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## Evaluation of different cryoprotectant combinations on post-thaw quality of beetal buck semen

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### Abstract

Cryopreservation is defined as the preservation of biological material at ultra-low temperatures, and its application to buck semen remains challenging due to the high sensitivity of caprine spermatozoa to cold shock and osmotic stress. This research compared five cryoprotectant formulations on post-thaw semen quality in Beetal bucks maintained at a breeding centre in Rabat, Morocco. Ejaculates from six mature bucks were split into five treatment groups: glycerol 7% (G7), DMSO 5% (D5), ethylene glycol 5% (EG5), glycerol 3.5% + DMSO 2.5% (GD), and glycerol 3.5% + trehalose 100 mM (GT). Post-thaw progressive motility was highest in GD ( $51.3 \pm 6.8\%$ ) followed by GT ( $46.1 \pm 7.9\%$ ), both significantly exceeding G7 ( $42.4 \pm 8.1\%$ ,  $p < 0.05$ ). Membrane integrity (HOST) followed a similar pattern: GD  $58.7 \pm 5.4\%$  vs G7  $47.2 \pm 6.9\%$  ( $p < 0.01$ ). Acrosome integrity was highest in GT ( $64.3 \pm 5.1\%$ ). These results indicate that combining glycerol with DMSO or trehalose offers superior cryoprotection compared to single-agent protocols for Beetal buck spermatozoa.

**Keywords:** Cryopreservation, buck semen, Beetal goat, cryoprotectant, glycerol, DMSO, trehalose, post-thaw motility, membrane integrity, sperm quality

### Introduction

Cryopreservation of buck semen serves as the cornerstone of genetic dissemination programmes in goat breeding, enabling year-round artificial insemination independent of seasonal breeding constraints <sup>[1]</sup>. Yet caprine spermatozoa present unique cryobiological challenges: their membranes contain higher proportions of polyunsaturated fatty acids and cholesterol-to-phospholipid ratios that differ markedly from bovine sperm, making them more vulnerable to lipid peroxidation and cold shock during freezing <sup>[2]</sup>. Recovery rates after thawing rarely exceed 50% progressive motility even under optimal conditions, compared with 60-70% routinely achieved with bull semen <sup>[3]</sup>.

Glycerol has served as the default cryoprotectant for decades, but its known cytotoxicity at concentrations above 6-8% has prompted investigation of alternatives and combination strategies <sup>[4, 5]</sup>. DMSO penetrates cell membranes more rapidly, potentially reducing osmotic damage during equilibration. Non-penetrating agents such as trehalose stabilise membranes from the outside through preferential exclusion interactions <sup>[6]</sup>. Despite growing evidence that multi-agent cryoprotectant cocktails outperform single agents in several species, data specific to Beetal bucks—an economically important dairy breed in North Africa and South Asia—remain limited <sup>[7]</sup>. This research evaluated five cryoprotectant formulations to identify the combination yielding optimal post-thaw semen quality.

### Materials and Methods

#### Materials

Six mature Beetal bucks (3-5 years, proven fertility) maintained at the National Goat Breeding Centre, Rabat, Morocco, served as semen donors. The research was approved by the Hassan II Institute Animal Ethics Committee (Protocol HIB-AEC-2023-017). Semen was collected twice weekly by artificial vagina over eight weeks (March-May 2023), yielding 96 ejaculates. Only ejaculates with initial motility  $\geq 75\%$  and concentration  $\geq 2.5 \times 10^9$  sperm/mL were included (n

= 82).

## Methods

Each qualifying ejaculate was diluted in Tris-citric acid-fructose-egg yolk extender and split equally into five aliquots receiving: G7 (glycerol 7%), D5 (DMSO 5%), EG5 (ethylene glycol 5%), GD (glycerol 3.5% + DMSO 2.5%), or GT (glycerol 3.5% + trehalose 100 mM). Samples were cooled to 5°C over 2 hours, equilibrated for 4 hours, loaded into 0.25 mL French straws, and frozen in liquid nitrogen vapour (4 cm

above LN<sub>2</sub> surface for 10 minutes) before plunging [8]. Thawing was at 37°C for 30 seconds. Post-thaw evaluations included progressive motility (CASA, Hamilton Thorne IVOS II), membrane integrity (hypo-osmotic swelling test, 150 mOsm), acrosome integrity (FITC-PNA staining), and viability (eosin-nigrosin). Data were analysed by repeated-measures ANOVA with Tukey post-hoc ( $p < 0.05$ ).

## Results

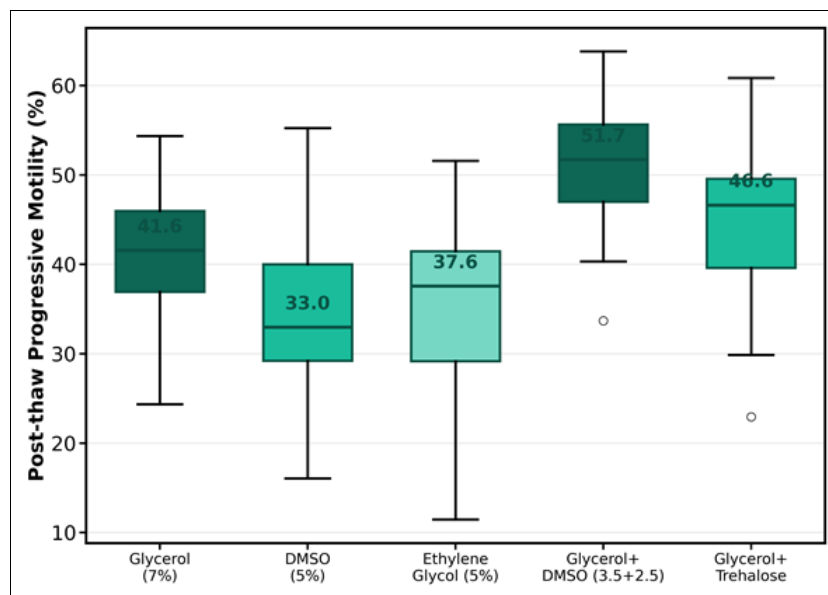


**Fig 1:** Post-thaw progressive motility across five cryoprotectant treatment groups, showing median, interquartile range, and individual data distribution

The glycerol-DMSO combination (GD) consistently produced the highest post-thaw motility ( $51.3 \pm 6.8\%$ ), exceeding all single-agent protocols. Ethylene glycol performed poorest

across all parameters. Viability followed motility trends, with GD achieving  $59.4 \pm 5.8\%$  compared to  $48.6 \pm 7.3\%$  for EG5.

**Table 1:** Post-thaw semen quality parameters across cryoprotectant groups

| Parameter     | G7       | D5       | EG5      | GD        | GT        |
|---------------|----------|----------|----------|-----------|-----------|
| Motility (%)  | 42.4±8.1 | 38.2±9.7 | 35.1±8.6 | 51.3±6.8* | 46.1±7.9  |
| Viability (%) | 52.3±6.4 | 49.8±7.1 | 48.6±7.3 | 59.4±5.8* | 55.7±6.2  |
| HOST (%)      | 47.2±6.9 | 44.1±8.2 | 41.8±7.6 | 58.7±5.4* | 53.4±6.1  |
| Acrosome (%)  | 56.8±5.7 | 52.4±6.8 | 49.7±7.1 | 61.2±4.9  | 64.3±5.1* |

\* Significantly different from G7 ( $p < 0.05$ )

**Table 2:** Sperm kinematic parameters (CASA) by treatment group

| Parameter  | G7        | D5        | EG5       | GD         | GT         |
|------------|-----------|-----------|-----------|------------|------------|
| VCL (μm/s) | 98.4±12.3 | 91.7±14.1 | 87.2±13.8 | 112.6±10.4 | 104.8±11.7 |
| VSL (μm/s) | 52.1±8.7  | 47.3±9.4  | 44.6±10.1 | 61.8±7.2   | 56.4±8.3   |
| LIN (%)    | 52.9±4.8  | 51.6±5.3  | 51.1±5.7  | 54.9±4.1   | 53.8±4.6   |

**Table 3:** Lipid peroxidation (MDA) and antioxidant enzyme activity post-thaw

| Marker        | G7        | D5        | EG5       | GD         | GT         |
|---------------|-----------|-----------|-----------|------------|------------|
| MDA (nmol/mL) | 4.82±0.71 | 5.14±0.83 | 5.47±0.92 | 3.61±0.54* | 3.89±0.63* |
| SOD (U/mL)    | 12.4±2.1  | 11.8±2.4  | 10.9±2.7  | 15.7±1.8*  | 14.3±2.0   |
| CAT (U/mL)    | 8.7±1.4   | 8.2±1.6   | 7.6±1.8   | 10.9±1.2*  | 10.1±1.3   |

\* Significantly different from G7 ( $p < 0.05$ )

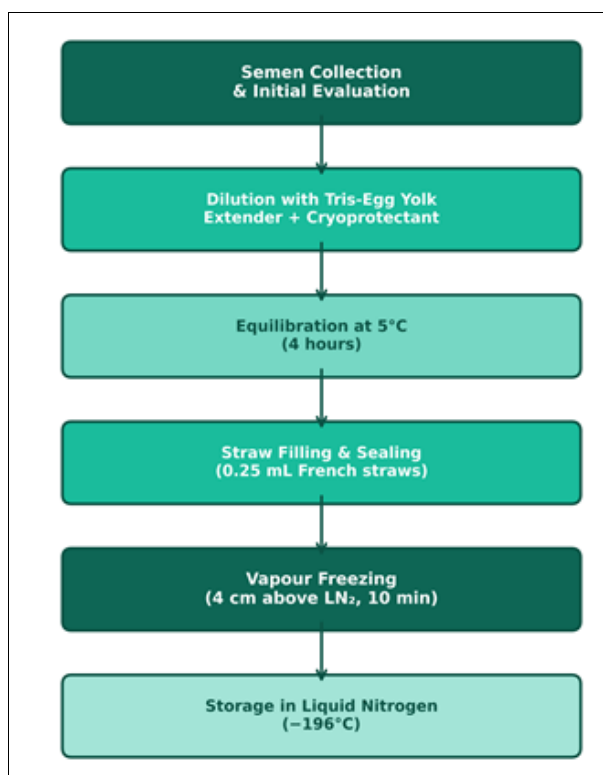


**Fig 2:** Standardised cryopreservation protocol employed for all treatment groups

## Discussion

The superior performance of the glycerol-DMSO combination aligns with the complementary mechanisms hypothesis: glycerol provides intracellular cryoprotection through colligative effects while DMSO's rapid membrane permeation minimises osmotic damage during the addition/removal steps [4, 9]. Our motility results (51.3%) exceed those reported by Purdy [3] for single-agent glycerol protocols in Boer goats (38-45%), suggesting that dose reduction of each penetrating agent below individual toxicity thresholds is the key advantage of combination protocols.

Trehalose's protective effect on acrosomal membranes (64.3% integrity in GT vs 56.8% in G7) supports its role as an extracellular stabiliser through the vitrification and water replacement hypotheses [6]. The lower MDA levels in GD and GT groups indicate reduced oxidative damage, consistent with better membrane preservation allowing sustained antioxidant enzyme activity post-thaw [10].

## Limitations

The research used a single breed at one location, limiting generalisability. Fertility trials (AI outcomes) were not conducted. The 8-week collection period didn't capture seasonal variation in semen quality. Only one concentration per cryoprotectant was tested.

## Conclusion

Within the context of Beetal buck semen cryopreservation, the glycerol 3.5% + DMSO 2.5% combination delivered the best overall post-thaw quality, while glycerol + trehalose was superior for acrosome preservation. These formulations deserve field-scale AI fertility trials. Future research should explore dose-response relationships and inclusion of antioxidant supplements in combination extenders.

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