



## Principles and perspectives for the conservation of goat buck spermatozoa<sup>☆</sup>

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### ABSTRACT

This paper is focused on the use of alternative protocols for goat buck sperm cryopreservation and other current approaches to study sperm cryosurvival, considering theoretical principles for the preservation of cells at freezing temperatures. Simple modifications to traditional freeze-thawing protocols may improve sperm cryosurvival; thus, cooling to  $-5^{\circ}\text{C}$  instead of  $+5^{\circ}\text{C}$ , before freezing, improves buck, ram and boar sperm cryosurvival. Premature capacitation induced by cryopreservation may be used as an additional tool to: (1) assess new cryopreservation protocols; i.e. the least aggressive one would induce the lowest proportion of premature capacitated spermatozoa; (2) to estimate the subpopulation of frozen-thawed spermatozoa that remain fertile (live cells showing Pattern F or B; Chlortetracycline Assay). Freezing and storage of spermatozoa using ultra-low freezers ( $-150^{\circ}\text{C}$ ) represents a feasible alternative to the use of liquid nitrogen, at least in both short and medium term. Simple tests based on simulation of osmotic stress that occurs during freeze-thawing are promising approaches to predict sperm cryosurvival. Assessment of spermatozoa during hyperosmotic and isosmotic conditions at  $23^{\circ}\text{C}$  has revealed that proportions of plasma membrane-intact and acrosome-intact spermatozoa are similar to those observed after cryopreservation (after freeze-thawing). Inter-male differences in sperm freezability are an ever present variable influencing sperm cryosurvival.

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### 1. Introduction

During cryopreservation (cooling, freezing, and thawing) spermatozoa have to face and overcome a number of physicochemical events to survive (Mazur, 1984; Hammerstedt et al., 1990; Watson, 1995):

- Plasma membrane–lipids phase transition due to temperature changes that occurs in a wide range of

temperatures, even below  $0^{\circ}\text{C}$ , with the consequent reduction in membrane elasticity.

- Changes in volume due to exposition to freezing extenders containing glycerol and the osmotic changes associated to ice formation and melting.
- Osmotic stress due to exposition to hyper- (ice formation and solute concentration) and hypo-osmotic conditions (ice melting).
- Ice formation, both intra- and extra-cellular.
- Drastic changes in temperature due to release of latent heat of fusion produced by ice formation and dissipation of such heat.
- The eventual presentation of supercooling that occurs when diluted spermatozoa pass through their theoretical freezing point without freezing.

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- The risk of recrystallization when thawing is too slow.
- The phenomenon of cryocapacitation or premature capacitation that renders sperm membranes to fuse prematurely reducing their fertile life.

In addition, goat buck spermatozoa display a limited tolerance to the inclusion of either egg yolk or skimmed milk in the freezing medium. Moreover, the goat is a seasonal breeder that shows variations in semen quality throughout the year, and those variations may affect semen freezability (Leboeuf et al., 2000).

Since modifications to sperm cryopreservation protocols usually produce marginal improvements in sperm survival, different approaches based on that knowledge have been used to try to overcome poor sperm cryosurvival.

## 2. Materials studied

### 2.1. Cooling to $-5^{\circ}\text{C}$ before freezing

Traditionally, diluted semen is slowly cooled to  $+5^{\circ}\text{C}$  before freezing in order to increase sperm cryosurvival; in addition, during cooling to  $+5^{\circ}\text{C}$ , holding of diluted semen at  $15^{\circ}\text{C}$  for 3 h improves pig sperm cryosurvival (Maxwell and Johnson, 1997). Another approach to improve sperm cryosurvival involves cooling to temperatures below  $0^{\circ}\text{C}$ , around ice formation of standard freezing media, before freezing.

Semen from three Canary bucks was collected, diluted, packaged in 0.5 ml plastic straws, and slowly cooled (approximately  $0.1^{\circ}\text{C}/\text{min}$ ) from  $22^{\circ}\text{C}$  to either: (1)  $+5^{\circ}\text{C}$ , control group or (2)  $-5^{\circ}\text{C}$ , experimental group. Straws were put into insulated containers; refrigerators (one for each treatment) were set to the target temperature,  $+5$  or  $-5^{\circ}\text{C}$ , and the cooling process was started from  $22^{\circ}\text{C}$ . When the straws from each cooling treatment reached target temperatures, straws were then exposed to nitrogen vapour for 15 min, plunged in liquid nitrogen and stored for 2 days. Then, they were thawed at  $37^{\circ}\text{C}$  for 30 s and sperm progressive motility, plasma membrane integrity and acrosome membrane integrity were assessed.

### 2.2. Freezing and storage of spermatozoa using ultra-low freezers ( $-150^{\circ}\text{C}$ )

Liquid nitrogen provides the best-known conditions to freeze and preserve mammalian spermatozoa for long periods of time; however, in some cases liquid nitrogen is very expensive and difficult to obtain. Therefore, it is important to look for new alternatives to freeze and store frozen semen; ultra-low temperature freezers may represent such alternative.

A number of experiments were performed to test whether an ultra-low temperature freezer ( $-150^{\circ}\text{C}$ ) may be used to properly freeze and store semen. In the first part, crude sample handling was simulated to see whether temperature of stored samples was maintained within a safe range; other thermal variables such as the freezing point and latent heat of fusion plateau of a semen extender were also monitored. In the second part, semen from three Canary bucks (seven pooled samples) were (1) frozen in liquid nitrogen and stored in the ultra-low freezer, (2) frozen and stored in the ultra-low freezer, and (3) frozen and stored in liquid nitrogen, to compare sperm cryosurvival between freezing methods. Thawing was carried out after two periods: 2 days and 2 months.

### 2.3. Test of osmotic stress to predict sperm cryosurvival

During freeze-thawing, spermatozoa are exposed to hyper- and hypo-osmotic conditions as a result of a series of phenomena such as extra-cellular ice formation, solute concentration and ice melting. A simple test, based on simulation of osmotic stress, to predict sperm cryosurvival would allow identifying the so-called "good" and "bad freezers" without doing time-consuming protocols; thus, only semen from "good freezers" would be frozen.

In a preliminary experiment, buck spermatozoa from 12 males (20 ejaculates) were collected by artificial vagina, washed to remove seminal plasma, diluted in a standard freezing medium and exposed to hyper-osmotic solutions: (i) 300, (ii) 1200 and (iii) 2100 mOsm/kg at  $23^{\circ}\text{C}$  for

15 min; then iso-osmolarity was re-established by exposing spermatozoa to suitable hypo-osmotic solutions at  $23^{\circ}\text{C}$  for 15 min. At the end of each time plasma membrane and acrosome membrane integrity was assessed.

### 2.4. Inter-male differences in sperm freezability

Individual differences between males regarding sperm freezability are an ever present variable influencing sperm cryosurvival. In several species, especially in pigs, the phenomenon of "good" and "bad freezers" has been described (Holt, 2000; Holt et al., 2005). Spermatozoa from those individuals respond differently to modifications in cooling rate: "good freezers" spermatozoa survive better when cooled at "optimal" than "sub-optimal" cooling rate; in contrast, "bad freezers" sperm cryosurvival is similar regardless cooling rate (Holt et al., 2005). It has been suggested cryopreservation could be used to detect seasonal effects and difference between males.

Semen from six Canary bucks was collected by using an artificial vagina, twice a week for a year. However, only semen collected during spring, autumn or winter was frozen. Nine of 18 ejaculates collected from each male in every season were washed and the other nine were not; 18 ejaculates collected from each male in every season, three were washed and three non-washed for each of three dilutions in a Tris-based freezing medium with 1.5, 6 and 12% egg yolk. Frozen straws were thawed after 2 periods, 2 or 6 months of cryopreservation.

## 3. Results

### 3.1. Cooling to $-5^{\circ}\text{C}$ before freezing

There was a significant difference ( $P < 0.003$ ) between treatments ( $+5^{\circ}\text{C}$  vs.  $-5^{\circ}\text{C}$ ) in the proportion of plasma membrane-intact spermatozoa:  $51 \pm 1.8\%$  vs.  $58 \pm 2.0\%$ . However, there were no significant differences ( $P > 0.05$ ) regarding motile ( $37 \pm 1.5\%$  vs.  $40 \pm 1.8\%$ ) or acrosome-intact spermatozoa ( $49 \pm 3.2\%$  vs.  $52 \pm 3.1\%$ ) spermatozoa. Although not significant, results from the experimental group (cooling to  $-5^{\circ}\text{C}$ ) showed a tendency to be higher than those from the control group (Medrano et al., 2001).

### 3.2. Freezing and storage of spermatozoa using ultra-low freezers ( $-150^{\circ}\text{C}$ )

Frequent removal of samples and long opening of the freezer door did not negatively affect stored sample temperature; freezing point of a buck freezing extender containing 4% glycerol was about  $-5^{\circ}\text{C}$  and latent heat of fusion plateau was 5 min long. This data suggests semen may be properly frozen and stored regardless that long latent heat of fusion plateau; survival of spermatozoa stored either at  $-150$  or at  $-196^{\circ}\text{C}$  was similar after 2 days and after 2 months of cryopreservation (Medrano et al., 2002).

### 3.3. Test of osmotic stress to predict sperm cryosurvival

Proportions of plasma membrane-intact and acrosome-intact spermatozoa decreased as osmolarity increased ( $P < 0.05$ ); a further decrease ( $P < 0.05$ ) was observed when iso-osmolarity was recovered (Tables 1 and 2). The largest difference in those variables at hyper- and iso-osmotic conditions was observed using the 2100 mOsm/kg solution (Rangel-Alonso, 2009).

**Table 1**

Buck plasma membrane-intact spermatozoa (%) under hyperosmotic conditions and after restoration of isosmolarity at 23 °C.

	Osmolarity value (mOsm/kg) <sup>a</sup>		
	300	1200	2100
Hyperosmotic condition	75.4a	69.4ab	65.5b
Restoration of isosmolarity	68.7a	63.1ab	55.5b

<sup>a</sup> Values are means. Different letters in rows indicate significant differences ( $P < 0.05$ ).

**Table 2**

Buck acrosome-intact spermatozoa under hyperosmotic conditions and after restoration of isosmolarity at 23 °C.

	Osmolarity value (mOsm/kg) <sup>a</sup>		
	300	1200	2100
Hyperosmotic condition	71.5a	63.6b	57.5b
Restoration of isosmolarity	57.9a	51.5b	47.7b

<sup>a</sup> Values are means. Different letters in rows indicate significant differences ( $P < 0.05$ ).

### 3.4. Inter-male differences in sperm freezability

Significant variation between samples of different seasons was observed in frozen-thawed semen (progressive motility and plasma membrane integrity,  $P < 0.01$ ). Moreover, season-processing interaction (washed or non-washed) was observed in all the frozen-thawed semen characteristics analyzed ( $P < 0.01$ ). However, a significant variation between males ( $P < 0.01$ ), male-method processing interaction ( $P < 0.01$ ) and male-season interaction ( $P < 0.05$ ) were observed in all the frozen-thawed semen characteristics analyzed (Cabrera et al., 2005).

## 4. Discussion

Regarding cooling to  $-5$  °C, similar results have been obtained in ram and boar spermatozoa, cooled to  $-2$  and  $-5$  °C, respectively. In those experiments, premature capacitation, induced by cryopreservation, was assessed in addition to common sperm assessment. In these species, cooling to temperatures below 0 °C reduced the shift from non-capacitated to capacitated spermatozoa; this suggests that slow cooling of diluted buck, ram and boar spermatozoa close to their freezing point favours sperm cryosurvival of rams (Rios et al., 2004) and boars (Flores and Medrano, 2005). Lipid phase transition that occur in a wide range of temperature during cooling and rewarming (Holt and North, 1984) could explain the beneficial effect of slow cooling since it provides plasma membrane the time to reorganize its three-dimensional structure after lipid phase transition takes place. There is evidence that this phenomenon still occurs at temperatures around 0 °C (Crowe et al., 1989; Noiles et al., 1995). More research is needed to see whether any improvement in sperm cryosurvival positively affect in vivo fertility.

With respect to the use of the ultra-low temperature freezer, one important concern is to know whether this sort of freezer may be used as a long-term semen bank. A similar work has provided evidence that is possible to store frozen buck spermatozoa for periods up to 1 year with-

out apparent changes in cryosurvival; results from artificial insemination trials indicate that fertility obtained by using frozen and stored spermatozoa in either liquid nitrogen or the ultra-low freezer is similar (Batista et al., 2009). This alternative to the use of liquid nitrogen deserves more research since it represents an easy way to freeze and store spermatozoa.

The osmotic stress test showed buck spermatozoa respond to increased osmolarity as well as restoration of isosmolarity in the same way as boar and ram spermatozoa (Medrano et al., 2006). Similar work in other species (pigs) has showed sperm viability from the osmotic stress test and that from freeze-thawing are not different (Garzon-Perez, 2008). These results suggest, this osmotic stress test simulates in some way the freeze-thawing process and it could be used as a predictor of sperm freezability.

Differences between males regarding sperm freezability have been observed since the early age of industrial cryopreservation; however, cellular and molecular basis for such differences are not understood. Hidalgo et al. (2007) have proposed sperm head dimensions could be used to predict buck sperm cryosurvival; apparently, sperm morphology from some individuals provides better resistant to cryopreservation than that from others.

## 5. Conclusion

Knowledge of fundamental aspects of cryobiology does not solve by itself poor sperm cryosurvival but provides the guide to elaborate protocols to suit sperm requirements for each stage: dilution, cooling, freezing and thawing. Goat buck semen is characterized by a special sensibility to extenders containing egg yolk and milk at high levels; in addition, buck spermatozoa have to suffer the inherent stress of freeze-thawing. Species-specific sperm characteristics determine the degree of damage in the sperm structural and physiological attributes needed to fertilize; thus, preservation protocols should provide spermatozoa the best conditions to survive. More research is needed for understanding the sources of variation between ejaculates and males at cellular level regarding sperm freezability.

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