



## Optimum Level of Liquid Nitrogen in Container Required for Maintenance of Semen Quality

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### Abstract

This study was conducted to determine the effect of different level of liquid nitrogen on post thaw seminal motility to estimate optimum amount of liquid nitrogen required in container for maintainance of semen quality. Semen of the three bulls (Murrah Breed) were collected in weekly rotation and semen straw were produced and frozen as per the protocol of NLBO (National Livestock Breeding Office) at Pokhara, Kaski, Nepal. The semen straws were stored in 3 litre container to study the effect of different level of liquid nitrogen on post thaw motility of sperm. Overall mean value of post thaw motility were  $56.88 \pm 8.05$ ,  $53.54 \pm 6.83$ ,  $48.96 \pm 8.72$ , and  $30.21 \pm 9.49$  when the level of liquid nitrogen was full, 3/4th, 1/2 and 1/4th of the level of frozen semen straw. The mean value of post thaw motility was best observed during the level of liquid nitrogen at full, 3/4th and 1/2 and decline significantly when the level of liquid nitrogen reached to 1/4th level of semen straw in container. Therefore, nitrogen level should not fall below 1/2 of the level of straw during storage even despite the fact that longevity of freezing has no effect in PTM (Post Thaw Motility).

**Keywords:** Liquid nitrogen; Quality; Semen; PTM; NLBO

### Introduction

Spermatozoa of the bull can be stored at liquid nitrogen temperature ( $-196^{\circ}\text{C}$ ) for indefinite periods and after thawing retain relatively high rate of fertility [2]. There is no further loss in quality of frozen semen which is continuously stored at  $-196^{\circ}\text{C}$  under the surface of liquid nitrogen [8]. Maintaining optimum liquid nitrogen level in container is very essential during prolonged storage and transportation of semen [7]. It is considered that storage period under deep freezing does not impair sperm viability. There is an argument that spermatozoa keep their fertilizing capacity eternally whether stored at  $-79^{\circ}\text{C}$  in dry ice or at  $-196^{\circ}\text{C}$  in liquid nitrogen [10]. In a contrasting perspective, certain observations have led to the conclusion that cryopreserved semen are reactively equal to capacitated fresh sperm, however the surviving popula-

tion showed poor fertility after insemination in the reproductive tract compared to an equivalent amount of fresh motile spermatozoa [6]. But, lack of optimum liquid nitrogen even for few hours in the same container may result in complete destruction of sperm [3].

### General objective

- To estimate the optimum level of liquid nitrogen in container (referee) for the maintenance of semen quality.

### Specific Objective

- To study the effect of different levels of liquid nitrogen on post thaw seminal motility.
- To find the best level of liquid nitrogen for storage of semen in container.

**Literature Review**

The post thaw motility was 45-60%, 45-55%, 40-50% and 20-40% at full, 3/4<sup>th</sup>, 1/2<sup>th</sup> and 1/4<sup>th</sup> level of liquid nitrogen respectively [4]. Overall percent of post thaw motility was significantly higher (P<0.05) when the level of liquid nitrogen was full and 3/4<sup>th</sup> of the level of straws as compared to 1/2<sup>th</sup> and 1/4<sup>th</sup> level of straws [9]. The overall percentage of post thaw motility was significantly higher (P < 0.05) when the level of straws as compared to 1/4<sup>th</sup> level of straws in Sahiwal and Red Sindhi bulls [5]. However, frozen semen straws can be stored in 2 litre liquid nitrogen container without refilling liquid nitrogen and showed a gradual decrease in percent post thaw motility after 10 days of storage [1].

**Materials and Method**

The study was conducted on three bulls (Murrah Breed) at NLBO Farm. Bull ID: - 107, 108 and 109.

**Materials**

- Container filled with liquid nitrogen (3 litre)
- Frozen semen straw.
- Scale to measure liquid nitrogen level.
- CASA system (Computer-Assisted Semen Analysis)
- Water bath
- Thawing thermometer
- Slide and cover slip.
- Tissue paper and Notebook

**Method**

All the bulls were maintained in identical feeding and management regimen according to the standard protocol of NLBO. Semen from experimental bulls was collected twice a week (Monday and

Thursday) in the morning hours in between 6-8 am before feeding. Immediately after collection, the semen was kept at 37°C in a water bath placed inside the pass box. The procedure for semen collection, semen straw production and freezing were done as per the protocol of NLBO, Pokhara, Kaski, Nepal.

**Frozen Semen Production Procedure using protocol of NLBO.**

Steps followed for producing frozen semen

- Preparation of Buffer solution
- Preparation of Semen Extender
- Collection of semen using AV.
- Evaluation and extension of neat semen.
- Filling, sealing, and printing of straws by automatic machine.
- Racking of filled semen straws and equilibration of semen.
- Freezing.
- Frozen semen storage.

Two liquid nitrogen container of volume 3 litres were taken, one for experimental unit and another for control. Thirty-two (32) semen straw from each bull was stored in liquid nitrogen container. These straws were evaluated for PTM (Post Thaw Motility) when the level of liquid nitrogen was full and when the level of liquid nitrogen reached to 3/4<sup>th</sup>, 1/2<sup>th</sup> and 1/4<sup>th</sup> level of straws. Frozen semen sample were thawed in water bath maintained at 37°C for 30 seconds and content will be evacuated in sterile tube by cutting seal. Then finally Post Thaw Seminal Motility was determined by CASA system (Palmary Software) as shown in image below.

**Results and Discussion**

The finding on the effect of different level of liquid nitrogen on post thaw motility percent are presented in given table 1.

Bull ID	Post Thaw Sperm Motility (Percentage) at different Level of liquid nitrogen in container			
	Full	3/4 <sup>th</sup>	1/2 <sup>nd</sup>	1/4 <sup>th</sup>
107	60.00 ± 8.01	55.63 ± 4.17	53.75 ± 4.43	34.38 ± 5.63
108	48.75 ± 4.43	45.63 ± 3.20	38.75 ± 5.82	19.38 ± 4.17
109	61.88 ± 3.72	59.38 ± 3.20	54.38 ± 4.17	36.88 ± 6.51
Total	56.88 ± 8.05	53.54 ± 6.83	48.96 ± 8.72	30.21 ± 9.49

**Table 1:** Effect of different level of liquid nitrogen on PTM.

The post thaw motility was  $56.88 \pm 8.05$ ,  $53.54 \pm 6.83$ ,  $48.96 \pm 8.72$  and  $30.21 \pm 9.49$  at full, 3/4<sup>th</sup>, 1/2 and 1/4<sup>th</sup> of the straw level of liquid nitrogen respectively. The overall percentage of post thaw motility was significantly higher (P value = 5.08E-19) when the level of liquid nitrogen was full and 3/4<sup>th</sup> as compared to 1/2 and 1/4<sup>th</sup> level of straw. PTM was significantly higher (P < 0.01) when the level of liquid nitrogen was full and 3/4<sup>th</sup> the level of straws as compared to 1/2 and 1/4<sup>th</sup> the level of straw (Sonar, *et al*, 2013). KEDIA., *et al*, (2013) reported that post thaw motility was significantly higher (P < 0.01) when the level of liquid nitrogen was full, 3/4<sup>th</sup> and 1/2 of the level of straw as compared to 1/4<sup>th</sup> level of straw. Similar effect of level of liquid nitrogen was observed in Sahiwal and Red Sindhi bull (Pathak, 2008). Das., *et al*. (2002) reported that frozen semen straws can be stored in 2 litre liquid nitrogen container without refilling liquid nitrogen and showed a gradual decrease in percent post thaw motility after 10 days of storage. A significant decline in motility may be due to decline in post thaw cytomorphological characteristics (Sonar, *et al*, 2013).

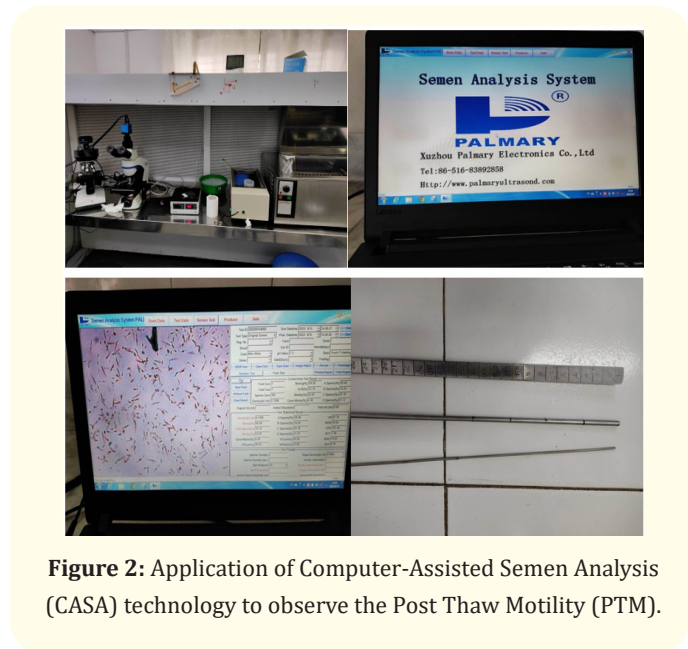


Figure 2: Application of Computer-Assisted Semen Analysis (CASA) technology to observe the Post Thaw Motility (PTM).

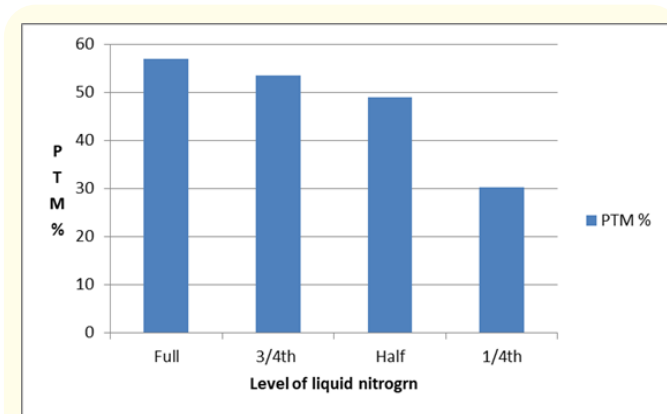


Figure 1: Bar diagram showing effect of level of liquid nitrogen on Post thaw motility.

### Conclusion and Recommendation

On the basis of percentage of post thaw motility at different level of liquid nitrogen in container, it was concluded that the post thaw

motility of sperms decreased on progressive decline in the level of liquid nitrogen in container. Therefore, to ensure better post thaw motility, the level of liquid nitrogen should not fall below 1/2 the level of straw while storage. Hypo-Osmotic Swelling Test (HOST), Cervical Mucus Penetration Test (CMPT) and Post-Thaw Viability Test are recommended for further study.

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Report					
Bull ID		full	3/4 <sup>th</sup>	Half	1/4 <sup>th</sup>
107	Mean	60.00	55.63	53.75	34.38
	N	8	8	8	8
	Std. Deviation	8.018	4.173	4.432	5.630
108	Mean	48.75	45.63	38.75	19.38
	N	8	8	8	8
	Std. Deviation	4.432	3.204	5.825	4.173
109	Mean	61.88	59.38	54.38	36.88
	N	8	8	8	8
	Std. Deviation	3.720	3.204	4.173	6.512
Total	Mean	56.88	53.54	48.96	30.21
	N	24	24	24	24
	Std. Deviation	8.050	6.833	8.720	9.496

Annex 1

Source of Variance	ss	df	MS	F	p-value	F-crit.
Between groups	10211.45833	3	3403.819444	49.02565775	5.08E-19	2.703594041
Within groups	6387.5	92	69.42934783			
Total	16598.95833	95				

Annex 2

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