

# Effects of Different Holding Temperatures before Freezing on the Quality of Bull Semen

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## Abstract

A study was conducted to evaluate the effect of different holding temperature before freezing on the motility, morphology and viability of chilled and frozen spermatozoa. Semen was performed from four mature bulls by electro-ejaculator. After initial evaluation and first dilution using Tris-egg yolk semen extender, four groups of diluted semen samples were kept in four different holding temperatures (4°C, 15°C, 25°C and 37°C) for 10 minutes. All semen samples were subsequently chilled in refrigerator at 4°C for at least 3 hours. Post chilling evaluation was done after second dilution with the same extender added with glycerol. The semen samples were packed into 0.25 ml straws before freezing into liquid nitrogen. Thawing of frozen semen samples were done on day 1 post cryopreservation in water maintained at 37°C for 30 seconds. Semen evaluation was conducted post thawing. The results showed that there were no significant difference among the four different holding temperatures for the post chilling and frozen-thawed sperm motility, morphology and viability. Based on the findings of this study, it can be concluded that 10 minutes holding time at different holding temperatures before freezing did not affect the quality of chilled and frozen bull spermatozoa.

**Keywords:** Semen, holding temperature, bull, freezing.

## Introduction

The development in cryopreservation technology is able to store frozen semen for extended period of time and used for insemination. Studies have been conducted in order to improve the techniques for collecting the maximum number of viable spermatozoa from the male through preservation of the semen quality by adding suitable media, providing appropriate cooling, freezing, storage and thawing procedures<sup>[1]</sup>.

Baieeetal<sup>[2]</sup> reviewed that cryopreserved spermatozoa are susceptible to the changes of their conditions during freezing, thawing and processing, in which causing reduced fertility as compared with raw semen<sup>[3]</sup>. Spermatozoa from many species such as buck<sup>[4]</sup>, boars<sup>[5]</sup>, stallions<sup>[6]</sup>, rams<sup>[7]</sup> and deer<sup>[8]</sup> can be maintained at 4-15°C for time periods up to 24 h, depending on their species, before cryopreservation and still have acceptable frozen-thawed sperm quality. Moreover, bull sperm held at 4°C for 4 h maintain acceptable motility (52.5%)<sup>[9]</sup>. It might be that holding time prior to cryopreservation processing can have a useful effect since it has been shown that the incubation of semen before freezing has a beneficial effect<sup>[5]</sup>. This incubation could improve resistance of spermatozoa to cold shock. Furthermore, the incubation of semen can result in a reversion of sperm capacitation processes<sup>[10]</sup>. However, documentation concerning cryopreserving bull sperm following holding in different temperature have been limited. Therefore, by manipulating different

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holding temperature of the semen before cryopreservation may produce different effect to the spermatozoa in terms of viability, motility and morphology before and after freezing. Thus, the main objective of this study was to determine the effect of different holding temperatures before freezing on the quality of cryopreserved bull semen.

### Materials and Method

**Animal:** Four sexually mature Brangus cross bulls from Taman PertanianUniversiti, Universiti Putra Malaysia cattle farm were used for semen collection. The age of the bulls were within the range of 3 to 7 years old with body weights between 300 to 400 kg. These bulls were kept semi-intensively, fed with palm kernel cake and allowed to graze freely during the day. Commercial mineral block and water were given ad libitum. The sample collection was approved by Institutional Animal Care and Use Committee (IACUC;FYP.2016/FPV13,31).

**Experimental design:** Semen samples were divided into four groups. First group, the controlled group was kept in refrigerator at 4°C. Second group was kept in a cool water bath at 15°C inside an insulated thermal box. The third group was put in a water bath at 25°C inside an insulated thermal box. The last group of samples was put in a warm water bath at 37°C. All groups were

maintained at its respective temperatures 10 minutes before chilling.

### Semen collection and fresh semen evaluation:

In day of semen collection two semen samples were collected from two bulls using an automated electro-ejaculation method; two time per week<sup>[11]</sup>. The collection was repeated four times to get a total of sixteen semen. The semen samples were evaluated immediately based on macroscopic (volume, consistency and colour) and microscopic (spermatozoa concentration, motility, morphology and viability) characteristics. The semen sample was diluted 1 to 40 ratios with normal saline at water bath of 37°C for initial evaluation. Evaluation of spermatozoa concentration and general motility were made by using a Computer Assisted Semen Analyzer (CASA). Spermatozoa morphology at 1000× magnification and viability at 400× magnification were evaluated using eosin-nigrosin stain<sup>[12]</sup>. A total of 200 spermatozoa were examined under a light microscope. Dead spermatozoa were stained purple and live spermatozoa were unstained. Sperm abnormality was determined by examining 200 spermatozoa for defects associated with sperm head, mid-piece and tail.

Only semen sample that achieved the standard minimum criteria can be used for the experiment (Figure 1).

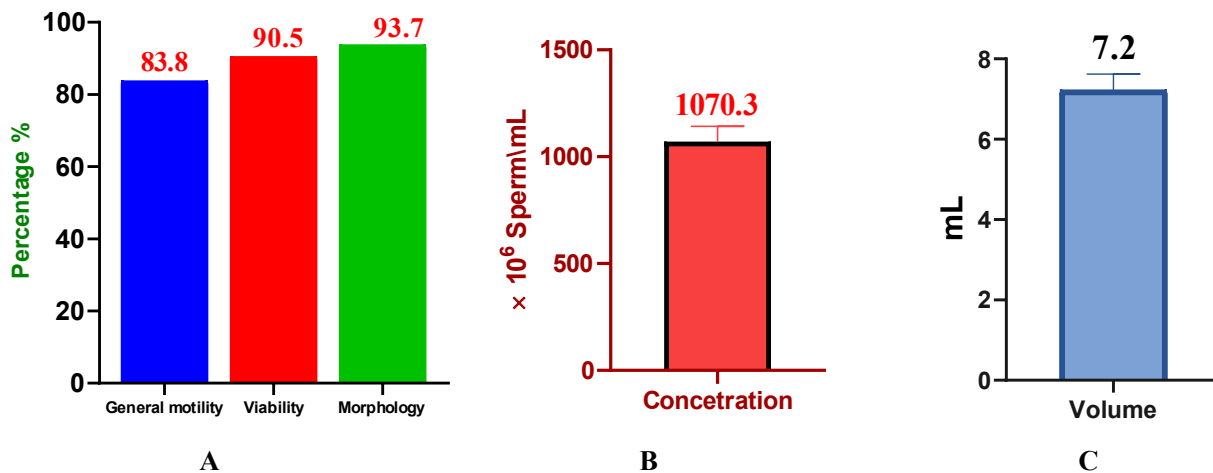


Figure 1: The initial evaluation of collected semen, A (sperm motility, viability and Morphology of sperm), B (Concentration of sperm) and C (volume of semen).

### Cryopreservation procedure

**Extender preparation:** Two extenders were prepared one without glycerol for chilling period and another with glycerol was added after the end of chilling period, 2.42g of Tris, 1.48g of citrate, 1g of fructose (Sigma-Aldrich) and 20 ml of fresh egg yolk were mixed with distilled water to produce 100 ml of Tris-egg yolk extender. The Same components with addition of 12.8ml glycerol were mixed with distilled water to produce 100 ml of Tris-egg yolk-glycerol extender<sup>[14]</sup>.

**Semen dilution, packing and freezing:** The semen sample was pre-diluted with Tris-egg yolk extender at water bath of 37°C. Four semen groups from each bull were kept at different holding temperature of 4, 15, 25 and 37°C respectively for 10 minutes. Then, all semen samples were transferred for chilling at temperature of 4°C and kept for more than 3 hours. After 3 hours, all the semen samples were evaluated using CASA and stain with eosin-nigrosin to determine the sperm general motility, progressive motility, viability and morphology.

The pre-diluted semen sample was then diluted with Tris-egg yolk-glycerol extender as final dilution under temperature of 4°C. The extended semen was packed into 0.25ml labelled French straws and sealed with sealing powder. Then after, the straws were subject to a slow gradual freezing process manually before they were stored in a liquid nitrogen tank following the method described by<sup>[15]</sup>.

**Frozen-thawed semen evaluation:** After 24 hours of storage, the straws were taken out from the liquid nitrogen tank and thawed in water bath with temperature of 37°C for 30 seconds. Then, the straws were cut and the semen samples were inserted into test tubes in the water bath with temperature of 37°C. All the post thawed semen samples were also evaluated using CASA and stain with eosin-nigrosin<sup>[12]</sup>.

**Statistical analysis:** All the data were tested for normality by using Shapiro-Wilk Test. The result of post chilling and post thawing evaluation were analysed by Kruskal-Wallis H Test using IBM SPSS Statistics 22 software (SPSS Inc., Chicago, IL, USA). Mann Whitney U Test was used to analyse the relationship between different pairs of treatment groups.

### Results

From the test of normality, Shapiro-Wilk Test showed that most of the data were not normally distributed,  $p < 0.05$ . Therefore, relevant non-parametric tests were selected to analyse the data from the experiment. The result of Kruskal-Wallis H Test for the post chilling evaluation revealed that there were no statistically significant difference ( $p > 0.05$ ) in sperm viability, sperm general motility, sperm progressive motility and sperm normal morphology between the different holding temperatures (Tables 1 and 2).

**Table 1: Mean value of post chilling evaluation of different holding temperature**

Temperature (°C)	Post Chilling Evaluation (% ± SEM)			
	Viability	General Motility	Progressive Motility	Normal Morphology
4	66.38 ± 4.74	58.88 ± 12.42	16.00 ± 4.23	94.56 ± 3.10
15	66.75 ± 6.45	62.38 ± 10.32	15.63 ± 3.47	93.56 ± 2.40
25	68.88 ± 5.90	65.25 ± 8.67	16.50 ± 3.15	95.50 ± 1.78
37	62.13 ± 5.17	62.50 ± 10.54	15.63 ± 3.04	96.00 ± 1.43

**Table 2: Mean Ranks for post chilling evaluation of different holding temperatures**

Post- Chilling	Temperature	N	Mean Rank
Viability	4	16	16.81
	15	16	17.88
	25	16	18.31
	37	16	13.00
	<b>Total</b>	<b>64</b>	

Post- Chilling	Temperature	N	Mean Rank
General Motility	4	16	17.69
	15	16	15.69
	25	16	16.94
	37	16	15.69
	<b>Total</b>	<b>64</b>	
Progressive Motility	4	16	18.19
	15	16	16.06
	25	16	17.25
	37	16	14.50
	<b>Total</b>	<b>64</b>	
Morphology	4	16	18.38
	15	16	13.38
	25	16	16.88
	37	16	17.38
	<b>Total</b>	<b>64</b>	

Besides, the result of Kruskal-Wallis H Test for the post thawing evaluation showed that there were no statistically significant difference in sperm viability,

sperm general motility, sperm progressive motility and sperm normal morphology between the different holding temperatures,  $p > 0.05$  (Tables 3 and 4).

**Table 3: Mean value of post thawing evaluation of different holding temperature**

Temperature (°C)	Post Thawing Evaluation (% ± SEM)			
	Viability	General Motility	Progressive Motility	Normal Morphology
4	58.25±7.29	32.13 ±9.06	6.25±2.34	96.00±2.37
15	56.63±4.18	27.63 ±6.74	4.63 ±1.48	96.25 ±0.97
25	56.38±5.05	31.88 ±6.84	5.13 ±1.77	95.69 ±1.77
37	55.88±5.58	36.63 ±9.61	7.00 ±2.26	95.06 ±2.23

**Table 4: Mean Ranks for post thawed evaluation of different holding temperatures**

Post thaw	Temperature	N	Mean Rank
Viability	4	16	16.69
	15	16	16.81
	25	16	16.63
	37	16	15.88
	<b>Total</b>	<b>64</b>	
General Motility	4	16	16.13
	15	16	15.00
	25	16	16.56
	37	16	18.31
	<b>Total</b>	<b>64</b>	

Post thaw	Temperature	N	Mean Rank
Progressive Motility	4	16	17.19
	15	16	15.25
	25	16	15.75
	37	16	17.81
	<b>Total</b>	<b>64</b>	
Morphology	4	16	19.25
	15	16	15.31
	25	16	15.69
	37	16	15.75
	<b>Total</b>	<b>64</b>	

On the other hand, the result of Wilcoxon Signed Rank Test for the fresh semen evaluation and the post thawing evaluation showed that there were statistically significant differences ( $p < 0.05$ ) in sperm viability, sperm general motility and sperm normal morphology. However, there was no statistically significant difference ( $p > 0.05$ ) in sperm progressive motility.

From the result of Mann-Whitney U Test for the post chilling evaluation, there were no significant differences ( $p > 0.05$ ) in sperm viability, sperm general motility, sperm progressive motility and sperm normal morphology between the holding temperature of 4°C and 15°C, 4°C and 25°C, 4°C and 37°C, 15°C and 25°C, 15°C and 37°C, 25°C and 37°C. Furthermore, the result of Mann-Whitney U Test for the post thawing evaluation showed that there were no significant differences ( $p > 0.05$ ) in sperm viability, sperm general motility, sperm progressive motility and sperm normal morphology between the holding temperature of 4°C and 15°C, 4°C and 25°C, 4°C and 37°C, 15°C and 25°C, 15°C and 37°C, 25°C and 37°C.

## Discussion

In this study, Tris-egg yolk extender containing citrate and fructose as energy supplement to the spermatozoa were used to extend their life spans [16]. Approximately 20% of egg yolk in the extender is used as standard level for most case of bull semen cryopreservation [17]. Tris-egg yolk extender was not used as diluent during fresh semen evaluation because CASA may count the egg yolk granules from the Tris-egg yolk extender as individual sperm in which will affect the outcome.

After cooling the semen sample at 4°C in the refrigerator for more than 3 hours, Tris-egg yolk-glycerol extender was used to dilute the semen before packing for cryopreservation. Tris-egg yolk-glycerol extender gives excellent protection for sperm either frozen or unfrozen [18]. Spermatozoa are sensitive to the changes in the osmolality of the surrounding solution, in which they will lose their motility irreversibly if the osmolality is intolerable [19]. Therefore, we have to be aware of the dilution factor used to dilute the semen with extender for cryopreservation.

There were statistically significant differences in the quality of bull semen before and after cryopreservation. Cooling and freezing of spermatozoa during process of cryopreservation causes reduction of temperature of the spermatozoa and its surrounding solution. This change in temperature induces sperm plasma membrane damage which leads to a state of sperm abnormal morphology such as abnormal acrosome [13]. The damaged sperm plasma membrane allows influx of calcium ions which resulted in abnormally high amount of intracellular calcium ions in the spermatozoa. This phenomenon contributes to the premature capacitation of spermatozoa [20]. It had been reported that there was variation among individuals in the extent to which their spermatozoa are damaged by freeze-thawing [21]. This individual differences particularly for bull semen have been recorded and to cover this difference, freezing protocols were adjusted for individual bulls or packaging straws with more spermatozoa for “poor freezers”. The effect of different temperature to the quality of spermatozoa may be varies for different individuals from the same species. Therefore, the accuracy of result may be reduced due to this factor.

## Conclusion

In this study, it can be concluded that different holding temperatures before freezing do not affect the quality of bull semen significantly. However, the process of cooling and freezing during cryopreservation affects the quality of bull semen significantly. Besides, more time and numbers of suitable samples should be required to improve this study. These will allow for more tests to be conducted for different parameters and more samples can be processed. Further investigation regarding the effect of different holding temperature before freezing should be done.

**Ethical Clearance:** The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

**Conflict of Interest:** Non

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