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High pre-freezing dilution improves post-thaw function of ram spermatozoa

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ABSTRACT

Despite considerable cryobiology research there is no industry standard for the concentration to which ram spermatozoa should be diluted before freezing. Ram semen is highly concentrated and often frozen at a high sperm concentration, necessitating the use of small laparoscopic insemination doses. The aim of this paper was to ascertain the effect of dilution on the integrity of frozen-thawed ram spermatozoa. In the first experiment, spermatozoa were extended with a Tris-buffered diluent before freezing or after thawing to yield a final sperm concentration of 20×10^6/ml, or were not diluted. Motility characteristics, viability and acrosome integrity of spermatozoa were analysed over a 6 h incubation period at 37 °C. In the second experiment, spermatozoa were either diluted before freezing, subjected to sex-sorting or not diluted before freezing. Thawed spermatozoa were separated into sub-populations using centrifugal counter-current distribution (CCCD) and the profile of partition and functional integrity (viability, chlortetracycline status and Annexin-V binding) in the sub-populations assessed. Dilution before freezing significantly improved post-thaw viability, acrosome integrity and total motility whereas dilution post-thaw decreased viability and motility of spermatozoa. Sperm heterogeneity, as assessed by CCCD profile, was not different for control, diluted and sex-sorted spermatozoa. Analysis of CCCD sub-populations showed the proportion of functional cells (displaying the F-Pattern or no PS translocation) was similar for all sperm types. The results show that ram spermatozoa retain normal function at higher pre-freeze dilution rates than are commonly used in the sheep industry. The application of these findings would result in more practicable and functional artificial insemination doses.

1. Introduction

Cryopreservation is one of the most important biological technologies available to the animal industry as it permits long-term storage and efficient dissemination of superior genetics. Frozen storage suspends the activity of spermatozoa but, when they revive upon re-warming, the status of their membranes is changed (Watson, 1995). Damage during cooling, freezing and thawing is primarily caused by intracellular ice formation and cell dehydration which exposes the spermatozoon to high solute concentrations (Lovelock, 1953). Freeze–thaw damage invokes a plethora of changes in spermatozoa including capacitation-like effects (Gillan et al., 1997), reduction in integrity of the plasma and acrosome membranes (Salamon and Maxwell, 1995b), reduced heterogeneity of the sperm population (Ollero et al., 1998), and diminished motility and ability

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to penetrate cervical mucus *in vitro* (Gillan et al., 1999). These changes impair the ability of spermatozoa to traverse the female tract and fertilise the oocyte(s), resulting in decreased *in vivo* fertility. Moreover, the freeze–thaw process inflicts significant sub-lethal damage on spermatozoa, as fertility of ewes is poor after cervical insemination, even if a high number of motile and viable frozen-thawed ram spermatozoa are used (Salamon and Maxwell, 1995b). This may be due to the selective pressure of the cervix on spermatozoa, and other interactions between spermatozoa and the reproductive tract of the ewe, that cannot be assessed *in vitro*.

There has been considerable empirical research performed to optimise cryopreservation protocols and minimise injury associated with freezing and thawing, and this information has been extensively reviewed (Parks and Graham, 1992; Royere et al., 1996; Holt, 2000). In the ram, research has been centred around diluent composition, cooling and dilution rates, as well as the use of additives to protect spermatozoa during freezing and thawing (Salamon and Maxwell, 1995a; Maxwell and Watson, 1996). Nevertheless, cryopreservation remains a highly damaging technology (Maxwell and Watson, 1996) and deposition of spermatozoa close to the site of fertilisation, via laparoscopic insemination, is the only reliable method to achieve acceptable fertility with frozen-thawed ram spermatozoa. However, the low monetary value of ewes and the extensive range conditions of the sheep industry in many countries, does not allow for the widespread uptake of this technology. Nevertheless, laparoscopic AI is used to inseminate a significant proportion of the genetically superior stud ewe population in Australia, and thus has important genetic effects that flow on to the rest of the sheep industry (Maxwell and Watson, 1996).

Intrauterine insemination by laparoscopy has allowed more economical use of superior genetics, as spermatozoa are placed closer to the site of insemination than for cervical insemination, and thus lower numbers are required to achieve acceptable fertility. However, ram spermatozoa are still frozen at relatively high concentrations. While there is no clear industry standard, ram semen is commonly frozen after only 4–5-fold (v/v) extension with cryodiluent (approximately 800–1000 million spermatozoa per ml) or may be extended to a specific concentration (Evans and Maxwell, 1987). This is most likely for convenience and to avoid negative effects associated with high dilution rates of spermatozoa. Mann (1964) described the "dilution effect" as the loss of motility and viability when spermatozoa are highly diluted. Excessive dilution has been reported to cause membrane destabilisation and capacitation-like changes in spermatozoa (Maxwell and Johnson, 1999) and cryopreservation may have an additive effect, further injuring the cells. It is thought the "dilution effect" is due to the removal of protective factors in seminal plasma (Harrison et al., 1982). However, there is a lack of information on the pre-freezing rate to which spermatozoa can be diluted without a reduction in their post-thaw survival. Sex-sorted spermatozoa undergo extremely high dilution during processing (5000×) and, even after concentration by centrifugation, are still frozen at a relatively low concentration (approximately 20 million per ml) in comparison to non-sorted spermatozoa (Maxwell et al., 2004). Dilution of this magnitude is thought to stress the gametes but it is not known how it affects the final quality of frozen-thawed sex-sorted ram spermatozoa, as they are also exposed to numerous other stressors during sorting and freezing.

Centrifugal counter-current distribution (CCCD) can be used to partition sperm populations according to differences in cell surface affinity to immiscible aqueous solutions of polymers, and can reveal differences in both the membrane surface and its coating components (Walter et al., 1985; Albertsson, 1986; Sharpe, 1988). The functional variability of spermatozoa in their response to stimuli is a known indicator of fertilization success (Amann et al., 1993) and CCCD has been used to assess heterogeneity of bull (Pascual et al., 1992) and ram semen (Pascual et al., 1993). In addition, sperm separation during the CCCD process is correlated with viability (Pascual et al., 1993), maturity (Ollero et al., 1994) and acrosome integrity of spermatozoa (Marti et al., 2000) as well as *in vivo* fertility (Perez-Pe et al., 2002; Grasa et al., 2005).

The aim of this paper was to ascertain the effect of dilution on the function and integrity of ram spermatozoa and elucidate how it contributes to the stress of sex-sorting and cryopreservation. This information could then be used to develop semen handling protocols which minimise sperm damage and facilitate the efficient use of gametes.

2. Materials and methods

2.1. Experimental design

The University of Sydney's animal ethics committee approved procedures herein. In Experiment 1, the effect of dilution of spermatozoa before or after cryopreservation was compared. Fresh semen was diluted to 100×10^6 spermatozoa/ml with a Tris-buffered diluent or not diluted. All samples were then extended 1 + 4 (semen or diluted spermatozoa + cryodiluent, v/v) to yield a final pre-freeze sperm concentration of 20×10^6/ml (pre-freeze dilution) in diluted samples and approximately 800×10^6/ml in non-diluted samples. After freezing and thawing, non-diluted samples were further split and either diluted to 20×10^6 sperm/ml (post-thaw dilution) or not diluted (control). All samples were then extended 1 + 1 (v/v) with Androhep (containing 0.25% BSA; Minitüb, GmbH, Landshut, Germany; adjusted to pH 7.4). Motility characteristics were objectively assessed using computer-aided sperm analysis (CASA), acrosome integrity was determined by fluorescein-conjugated peanut agglutinin staining (FITC-PNA) and membrane integrity determined by dual staining with carboxyfluorescein diacetate (CFDA) and propidium iodide (PI). Three ejaculates per ram from three individual rams were collected and processed and these functional assessments were conducted immediately upon thawing and after 2, 4 and 6 h of post-thaw incubation (37 °C).

Experiment 2 was conducted to examine in further detail the effect of dilution prior to freezing. Sex-sorted spermatozoa were also included as a positive control, as they are highly diluted during processing and are frozen at low concentrations (20×10^6 sperm/ml). Centrifugal counter-current distribution (CCCD) was employed on one