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SHORT COMMUNICATION
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Viability of Cryopreserved Goat Sperm Cells as Influenced by Proline Supplemented Semen Extender

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ABSTRACT

Artificial insemination has transformed animal production. The technology facilitated rapid genetic improvement, maximizing the potential of male animal breeders. One component of the technology is cryopreservation. The process involves several techniques for cooling, freezing, and thawing. Exposing sperm cells to different temperatures can reduce sperm viability, thus affecting fertility. The supplementation of amino acids in the semen freezing medium served a crucial biological role in preventing cell damage. The current study aimed to investigate the effect of proline supplementation on the motility and velocity of cryopreserved goat sperm. Various concentrations of medium supplementation were used to cryopreserve goat semen (0, 0.001, 0.002 and 0.003g). The processed goat sperm cells were subjected to the slow-freezing method of cryopreservation. Post-thaw sperm motility and velocity were assessed using the Computer Aided Sperm Analyzer Machine. Based on the results obtained from the experiment, it was noted that the addition of 0.001 and 0.002 g of proline significantly ($P<0.05$) enhanced overall motility, progressive motility, and rapid motility, while reducing immotile sperm cells. The velocity, on the other hand, remained constant across treatments. According to these results, proline supplementation rates in the freezing medium, ranging from 0.001 to 0.002g, enhanced the quality of post-thaw goat sperm during cryopreservation.

Keywords: Cryopreservation; Goat, Frozen sperm; Motility, Proline, Amino acid.

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INTRODUCTION

The use of sperm cryopreservation technology on artificial insemination in domestic animals is highly encouraged, as it significantly aids in the preservation of genetic materials using various cryoprotective agents. Sperm cell cryopreservation is a process that includes cooling, freezing, and thawing (Zhang et al., 2022). When the cells are subjected to these processes, they are exposed to irreversible damage due to cold shocks (Azimi et al., 2020), osmotic stress due to extracellular ice formation (Salmon et al., 2016) and oxidative stresses (Farshad & Hosseini, 2013) which lead to reduce fertilization capability (Merati et al., 2018). Zhang et al. (2022) reported that sperm experience reactive oxygen species (ROS) stress during cryopreservation. Excessive ROS can harm sperm quality by disrupting mitochondrial function (Gadea et al., 2004) which reduces sperm motility. Studies have shown that ROS-induced oxidative stress is a primary cause of low post-thaw survival and quality, compromising its functional capacity in assisted reproductive technologies (Bucak et al., 2009; Bansal & Bilaspuri, 2011; Zhu et al., 2019) including artificial insemination. In addition, ROS can trigger apoptosis pathways affecting sperm motility, DNA integrity, and overall survival rates after thawing (Said et al., 2010).

The building blocks of peptides and proteins are amino acids. The supplementation of amino acid has already shown to improve growth performance and reproductive performance of ruminant species such as cattle, sheep and goats (Ny et al., 2022). Amino acids play a vital role in preventing cell damage during cryopreservation (Kutluyer & Kocabas, 2016). The antioxidant properties of amino acids may help achieved equilibration time during freezing and shield the sperm from cold shock (Sangeeta et al., 2015; Zhang et al., 2022). The role of amino acids in sperm freezing has been investigated in stallions, humans, and rams. Moreover, multiple studies found that adding amino acids (e.g., taurine, proline, glutamine, glycine to histidine,

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and methionine) to extenders reduced sperm damage and DNA fragmentation while improving post-thaw motility (Ros-Santaella & Pintus, 2021; Pintus & Ros-Santaella 2021). One of the amino acids that has been studied is proline. This particular amino acid has been identified as a potent antioxidant (Zhang et al., 2022) and an effective intervention for reducing the production of reactive oxygen species (ROS) during cryopreservation.

During cryopreservation, proline has been shown to improve the quality of sperm in rams, stallions, cynomolgus monkeys, donkeys, and canines. Proline also reduced ROS levels during liquid storage and enhanced boar sperm motility, acrosome and membrane integrity (Feng et al., 2020). This indicates that proline has been demonstrated to enhance post-thaw sperm motility, viability, and membrane integrity when added to cryopreservation medium. It also helps to regulate osmotic balance, preventing cellular dehydration and excessive shrinkage (Bucak et al., 2009).

Due to limited studies on cryopreserved goat sperm cells, the viability in terms of motility and velocity of the cells with different levels of proline supplementation on semen extender was studied.

MATERIALS & METHODS

The Boer bucks at the age of 3 to 4 years old served as semen donors. The animals were raised under a confinement production system following the management program of Cagayan Valley Small Ruminants Research Center, Isabela State University, Echague, Isabela, Philippines. In this production system, the animals were stall-fed and provided with diet composed of grasses and legumes. Each experimental animal received fresh forages at a rate of 30% of its body weight. Roughage was further separated into a 60:40 (grass: legume) ratio. The ration was divided into four equal portions and distributed to the animals at 8 and 11 a.m., and 2 and 4 p.m.

Semen collection was scheduled three times a week on alternate days. The collection took place at 6:00 a.m. using an artificial vagina (AV). The collected semen was immediately submitted to the laboratory for analysis. The materials used in the evaluation were pre-warmed at 37°C, and the collected sample was held in a water bath at the same temperature throughout the evaluation. The raw semen was analyzed at 40x magnification under the video microscope Computer Aided Sperm Analyzer, or CASA Machine, installed with AndroVision™. Semen with motility score of 85% is divided into four equal parts and is diluted using semen extender utilized composed of 3.028 grams of Tris, 1.675 grams of citric acid, 1.25 grams of fructose, 100 mL of distilled water, 20 g of soybean lecithin, and 12 mL of glycerol. Four different levels of proline supplemented to the semen extender were studied to include without proline (Treatment 1), 0.001g proline (Treatment 2), 0.002g (Treatment 3), and 0.003g (Treatment 4).

Slow-freezing method was followed in cryopreservation. The frozen sperm cells were stored for 1 week inside the cryobank. For 30 seconds, the frozen sperm cells were submerged in a water bath at 37°C. The viability of the sperm cells was evaluated in terms of overall motility (%), fast progressive motility (%), slow progressive motility (%), local motility (%), immotile sperm cells (%), and velocity (um/s). The Statistical Tool for Agricultural Research (STAR) Program was used to evaluate the data using One-way analysis of variance (ANOVA).

RESULTS

Motility

The response of the proline supplementation at different levels on viability of the post-thawed goat sperm cells is significant. The total motility of Treatments 2 and 3 varies significantly ($P<0.05$) from that of the other treatments, with averages of 89.78 and 86.78%, respectively. The same observation is also noted in progressive motility at 78.29 and 76.49%; fast motility, with rates of 60.25 and 58.43%; and immotile sperm cells at 10.22 and 13.20% (Table 1). On the other hand, no significant difference on slow and local motility is observed. Slow motility across treatments ranges from 17.69 to 19.30%; while local motility rates range from 10.30 to 14.91% (Table 1).

Velocity

To assess the viability of the sperm cells, their velocity was measured using Straight-Line Velocity (VSL) and Average Path Velocity (VAP). As shown in the Table 2, there is no significant difference in the speed of the sperm cells. The computed VSL ranges from 30.29 to 40.19; while VAP ranges 40.05 to 51.07um/sec.

DISCUSSION

Sperm cryopreservation technology facilitates artificial insemination in domestic animals and significantly aids in preserving genetic material, particularly for breeding animals and endangered species. The technology of sperm cryopreservation comprises various processes, including cooling, freezing, and thawing (Zhang et al., 2022). When semen is successfully cryopreserved, long-term storage and wider distribution can be done and can increase reproductive efficiency.

During cryopreservation, spermatozoa are inevitably exposed to drastic changes in temperature, ice crystal formation and diverse types of stresses whether physical, chemical, osmotic and oxidative including physical, chemical, osmotic and oxidative, all have a negative impact on sperm quality and fertility (Ezzati et al., 2020). The traditional method of cryopreservation reveals that only around 50% of spermatozoa retain their viability after cryopreservation. Several scientific studies claimed that claimed that adding amino acids to semen increased post-thawing sperm quality. Alanine, glycine, glutamine, histidine, and proline are examples of amino acids that have been employed as cryoprotectants for a variety of species. These amino acids have the ability to alter the osmotic process or prevent lipid peroxidation (Sangeeta et al., 2015).

Proline, a proteinogenic amino acid primarily involved in protein synthesis (Moradi et al., 2021) and an osmoprotectant (Abdelnour et al., 2024). It is classified as a nonessential amino acid. It has an important role in cell

Table 1: Motility of cryopreserved goat sperm cells

Treatment	Total Motility, %	Progressive Motility, %	Fast Motility, %	Slow Motility, %	Local Motility, %	Immotile, %
Treatment 1	76.12[b]	61.27[b]	41.82[b]	19.30	14.91	23.47[a]
Treatment 2	89.78[a]	78.29[a]	60.25[a]	17.69	11.50	10.22[b]
Treatment 3	86.78[a]	76.49[a]	58.43[a]	17.80	10.30	13.20[b]
Treatment 4	74.02[b]	60.66[b]	41.73[b]	18.49	13.91	25.96[a]
ANOVA	*	*	*	Ns	ns	*
CV	15.92	26.31	42.27	39.24	71.60	71.71

Note: Means labelled with the same letter are not significantly different from each other. ns- not significant *- significant at 5% level.

Table 2: Velocity of the cryopreserved goat sperm cells

Treatment	VSL, um/sec	VAP, um/sec
Treatment 1	30.75	40.05
Treatment 2	30.29	47.15
Treatment 3	40.19	51.07
Treatment 4	32.97	43.11
ANOVA	Ns	Ns
CV	91.13	74.83

ns- not significant.

signaling, survival, and the metabolism of carbon, nitrogen, and osmotic (Alvarez et al., 2022). It appears to be an adaptive response to ROS stress and protects plant and mammalian cells from oxidative damage (Signorelli et al., 2014). L-proline has been reported to be useful in semen cryopreservation (Liu et al., 2023). Studies shown that proline acts as cryoprotectant of sperm cells collected from ram (Sangeeta et al., 2015), goat (Zhang et al., 2022; Zhao et al., 2023), Andalusian donkeys' (Li et al., 2021) and in stallion oocytes (Davoodian et al., 2017) and in human endothelial cells (Sun et al., 2014). It can effectively mitigate ROS production during freezing and cooling procedures, thus enhancing semen quality after cryopreservation process. The study of Heidari et al. (2018) discussed the capability of proline to act as an osmoregulator, scavenger of free radical species, protector of mitochondria, and promoter energy production.

The study's results reveal that supplementing proline at rates of 0.001g and 0.002g per 100mL of semen extender for goats effectively improves sperm cell viability by protecting them against rapid cooling and ice crystal formation (Liu et al., 2023). As noted in this study, proline rates from 0.001 to 0.002 g achieved sperm cell motility rates >80% indicates a high likelihood of successful fertilization. Moreover, the proportion of sperm cells with increasing motility and fast motility is higher at these rates. Progressive motility measures the total number of sperm cells moving forward in a mostly straight line, whereas fast motility measures how rapidly sperm cells move within that line. Both are essential indicators of male fertility since they directly affect the sperm's ability to reach and fertilize the egg. A lower percentage of immotile cells was also seen at these rates, demonstrating the presence of healthy viable sperm.

On the other hand, the velocity of the sperm cells did not differ significantly across treatments. The sperm cells that recorded VSL and VAP rates from the various treatments were considered motile, while proline-supplemented sperm cells had higher velocity rates, an indication that proline can improved post-thaw sperm motility and overall quality in goats during cryopreservation.

Conclusion

Proline supplementation at the rates 0.001 to 0.002g in the freezing medium effectively enhances the post-thaw quality of goat sperm.

DECLARATIONS

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Conflict of Interest: The authors declare no conflict of interest.

Data Availability: The data is available via corresponding author at the Semen Processing Laboratory of Cagayan Valley Small Ruminant Research Center, Isabela State University, Echague, Isabela, Philippines

Ethics Statement: The study was conducted in accordance to Philippines' Republic Act No. 8485 known as the Animal Welfare Act of 1998. In particular, the experiment was carried-out following the guidelines of the Philippine National Standard- Code of Good Animal Husbandry Practice (GAHP) for Goat (60:2008).

Author's Contribution: May Ann D. Fajardo gathered data from a laboratory experiment, while Aubrey Joy M. Balbin contributed data analysis and manuscript writing and Jonathan N. Nayga assisted in finalizing the paper.

Generative AI Statement: The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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