150 mM sucrose with 3.4% glycerol proved to be better than the same extender containing 300 mM sucrose without glycerol. Efforts were made to employ an extender devoid of glycerol but it was observed that a small amount of sucrose in combination with glycerol proved to be advantageous. We, therefore, tested glycerol-containing commercial extenders (Steridyl® and AndroMed®) with sucrose. In semen diluted in Steridyl® with 150 mM or 300 mM sucrose, relative motility was significantly decreased to 67 (SE 3) % in the former and 31 (SE 4) % in the latter. In AndroMed® extender with 150 mM sucrose, motility had significantly decreased to 70 (SE 5) % and with 300 mM sucrose to 42 (SE 6) %. Motility in Steridyl® and AndroMed® extenders containing 150 mM sucrose was better than that of the same extenders with 300 mM sucrose. However, motility in Tris-egg yolk extender containing 300 mM sucrose without glycerol was better than that of commercial extenders containing glycerol in combination with 300 mM sucrose. This proves that it is possible to completely replace glycerol with sucrose in the customized Tris-egg yolk extender and still retain some motility though not as good as in the extender with 150 mM sucrose and 3.4% glycerol. It, therefore, appears that sucrose could be used in semen extenders in order to reduce the amount of glycerol added to such extenders; thus, ameliorating the toxicity of glycerol when used in high concentrations.

Due to improvements in sperm cryobanking, storage of semen in liquid nitrogen (LN_2) has become the standard in developed countries. In developing countries, where access

to facilities for modern sperm cryobanking could be difficult or impossible, there is a need for an alternative approach, if the exchange of frozen semen between livestock breeding and improvement centres in developed and developing countries is to be facilitated. Transport of frozen semen on dry ice for immediate use could be a more practical approach in countries where facilities for liquid nitrogen storage are scarce or not available. For transporting frozen semen at liquid nitrogen temperature, a dry shipper has to be used and must be returned to the consigner. Due to the inconvenience of using a liquid nitrogen container, semen thawed by the technician at home or the sub-centre is carried to the farmer in some developing countries. In this situation, transporting the frozen semen in dry ice in a styrofoam box could prove less cumbersome when compared to a liquid nitrogen tank. This also has the added advantage of preserving semen quality in comparison with transporting already thawed semen under the prevailing unreliable public transport system in developing countries. Shipment of dry ice is far less problematic and requires merely a styrofoam box. Results show that neither a 1h nor a 6h sojourn on dry ice affected spermatozoon motility regardless whether semen was thawed immediately or after being returned to LN_2. Intensity of progressive motility was virtually unimpaired by the respective treatments. Post treatment motility rates were reduced by a factor of 10 as compared to semen not subjected to refreezing. No significant difference in percent post-thaw motility after refreezing was observed between semen samples that had been transiently stored on dry ice for 0, 1 or 6 hours. Semen that underwent refreezing on dry ice rather than in LN_2 vapour before being returned to LN_2 exhibited a significantly higher post-thaw motility rate. Although the proportion of motile spermatozoa was drastically reduced, the intensity of progressive forward motion was satisfactory. Temporary dry ice storage appears to have no adverse effect on percent motility of bovine spermatozoa. It is, therefore, possible to transport bull semen temporarily stored on dry ice for immediate use or subsequent storage in LN_2 where facilities exist.

5.2. Suggestions for further study

For further study, it is suggested that:

1. Motility should be evaluated using objective methods such as CASA.
2. Supplementation of extended semen with exogenous PGF2-alpha should be done on the basis of seminal PGF2-alpha concentrations in experimental bulls.
3. Replacement of glycerol with sucrose should be made on equimolar basis taking into consideration other components of the extender such as the buffer and egg yolk.
4. The extent of sucrose incorporation needs attention in order to clarify its optimal concentration when used alone as in glycerol-free extenders or in combination with glycerol in commercial extenders.
5. Period of temporary storage of semen on dry ice should be extended.
6. Results of studies presented in this dissertation should be subjected to *in vivo* fertility trials in order to determine their success rates at insemination.

Curriculum vitae

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