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Effects of Frozen Semen Extender for Goats (MJ Ex2™ and Tris-egg Yolk) on Sperm Motility Quality after Freezing

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ABSTRACT

Background: Semen extenders are crucially important when freezing animal semen to protect against sperm cell death and preserve the quality of the sperm cells. Semen diluents play a crucial role in the production process of freezing semen. Both pre-and post-freezing methods can be employed until the point of insemination.

Methods: This study aimed to compare the quality of sperm motility after freezing a newly produced semen Extender (MJ Ex2™) with a conventional formula (Tris-egg yolk) using Computer Assisted Sperm Analysis (CASA). This was achieved by dividing the semen into two portions, diluting each portion with a different formula and then freezing both semen specimens using the same process.

Result: The results indicate that the fresh goat semen samples in this study had a cream color, an average volume of 1.10±0.87 ml/time, a density of 4D (thick), a viscosity of 4°C (milky), a pH of 6.53±0.14, a concentration of 17,203±275.18 × 10^6/ml and a motility of ++++ (75.7±0.13). The MJ Ex2™ extender diluent had a movement percentage of 76.72±5.00, which was significantly higher than the Tris-egg yolk formula of 66.48±2.89 (P<0.01). As for the percentage of forward movement, VAP, VSP, ALH and BCF values were not different. The motility characteristics of the Tris-egg Yolk formula had a LIN value of 50.19±4.05, which was significantly high (P<0.01). The percentage of motility and STR values of sperm diluted with MJ Ex2™ extender was 78.14±5.04, which was significantly higher than the Tris-egg yolk formula 72.60±7.93 (P<0.05). The results of this study indicate that the MJ Ex2™ extender formula enhanced the quality of frozen goat semen. Considering the motility of the sperm; however, would suggest that other effects on those sperm cells would need to be furthers to investigate.

Key words: Extender, Goat artificial insemination, Goat semen, Sperm motility.

INTRODUCTION

Artificial insemination has been developed for use amongst many animal breeds (Ombeler and Van Robays, 2015). Reports of artificial insemination in goats and sheep date back to the 1950s (Bustani and Baiee, 2021). Reducing the temperature from 25°C to 5°C is the most important step in the frozen semen production process. Sperm cells also exhibit temperature sensitivity, which can result in cold shock due to the material's transformation from a substance to a liquid. Therefore, using something called a cryoprotectant is required. Egg yolk and skim milk with glycerol are common cryoprotectants that work well with a variety of animals including pigs, horses, buffalo and cows (Yanez-Ortiz *et al.,* 2022; Chaudhary *et al.,* 2018). Conversely, researchers have discovered that goat semen secretes bulbourethral gland secretion (BUS), a combination of phospholipase A (Iritani and Nishikawa, 1972) and egg yolk coagulating enzyme (EYCE), from the bulbourethral gland (Hossein *et al.,* 2014). Employing egg yolk as a diluent over 1.5 per cent (v/v) triggers a chemical reaction that produces lysolactins, which are a group of lysophospholipids that are known to be harmful to sperm cell membranes (Ritar and Salamon, 1982). This can lead to the death of sperm cells, their loss of motility and the formation of aberrant acrosomes. Furthermore, the breakdown of chromatin rods impacts the egg cell's capacity to fertilize (Nunes *et al.,* 1982; Sawyer and Brown, 1995). Consequently, this BUS material is combined with skim milk to make sperm hazardous (Sias *et al.,* 2005). According to certain findings, sheep may utilize up to 20 egg yolk. As a result, freezing goat semen involves a difficult process. Numerous factors influence the quality of the semen.

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To improve the rate of sperm survival and fertilization, proper diluent selection and freezing condition management are positively crucial (Tometo *et al.,* 2010; Roof *et al.,* 2012).

Currently, several commercially available semen diluent formulas are available, including Irvine TYB (containing egg yolk) and Bioxcell® (containing soy), which can be used to freeze goat semen without washing it (Roof *et al.,* 2012). Additionally, "IMIDA" (containing soya), skimmed milk diluent and egg yolk diluent with sodium dodecyl sulfate (SDS) are also available for this purpose. The development of this technology in the goat farming system can be considered a triumph, as it can replace the use of alternative goat breeding techniques for herd control or actual insemination. This determination is in line with the outcomes of earlier studies that compared the survival rate of goat sperm by analyzing the quality of sperm after freezing goat semen. That process involved examining the motility rate, evaluating the quality of the semen by dropping the sperm onto a warmed 37°C slide and examining the abnormalities and survival rate (live/dead) of the sperm under a phase contrast microscope. The quality of the semen could then be examined with a light microscope by utilizing Eosin-Nigrosin staining and frozen semen from Egg-yolk Tris semen freezing solution and MJ-Ex2™ Extender semen freezing solution, a commercial formula that has proven successful in frozen semen production for pigs and cattle (Thaworn and Pattanawong, 2014). The former is a widely used basic semen diluent formula. As a result, we are interested in expanding its use further to raise the rate of conception and pregnancy in the field of goat breeding, since this will add value to the nation's goat farming system and the commercialization of the goat production business.

This study developed the MJ Ex2™ formula for use in goat breeding to enhance the efficacy of research on goat semen diluent. Therefore, we used the accurate and well-recognized computer-assisted sperm analysis (CASA) as a semen quality analyzer and compared the outcomes with the basic Tris-egg yolk formula to detect sperm movement characteristics after freezing.

MATERIALS AND METHODS

Experimental animals

Three apparently healthy male Boer hybrid goats of 50 to 60 kg body weight each and 3 to 5 years old were used in this study. The animals were fed a ready-mixed diet known as TMR (Total Mixed Ration), which consisted of a concentrate and roughage combined in proper proportions and had a minimum of 14-16 CP. The goats always had access to fresh water and were provided with dry pangolin meat in twice-daily feedings (Institutional Animal Care and Use Committee [IACUC] approval number: RMUTL-IACUC 010/2023).

Semen collection

An artificial vagina (AV) was used to collect semen by invoking a method of semen collection that was similar to what typically occurs in nature. The goat semen was collected once a week for six consecutive weeks. A minimum of 80 mass movement and a minimum of 70 progressive motility were required of the fresh semen that was ultimately collected (Nunes and Salgueiro, 2011).

Evaluation of fresh semen quality

The collected semen must first be evaluated for quality before being used to make frozen semen. The semen was incubated in warm water at 33°C for further examination. Before diluting the semen in the next step, evaluation of semen quality was carried out by visual inspection as described by the Department of Livestock Development, (2006) and also by Computer Assisted Sperm Analysis (CASA).

Semen dilution

Freshly collected semen from each boer were divide into 2 portions. Each portion was either be diluted with Tris-egg yolk basic frozen semen diluent or diluted with frozen semen diluent formula MJ Ex2™. The final concentration was set to 20-30 $\times$ 10^6 spermetozoa/ml. After diluting the semen with either semen diluent formula, the specimens were transferred into 0.25 ml semen tubes. Subsequently, refrigeration of the semen at 4°C for 4 hour was positioned on a pedestal within an aluminum box that held 100 L of frozen tubes. Elevated above the box's bottom, a negative-temperature-resistant metal platform could hold liquid nitrogen up to a height of approximately 5 to 10 cm. The box's lid was closed for one to two minutes to maintain a steady temperature within. As was observed, the nitrogen vapor did not disperse. Subsequently, the semen tubes were uniformly spaced out such that after 13 minutes at a temperature of -121°C, the semen tubes are about 3-4 mm. above the liquid nitrogen level. Then, the contents of every frozen semen tube were gathered and placed in a cup of liquid nitrogen. After freezing and being placed in a frozen semen container, a quality inspection step was administered (Leboeuf *et al.,* 2000).

Evaluation of semen quality after 24 hours of freezing

After the semen was frozen for 24 hours, it was then thawed in 37°C water for 30 seconds and the semen quality was analyzed based on sperm motility using CASA (software: HT CASA II Goat version 1.12) by employing the following parameters: Motility, Progressive motility, Path velocity (VAP), Straight-line velocity (VSL), Curvilinear velocity (VCL), Straightness (STR), Linearity (LIN), Lateral head displacement (ALH) and Beat cross frequency (BCF).

Statistical analysis

All collected data were subjected to statistical analysis using one-way analysis of variance and mean differences among treatments were evaluated by Duncan's multiple range and post-hoc tests using the statistical software IBM SPSS Statistics Version 25 (SPSS Inc., Chicago, IL, USA). Significance was considered at a 5 probability.

RESULTS AND DISCUSSION

The fresh goat semen samples in this study had a cream color, an average volume of 1.10±0.87 ml/time, a density of 4D (thick), a viscosity of 4°C (milky), a pH of 6.53±0.14, a concentration of 17,203±275.18×10^6/ml and a motility of ++++ (75.7±0.13). The obtained fresh semen exhibited excellent characteristics, enabling the production of frozen semen with normal features such as a semen volume of 1-2 ml/time (Settergren, 1934), a motility of at least ++ or 30 and a progressive motility rate of more than 70. The osmotic values of Tris-egg yolk (1,611 Osmol/kg) and MJ Ex2™ (1,708 Osmol/kg) for both semen diluent formulas were measured using an Osmomat 3,000 basic freezing point osmometer, Go Notes brand and they were found to be normal when Nunes and Salgueiro's (2011) freezing method was employed (Table 1).

Table 1: Results of macroscopic and microscopic quality of fresh semen. Visual inspection (physical assessment).

Parameters	Fresh semen quality
Macroscopic	
Volume (ml)	1.10±0.87
Color	Cream
Density (D-4D)	Creamy-grainy
Consistency (C-5C)	Milky
pH	6.53±0.14
Microscopic	
Concentration (10^6/ml)	17,203±275.18
Mass movement (%)	75.7±0.13
Motility (%)	86.05±0.52
Osmomat (mOsmol/kg)	
Tris-egg yolk	1,611
MJ Ex2™	1,708

±SD; Standard deviation.

Table 2: Effects of frozen semen diluent for goats MJ Ex2™ extender formula and Tris-egg yolk extender formula on sperm motility quality after the semen was frozen for 24 hr., using CASA (software: HT CASA II Goat version 1.12).

Variables	Treatments	
	Tris-egg yolk	MJ Ex2™
Total motility (%)	66.48±2.89	76.72±5.00**
Progressive motility (%)	43.75±4.70*	40.33±4.62
VAP (μm/sec)	72.57±3.64	74.3±2.87
VSL (μm/sec)	56.34±6.65	56.88±7.97
VCL (μm/sec)	121.02±11.62	118.06±11.57
STR (%)	72.6±7.93	78.14±5.04*
LIN (%)	50.19±4.05**	46.48±3.36
ALH (Mm)	6.26±0.48	6.11±0.79
BCF (Hz)	26.42±2.49	25.24±1.99

Note: VAP= Path velocity, VSL= Straight-line velocity, VCL= Curvilinear velocity, STR= Straightness, LIN= Linearity, ALH= Lateral head displacement, BCF= Beat cross frequency.

±SD; Standard deviation*, **would indicate that values in the same in row with different superscripts differ significantly (P<0.05, P<0.01 respectively)

The results indicated that the motility percentage of MJ Ex2™ extender was 76.72±5.00, which was a lot higher than the motility percentage of Tris-egg Yolk extender (66.48±2.89) (P<0.01). However, the percentages of forward motility, Path velocity (VAP), Straight-line velocity (VSL), Lateral head displacement (ALH) and Beat cross frequency (BCF) did not change. The Tris-egg Yolk extender formula's motility was much higher, with a Linearity (LIN) value of 50.19±4.05 (P<0.01). The motility and Straightness (STR) values of the diluted sperm treated with MJ Ex2™ extender were much higher (78.14±5.04) than with the Tris-egg Yolk extender formula (72.60±7.93, P<0.05). When considering the sperm motility measurements, the research findings indicated that the MJ Ex2™ extender formula significantly enhanced the quality of frozen goat semen (Table 2), (Fig 1,2). In both groups of the experiment, sperm motility patterns showed that frozen goat semen mixed with MJ Ex2™ extender semen diluent contained more viable than Tris-egg yolk formula. When statistically analyzed, a significant statistical difference was observed. It is possible that the use of the MJ Ex2™ extender formula in frozen goat semen made a significant impact by increasing certain important components such as gallic acid, ellagic acid and tannin acid. This outcome is consistent with the study of Bo *et al*, (2010) who reported that gallic acid, ellagic acid and tannin acid could affect cells, all of which were related to the inhibition of cell growth and cell death and reduced free radicals. Furthermore, various semen diluent formulas for refrigeration or freezing, such as the Tris-fructose-egg yolk (TES-Tris) formula that uses fructose (Foote, 1964) and the Tris-egg yolk formula, have been reported to incorporate different sugars as key components. Using glucose (Province *et al.,* 1984) or lactose (Yubi *et al.,* 1987) as reactive molecules helps to resist or prevent oxidation, thereby effectively maintaining the condition of cells and membranes. Moreover, studies have shown that the MJ Ex2™ extender semen diluent, which does not separate the seminal fluid and sperm before the sperm is diluted, might not be harmful or may not stop the substances in Cowper's gland from reacting. Importantly, the production of frozen semen contradicts the findings of Nunes *et al*. (1982) and Sawyer and Brown (1995) in cattle. In sheep, up to 20 egg yolk can be used (Tometo *et al.,* 2010; Roof *et al.,* 2012). This can protect sperm from damage during the freezing process, reduce temperature and provide energy for sperm to move and survive (Vidal *et al.,* 2013). Mobility indicates the number of *via*ble sperm that are still able to move and are ready to travel in the female reproductive tract to the point of fertilization with an egg cell. The sperm can then complete all the processes of fertilization with the egg cell. The evaluation of the quality of semen after freezing would indicate that it is a good diluent.

The recipe for MJ Ex2™ extender goat semen cryotherapy is unique. The primary components are gallic acid, ellagic acid and tannin acid. This would align with the findings of Bo *et al*. (2010), who found that gallic acid, ellagic acid and tannic acid all had a positive effect on cells by slowing down cell proliferation and reducing cell death. When goat semen is frozen or refrigerated, however, each formula has been found to use different sugars. For example, the Tris-fructose-egg-yolk formula uses fructose (Foote, 1964), the Tris-egg-yolk formula uses glucose (Province *et al.,* 1984) and the egg-yolk formula uses lactose. These are reactive chemicals that help resist or inhibit oxidation, thereby protecting cells and membranes from freezing damage. This finding fits well with what Naing *et al*. (2010) reported when they studied how the concentration of trehalose, a disaccharide sugar used in a Tris-containing semen freezing solution, affected their results. This research study demonstrated that monosaccharides, specifically glucose and fructose, significantly influenced sperm motility more than disaccharides at the examined amounts. The experimental results clearly showed that adding trehalose in different amounts, up to 198.4 mM with 69.38 mM glucose, had the most significant effect on the quality of the sperm after being frozen. Similarly, Nunes and Salgueiro (2011) found that glucose is a useful energy source for sperm and enhances sperm motility and direction. As a result, glucose is an ideal sugar for enhancing the goat semen freezing solution. However, the chemical makeup of goat sperm determines the impact of each form of sugar supplementation on sperm quality during the semen freezing procedure. Accordingly, electrolytes, amino acids, phospholipids and other related chemicals have been utilized including proteins and certain specific enzymes. This list would also include plasma, inacetal, glycerylphosphorylcholine and ergothioneine. Examining the specific composition of sperm obtained from different species did not necessarily reveal any differences (Anel *et al.,* 2003).

Furthermore, it was dependent upon the type of animal and the temperature at which the semen was stored, leading to two phases of semen quality storage, as follows: Short-term semen storage involves storing semen in liquid form (liquid semen) at a temperature of 5°C for about one week with an acceptable fertilization rate. Long-term storage of semen quality involves storing it in a frozen state, which can be achieved in two ways: either packing it in straw tubes (ministraw) or freezing it at a temperature of -196°C using liquid nitrogen to maintain the desired temperature. Another method would be to freeze the semen in pellets (pellets) using dry ice (solid carbon dioxide) to keep the temperature at -79°C, which is not common at the moment because it can be cumbersome to thaw and involves combining diluted solutions and packing them in semen injection tubes. This liquid nitrogen cryopreservation method may keep sperm viable for an extended period of time; however, it has been observed that semen frozen for more than 40 years retains acceptable quality and has a high fertilization rate (Anel *et al*., 2003). Apart from the diluent solution, there is another solution known as cryoprotectant that can effectively prevent the loss of semen quality. For instance, Polge Smith and Parkes discovered glycerol in 1948. Up until now, the production of frozen semen has widely used glycerol as a cryoprotectant. Other methods involve ethyleneglycol, dimethyl sulfoxide and propylene glycol. However, for bovine semen, glycerol works best to prevent the loss of cell properties during the freezing process. In this case, it acts physically rather than chemically (Leboeuf *et al.,* 2000). Additionally, each ejaculation's properties can influence the semen, yielding distinctly different outcomes. Using the semen filtration method will help reduce the differences in semen quality caused by each ejaculation. This has been found to be important in ensuring consistent control standards for preservation and freezing (Nunes and Salgueiro, 2011). To maintain the stability of the sperm cell membrane, many breeders of animal species use egg yolk or skim milk as a basic component in semen diluent to preserve semen quality. Lipoproteins and lecithin, or phosphatidylcholine, are found in egg yolks. In skim milk, casein protein is present, which is an important substance that helps to stabilize the cell membrane, reduce the loss of enzymes in the acrosomal membrane and prevent cell damage from temperature drops during both refrigeration and freezing processes. Notably, freezing semen in goats can encounter the same problems as the freezing of semen in other species. This is true of ice crystals forming in the cells during temperature reduction, which can damage the sperm cells. A good semen diluent must be able to maintain the condition of the sperm cells during the freezing process, prevent sperm cell damage from temperature changes and adjust the appropriate acid-based balance for sperm cells or buffers to make the resulting product as *via*ble as possible and close to natural semen (Nunes and Salgueiro, 2011). It prevents microbial contamination from antibiotics being added to the semen diluent and is also an energy source for sperm cell movement (Garner and Hafes, 1993). The semen diluent must be able to maintain the integrity of the cell wall and acrosome, while ensuring the forward movement of sperm cells, the strength of the sperm cells, the *via*bility of the sperm cells and the ability to provide a high fertilization rate (Vidal *et al.,* 2013).

The MJ Ex2™ extender formula revealed that sperm motility had higher STR values than Tris-egg yolk semen dilution, indicating that average sperm velocity and forward movement are crucial for sperm motility in the female reproductive canal. In terms of sperm motility, it has been determined that sperm with high motility are stronger and can go farther than sperm with the same motility speed. There is no information on which motility speed is best suited, yet sperm motility has clearly been linked to fertilization rate (Correa *et al.,* 1997). Research teams can expect the

findings of this investigation to serve as a set of guidelines for future development and improvement in the production of frozen goat semen. We can conduct research by varying the number of ingredients until we achieve the desired amounts, or by incorporating additional compounds into the mixture. The cryoprotectant's beneficial characteristics must allow sperm cells to survive and fully fertilize egg cells. It can also prevent damage to the cell membrane and acrosomes (Nunes *et al.,* 1982 and Sawyer and Brown, 1995), lower the amount of harmful chemicals in cell metabolism or act as an antioxidant (Zhang *et al.,* 2022), stop cell shock after the temperature drops to -10°C (Ivan *et al.,* 2022) and offer various other advantages. To evaluate the effectiveness of a semen diluent, we must dilute the semen based on the amount of sperm required for frozen goat semen production. When sperm cells remain in the semen diluent, it would indicate that the substance does not cause them to lose certain properties, which could lead to cell death or toxicity when mixed with other substances, thereby altering the surrounding environment and causing cell

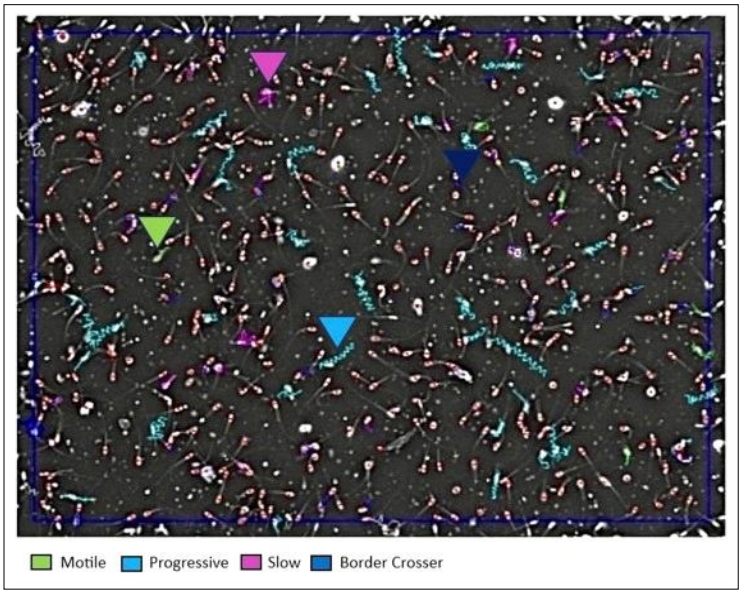


Fig 1: Sperm motility and motility characteristics after the semen was frozen for 24 hr. of Tris-egg yolk basic formula semen, using CASA (software: HT CASA II Goat version 1.12).

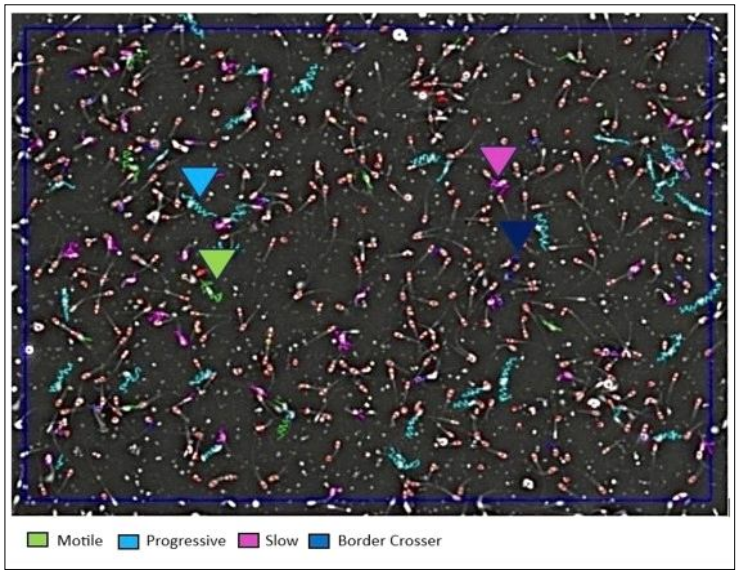


Fig 2: Sperm motility and motility characteristics after the semen was frozen for 24 hr. of MJ Ex2™ extender formula semen, using CASA (software: HT CASA II Goat version 1.12).

weakness. Researchers have been studying a limited number of cases where goats use semen diluent. Notably, Thailand's data has become increasingly examined worldwide. However, the study and research of semen diluent continues to be an intriguing topic worthy of further pursuit, as it will benefit goat farmers throughout the nation. In terms of the application of knowledge, understanding goat artificial insemination enables farmers to employ more goat artificial insemination techniques, thereby assisting farmers in developing goat breeds and producing enough goats to fulfill global population demands.

CONCLUSION

The MJ Ex2™ extender diluent formula had a significantly higher total sperm motility rate than the Tris-egg yolk formula (P<0.01). There were no significant differences in the percentages of forward motility, VAP, VSP, ALH and BCF values. The motility characteristics of the Tris-egg yolk formula had a significantly higher LIN value (P<0.01). It was also discovered that the STR values and the percentage of motility characteristics of sperm diluted with MJ Ex2™ were significantly higher than those of sperm diluted with Tris-egg yolk (P<0.05). Overall, we can conclude that the MJ Ex2™ extender formula, with its higher sperm motility values when compared with Tris-egg yolk semen diluent, influenced the quality of frozen goat semen. The development of this new goat semen diluent formula will be an important alternative method that could have a positive effect on the high survival rate of sperm.

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Artificial intelligent declaration

The authors utilized Institutional Animal Care and Use Committee (IACUC) approval number: RMUTL-IACUC 010/2023) for U1-067182560 in the preparation of this research work. Subsequently, the author(s) conducted a thorough review and editing process, taking full responsibility for the content of this publication.

Conflict of interest

All authors declare that they have no conflict of interest.

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