CHAPTER IV

RESULTS

In the present study, a total of 33 ejaculates (9 ejaculates in experimental I and 24 ejaculates in experimental II) were used for semen cryopreservation. Descriptive statistics (mean and standard deviation) of the sperm parameters of fresh semen used in experiment I and II are presented in Table 4 and 5, respectively.

The semen quality before and after cryopreservation was evaluated using four sperm parameters including total motility, viability, acrosome integrity and functional plasma membrane. In overall, it was revealed that all of the sperm parameters were significantly decreased after cryopreservation process.

Table 4. Descriptive data of semen characteristics of fresh semen (n=3 ejaculates per stallion) (*Experiment I*)

Stallion	Progressive motility (%)	Volume (ml.)	Concentration (x 10^6/ml.)	Total sperm per ejaculation (x 10^9/ml.)	Morphology % Normal head	Morphology % Normal tail
No.1	81.7±2.9	59.0±27.1	88±13.5	4.9±1.2	79±2.4	73.2±2.7
No.2	81.7±2.9	37.3±12.5	117±28.9	4.3±0.8	84.5±3.9	75.8±3.7
No.3	83.3±2.9	38.3±16.1	113.3±15.8	4.2±1.3	78.1±2.7	75.6±3.8

Table 5. Descriptive data of semen characteristics of fresh semen (n=4 ejaculates per stallion) (*Experiment II*)

Stallion	Progressive motility (%)	Volume (ml.)	Concentration (x 10^6/ml.)	Total sperm per ejaculation (x 10^9/ml.)	Morphology % Normal head	Morphology % Normal tail
No.1	80±4.1	45.8±11.8	187.5±117	7.6±2.2	82.7±3.9	75.2±3.4
No.2	82.5±2.9	44±4.2	141.3±62.5	6.3±3.3	82.3±1.8	78.6±2.1
No.3	83.8±2.5	45.5±13.8	167.5±69	7.2±2.5	83.1±2.6	79.4±3.7
No.4	82.5±2.9	32.5±13.2	277.5±91.1	8.1±0.8	85.6±2.7	80.1±2.5
No.5	82.5±2.9	35±12.9	200±118.6	6.2±2.9	85.1±1.6	79±4.3
No.6	82.5±2.9	25±10.8	175±48	5.2±3.2	82±2.2	77.8±3.3

Experiment 1: effect of freezing techniques (conventional versus controlled-rate freezer) on post-thawed sperm motility and viability

The stallion semen quality including total motility, viability and longevity of fresh and frozen thawed semen between conventional and controlled-rate freezing techniques was presented in Table 6 and 7. The mean percentage of total motility (TM%) significantly decreased after cryopreservation process($P<0.05$). The post-thawed motility of all stallions was not significantly different ($P>0.05$) between both techniques. However, TM% in stallion No.3 was significant greater than others stallions when observed at 2 hrs post-thawing. At 4 hrs post-thawing, TM% of stallion No.3 was significantly greater than stallion No.1.

The mean percentage of sperm viability (SV%) decreased after cryopreservation process (Table 7). SV% was significantly greater ($P<0.05$) in controlled-rate freezing technique of all stallions when compared with conventional technique at 2 and 4 hrs post-thawing. However, this did not significantly differ in stallion No.3.

Table 6. Mean ± standard deviation of sperm motility (%) of cryopreserved semen in each stallion (n=3 ejaculates per stallion)

Time	Freezing technique	Percentage of progressive motility		
		Stallion 1	Stallion 2	Stallion 3
After collection	-	81.7±2.9	81.7±2.9	83.3±2.9
After equilibration at 4 °C	-	66.7±5.8	66.7±5.8	70±0
10 min post-thawing	Conventional	40±10	43.3±11.5	60±0
	Controlled-rate	53.3±5.8	56.7±5.8	60±0
2 hr. post-thawing	Conventional	16.7±5.8	16.7±5.8	40±0
	Controlled-rate	33.3±11.5	33.3±11.5	43.3±5.8
4 hr. post-thawing	Conventional	5±0	5±0	13.3±5.8
	Controlled-rate	8.3±2.9	11.7±7.6	20±0

[a,b] Different superscripts within rows indicated significant difference ($P<0.05$).

Table 7. Mean ± standard deviation of sperm viability (%) of cryopreserved semen in each stallion (n=3 ejaculates per stallion)

Time	Freezing technique	Percentage of sperm viability		
		Stallion 1	Stallion 2	Stallion 3
After equilibration at 4 °C	-	70.4±6.6	75.4±4.5	76±3.5
10 min post-thawing	Conventional	52.3±7.5	54.5±7.1	62.5±2.9
	Controlled-rate	64.7±5.3	65.7±2.2	68.1±2.4
2 hr. post-thawing	Conventional	39.1±2.5[a]	40.2±2.2[a]	56.3±1.3
	Controlled-rate	49.4±3.9[b]	49.4±2.3[b]	59.2±1.7
4 hr. post-thawing	Conventional	31.8±1.2[†]	32.2±2.7[†]	48.4±2.4
	Controlled-rate	40.2±3.2[‡]	40.7±3.1[‡]	52±1.9

[a,b,†,‡] Different superscripts within column indicated significant difference ($P<0.05$).

Experiment 2: influence of freezing extenders supplemented with different sugars on post-thawed stallion semen quality

The stallion semen quality after cryopreservation including total motility, viability, acrosomal status, and plasma membrane function are present in Table 8.

Sperm motility

The mean TM% in freshly pooled semen was 82.3±2.9 (n=24). TM% decreased over the incubation time in all treatment groups (Table 5 and 8). There were significantly different in TM% between sugars at 10 min post-thawing. However, no significant difference was found in all extenders when observed at 2 and 4 hrs. The addition of sorbitol to EY extender significantly improved TM% than the other two extenders ($P<0.001$). Incubation time of frozen-thawed had a detrimental effect on both TM% and PM%. The type of monosaccharide sugar influenced TM% such that the TM% were 40±8.8, 40.6±8.5 and 50.2±7.5 for fructose, glucose and sorbitol, respectively.

Sperm viability

In general, freezing and thawing significantly reduced the number of viable spermatozoa ($P<0.05$). At 10 min post-thawing, mean percentage of sperm viability treated with sorbitol-based extenders was significantly greater than glucose- and fructose-based extenders ($P<0.001$) (Table 8, Fig. 6). Moreover, at 2 and 4 hr post-thawing, sorbitol-based extender resulted in a significant difference when compared with fructose-based extender. It is therefore clearly indicated that the type of monosaccharide sugar plays an important role in protecting equine sperm against cryoinjury during freezing and thawing.

Acrosome integrity

This study revealed that cryopreservation potentially disrupted acrosomal integrity of the stallion spermatozoa. Sorbitol-based extender preserved acrosomal integrity (%intact: 40.6±6.4) better than fructose and glucose-based extenders

(33.9±5.5 and 35.9±5.8), respectively. However, percentage of intact acrosome, irrespective the sugars types used, was significantly lesser than non-cryopreserved sperm (62.3±4.3). In sorbitol-based extenders, acrosome integrity was significant difference when compared with fructose-based extenders ($P<0.05$) at every time points (10 min, 2 and 4 hrs) post-thawing (Table 8, Fig 7).

Functional integrity of sperm plasma membrane

Cryopreservation, thawing and also incubation period decreased significantly the function of plasma membrane ($P<0.05$) of stallion spermatozoa. The mean percentage of spermatozoa with intact functionally plasma membrane in freshly pooled semen was 34.4±8.8. After thawing, the percentage of functional sperm plasma membrane as determined by sHOS test was decreased over the time ($P<0.05$) post-thawing (Table 8, Fig 8). When data was pooled across sugar type, the least square means of spermatozoa with intact plasma membrane (positive sHOS test) after 10 min post-thawing were: 16.1±4.9 (Fructose), 17.2±5.3 (Glucose), and 21.3±5.8 (Sorbitol). Sorbitol-based extender had significantly a better plasma membrane functionality than fructose-based extender ($P<0.05$), but no significance was found between sorbitol and glucose. Type of sugar therefore provided a protective effect on plasma membrane functionality.

Fertility assessment of cryopreserved-thawed stallion semen

According to the result of *in vitro* sperm quality assessment, sorbitol-based extender provided a better in sperm quality than glucose and fructose-based extenders, in terms of sperm motility, viability, acrosome integrity and plasma membrane functionality. The sorbitol-based extender was therefore used to test *in vivo* fertility of frozen semen. A total of 4 mares were inseminated with cryopreserved semen using sorbitol in freezing extender, two mares (50%) were pregnant on day 14-35.

Table 8. Least Square Means of post-thawed stallion semen quality in freezing extenders (Ext.) containing fructose (F) or glucose (G) or sorbitol (S) at various times (n=24 ejaculates per groups)

Parameter / Time	Motility (%)	Viability (%)	Intact acrosome (%)	sHost (%)[1]
10 min post-thawing				
Ext.F	40.0±8.8[a]	46.9±5[a]	33.9±5.5[a]	16.1±4.9[a]
Ext.G	40.6±8.5[a]	48.6±5.3[a]	35.9±5.8[ab]	17.2±5.3[ab]
Ext.S	50.2±7.5[b]	54.3±6.1[b]	40.6±6.4[b]	21.3±5.8[b]
2 hrs post-thawing				
Ext.F	22.3±8.4	34.3±4.8[a]	24.8±5[a]	11.2±3.6[a]
Ext.G	24.6±8.7	37.2±4.4[ab]	27.3±5.7[ab]	12.3±3.7[ab]
Ext.S	26.5±8.4	40.1±4.4[b]	30.3±5.2[b]	15.5±4.3[b]
4 hrs post-thawing				
Ext.F	9.8±5.2	26.5±5.1[a]	18.1±3.5[a]	8.5±2.8[a]
Ext.G	11.9±5	29.8±5.1[ab]	19.9±4.3[ab]	9.3±3.2[ab]
Ext.S	12.5±5.1	31.8±4.7[b]	22.5±4[b]	12.3±3[b]

[1] sHost = functional integrity of sperm plasma membrane

[a,b] values with different superscripts within column differ significantly ($P<0.05$)

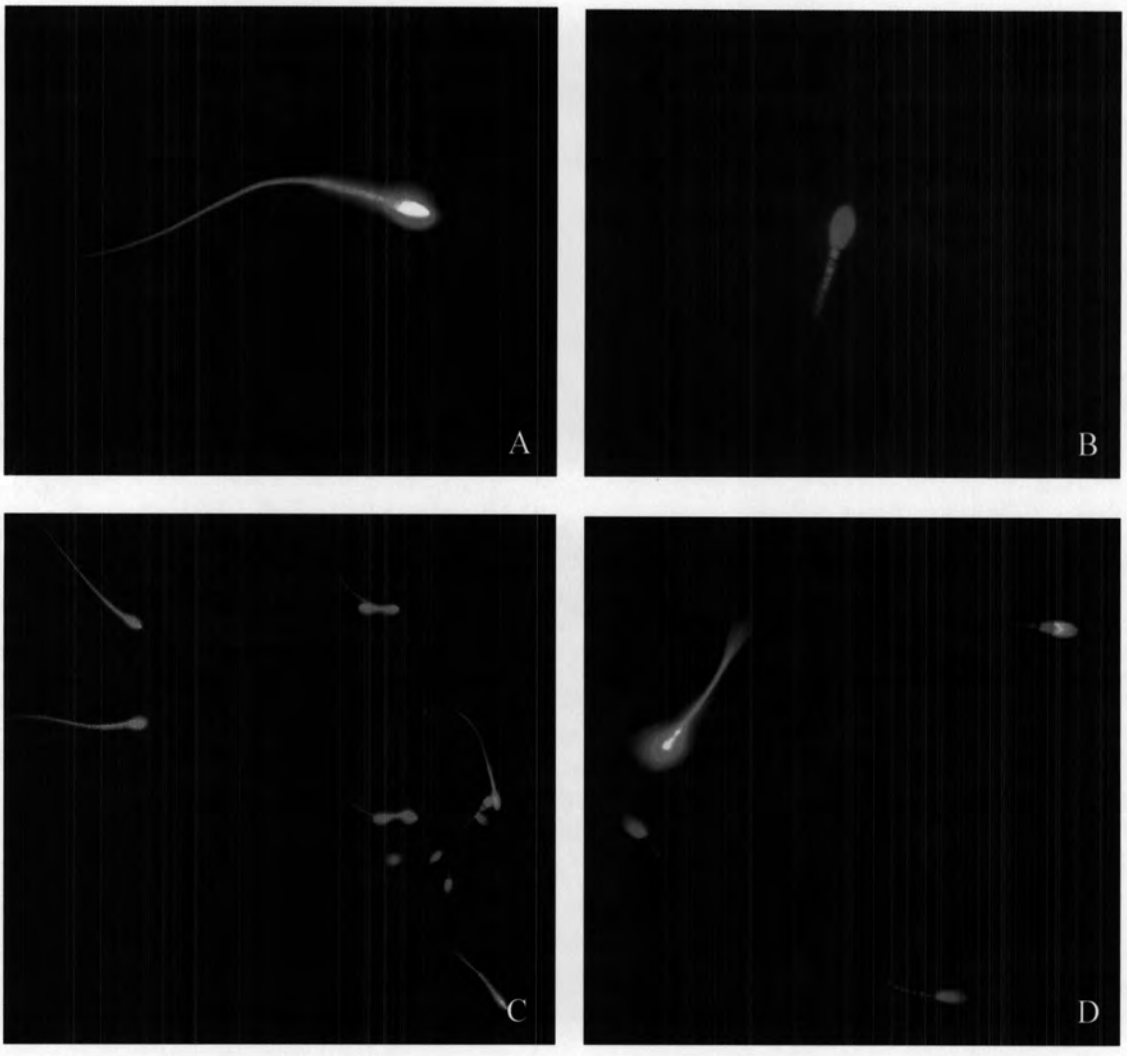

Figure 6 Photomicrographs of stallion spermatozoa stained with Calcein-AM combined with Ethidium homodimer-1. The spermatozoa with intact plasma membrane (A; live) were stained green with calcein-AM, while EthD-1(red) positive spermatozoa indicated the damage of plasma membrane (B).

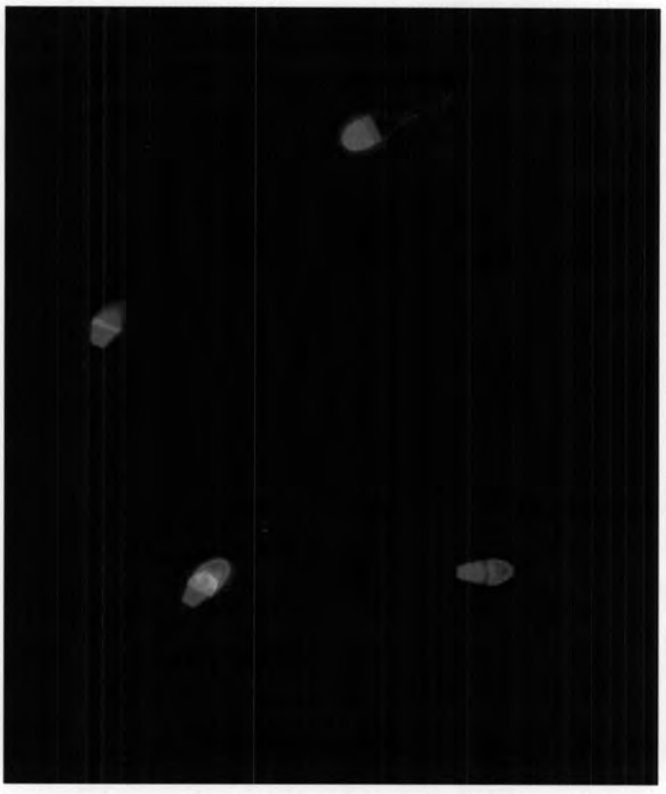

Figure 7 A photomicrograph of stallion spermatozoa stained with FITC-PNA/Ethidium homodimer-1. The spermatozoa with intact acrosome were stained green with FITC-PNA, while the spermatozoa with damaged or loss acrosome were stained red with EthD-1 (1000x magnification).

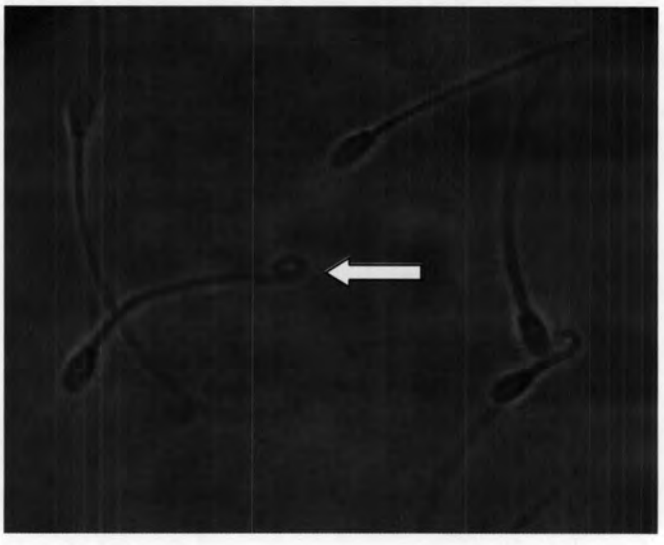

Figure 8 A photomicrograph of stallion spermatozoa incubated with a hypo-osmotic solution (100 mOsm/kg). The types of typical tail abnormalities indicative of swelling (sHOST positive, white arrow) were evaluated under light microscopy at 400x magnification.