# EVALUATION OF SPERMATOZOA QUALITY TRAITS USING TWO DIFFERENT CHILLING METHOD IN BULLS

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**ABSTRACT.** This study was conducted to evaluate the spermatozoa quality in bulls before and after freezing using two different chilling methods (chiller cabinet versus refrigerator). Six parameters were selected: progressive motility, motility, viability of spermatozoa (live), viability of spermatozoa (dead), post-thawed progressive motility and post-thawed motility. A total of 54 semen samples were collected and examined. The percentage of spermatozoa quality traits were compared between using a chiller cabinet (4 °C to 5 °C) and a refrigerator (0 °C to 10 ˚C). The results showed significant differences ($p$<0.0.5) in progressive motility, viability of live spermatozoa, viability of dead spermatozoa, post-thawed progressive motility and post-thawed motility. Although, there was no significant difference in motility between both methods, a higher percentage of spermatozoa quality from semen stored in the chiller cabinet than the refrigerator was observed. The results of chiller cabinet versus refrigerator, respectively, for progressive motility were 65.2 ±1.73% versus 59.6±1.38%, viability of live spermatozoa 70.0±3.41% versus 63.1±2.88%, viability of dead spermatozoa 30.0±3.41% versus 36.9±2.88%, post-thawed progressive motility 52.5±1.83% versus 44.9±2.23% and post-thawed motility 74.3±1.79% versus 57.8±2.27%. Despite the significant differences between both chilling methods, the refrigerator can still be used as an alternative solution in the field for chilling of semen chilling due to its size, ease of maintenance, low economic value and practicality.

*Keywords*: spermatozoa quality, progressive motility, cryopreservation, bull, refrigerator

## INTRODUCTION

Artificial insemination (AI) of cattle in the field of reproductive biotechnology is expanding rapidly with increasing demand for cryopreserved semen using genetically superior bulls. Since AI contributes to genetic improvements, only successful freezing of spermatozoa can make AI possible. The contributory factors for success depends on the management of semen collection, storage and use (Nilani *et al*., 2012). In addition, technical skills in AI and targetting the oestrus cycle of females are also important factors for success.

Cryopreserved semen of ruminants for the livestock industry in Malaysia including cattle, sheep, goat and buffalo are sourced from the National Institute of Veterinary Biodiversity (NIVB) in Jerantut, Pahang. The production of cryopreserved semen in NIVB complies with Malaysian Standard (MS ISO 9001:2008) procedures.

A crucial step in extending the life span, and hence viability, of spermatozoa is through cryopreservation. The first bovine cryopreservation protocol was introduced in the 1950s.

Cryopreservation slows down the metabolic rate of spermatozoa at which substrates are used and toxins produced (Lemma, 2015). It involves several steps such as dilution, cooling, freezing and thawing (Hamed *et al.,* 2013; Anghel and Zamfirescu, 2010). Each of these steps can damage or alter the membrane structure and function of normal spermatozoa (Alok *et al.,* 2018). The survival of spermatozoa of all warm blooded organisms vary according to the temperature at which they are stored (Amnon *et al.,* 1981).

In NIVB, fresh bull semen is diluted with commercial semen extenders (Bioxcell), then stored in a chiller cabinet (4 °C to 5 °C) for 2 to 3 hours before semen is filled into a straw and frozen at -196 °C in liquid nitrogen.

In conducting the cryopreservation work in the field, an alternative method needed to be considered because of limited facilities and variation in temperature due to environment and weather.

The most basic and available chilling medium in the field was a refrigerator. This study evaluates the effects caused by two chilling methods (chiller cabinet versus refrigerator) on semen quality based on six parameters: motility, progressive motility, viability of live spermatozoa, viability of dead spermatozoa, post-thawed motility and post-thawed progressive motility.

## MATERIALS AND METHODS

### Sample Collection

The study was conducted at NIVB in Jerantut, Pahang, from May to July 2018. 54 semen samples were collected from selected bulls such as Jersey, Mafriwal, Sahiwal and Belgian Blue. Semen was collected using an artificial vagina (AV). Each bull contributed one to three ejaculations. Each ejaculate was stored in an insulated jacket and immediately transported to the laboratory for processing and evaluation.

### Semen Processing and Analysis

At the NIVB laboratory, the concentration of fresh semen from each jacket was calculated using SpermaCue (Minitüb GmbH, Germany) photometer. A test sample of 10 µl fresh semen was diluted with 90 µl BioXcell™ extender (IMV Technologies, France). A few drops of the mixture was evaluated using Computed Assisted Sperm Analyser (CEROS System, Hamilton Thorne Inc., USA) and recorded. Fresh semen showing progressive motility greater than 60% were selected for the study.

The selected fresh semen was diluted with BioXcell™ extender. 3.0 ml of diluted semen was put into two test tubes, each containing 1.5 ml of diluted semen. One tube was put into a chiller cabinet (4 °C to 5 °C) as control while the other was put into a refrigerator (0 °C to 10˚C) for the chilling process.

In order to prevent cold shock, the test tubes were put in a beaker of water placed in a water bath at 37 °C (called water jacket) for

the first hour. After 2 hours, semen samples were taken for post-chilling tests.

Progressive motility and motility of the semen were evaluated and recorded. At the same time, a viability test of live and dead spermatozoa was carried out using 1% eosin-nigrosine stain and then viewed under a microscope. The percentage of live and dead spermatozoa were recorded. Semen was filled and packed into 0.25 ml straws.

Semen straws were arranged on a cold rack in a chiller cabinet to maintain its temperature. The rack was placed above the surface of liquid nitrogen in a freezing vapour container and gradually frozen in liquid nitrogen vapour at temperatures between -70 °C to -120 °C for nine minutes. After that, the straws were stored by immersing into liquid nitrogen at -196 °C.

On the fifteenth day the straws were thawed in a water bath at 37 °C for 45 seconds and then assessed. Progressive motility and motility of post-thawed semen were evaluated and recorded.

**Semen Evaluation**

Evaluations were carried out on fresh semen, before and after freezing (15th day). Parameters involved in this study were motility, progressive motility, viability of live spermatozoa, viability of dead spermatozoa, post-thawed motility and post-thawed progressive motility. Motility and progressive motility for both states were analysed using Computed Assisted Sperm Analyser (CEROS System, Hamilton Thorne Inc., USA). The viability test was using 1% eosin-nigrosine stain. A total of 100 spermatozoa were observed and calculated under a light microscope for evaluation of live and dead spermatozoa based on their stain characteristics. Dead spermatozoa are pink-purple stained due to absorption of eosin-nigrosine while those in white are considered alive.

**Statistical Analysis**

The data were analysed with the Statistical Package for the Social Sciences (SPSS, Version 20; IBM, Chicago, IL, USA) using the Mann-Whitney test. The comparision between the two chilling methods were assessed to determine the significant differences. The results were expressed as median ± IQR (interquartile range). *p*-values less than 0.05 were considered as statistically significant.

**RESULTS AND DISCUSSION**

The mean values of spermatozoa quality parameters are presented in Table 1. Fresh semen with greater than 60% in progressive motility was selected for the study.

There was no significant difference in the percentage of motility between semen stored in chiller cabinet (84.50±8.00%) and refrigerator (84.00±14.25%) at $p>0.05$. Progressive motility of spermatozoa was higher for semen stored in chiller cabinet (69.00 ±11.00%) than refrigerator (61.00±11.50%) at *p*-value less than 0.05 ($p<0.000$). Other parameters also showed a higher percentage of spermatozoa quality for semen in chiller cabinet than refrigerator, respectively as follows: viability of live spermatozoa was 77.00±22.00% versus 71.00±22.00%, viability of dead spermatozoa was 23.00±22.00% versus 29.00±22.00%,

**Table 1**: The effect of chilling methods on spermatozoa quality traits (%) using chiller cabinet and refrigerator.

| Semen Quality Traits | No.of sample | Mean (sem) | | Z statistic | P-value* | Significant |
|---|---|---|---|---|---|---|
| | | Chiller Cabinet | Refrigerator | | | |
| Progressive Motility | 54 | 69.00 (11.00) | 61.00 (11.50) | -3.599 | 0.000** | Yes |
| Motility | 54 | 84.50 (8.00) | 84.00 (14.25) | -1.247 | 0.213 | No |
| Alive Sperm | 54 | 77.00 (22.0) | 71.00 (20.00) | -2.419 | 0.016** | Yes |
| Dead Sperm | 54 | 23.00 (22.00) | 29.00 (20.00) | -2.419 | 0.016** | Yes |
| Post-thawed Progressive Motility | 54 | 52.50 (24.25) | 46.00 (29.75) | -2.202 | 0.028** | Yes |
| Post-thawed Motility | 54 | 76.50 (16.75) | 61.00 (29.25) | -4.792 | 0.000** | Yes |

* Mann-Whitney test

** Test significant when $p<0.05$

post-thawed progressive motility was 52.50±24.25% versus 46.00±29.75% and post-thawed motility was 76.5±16.7% versus 61.00±29.25%). Overall, the results showed significant difference ($p<0.05$) in progresive motility, viability of live spermatozoa, viability of dead spermatozoa, post-thawed progressive motility and post-thawed motility between the two storage modes.

In a cryopreservation process, the crucial steps are dilution, chilling, freezing and thawing. Previous researchers found that each of these steps can damage the membrane plasma structure and negatively affect the normal function of spermatozoa such as spermatozoa viability, acrosome integrity, formation of toxic compounds and metabolic byproducts (Nilani *et al.,* 2012, Amnon *et al.,* 1981, Angelica *et al.* 2016, Hamed *et al.,* 2013). The storage of diluted semen at low temperatures helps to extend spermatozoa lives by slowing down the metabolic activities and inhibiting bacterial growth (Nilani *et al.,* 2012). Amnon *et al.* (1981) also reported varing the temperatures (0 °C to 48 °C) of storage medium can immediately change the motility of spermatozoa. According to current and previous studies, this explains the better spermatozoa quality of semen stored in the chiller cabinet, as its temperature is lower with a smaller range than the temperature of the refrigerator used (Nilani *et al.*, 2012; Amnon *et al.*, 1981; Angelica *et al.*, 2016; Hamed *et al.*, 2013).

According to the National Dairy Development Board (NDDB), the minimum requirement for semen post-thawed motility that can be used in AI is 50%. This study showed both methods had successfully generated spermatozoa quality above the NDDB standard, that is 74.3% for chiller cabinet and 57.8% for refrigerator. Therefore, this study shows that the usage of a refrigerator can be an alternative to a chiller cabinet for use in a cryopreservation proccess in the field. This finding is also support by Aboagle and Terada (2003) where it was recommended for the usage of the refrigerator as a storage medium

of diluted semen as it helps to stop the metabolic process of stored liquid semen, thus extending the lives of spermatozoa.

## CONCLUSION

This study found that a refrigerator can be used as an alternative method in the initial process of bovine semen cryopreservation. Therefore, its use may be recommended for work in the field, instead of a chiller cabinet, due to its size, ease of maintenance, economic and practical value. It is hoped that better semen management may contribute to higher success rates in AI programmes and thus, improved cattle productivity in Malaysia.

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