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Original Article

Effect of Different Extenders on Sperm Motility and Vitality in Goose Semen Cryopreservation

■Author(s)

Taskin A[I] https://orcid.org/0000-0001-5897-2062
Ergun F[II] https://orcid.org/0000-0001-5587-1581
Karadavut U[I] https://orcid.org/0000-0001-5362-7585
Ergun D[I] https://orcid.org/0000-0002-4047-0512

[I] Kirsehir Ahi Evran University, Faculty of Agriculture, Department of Animal Science, Kirsehir-Turkey
[II] Kirsehir Ahi Evran University, Faculty of Health Science Kirsehir- Turkey.

■Mail Address

Corresponding author e-mail address
Atilla Taskin
Kirsehir Ahi Evran Universitesi – Animal Science 40100, Kirsehir, Turkey.
Phone: 90386 280 48 05
Email: ataskin@ahievran.edu.tr

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ABSTRACT

This study aimed to investigate the usability of different diluents containing 6% Dimethylformamide (DMF) for cryo-preservation of the semen of geese (Anser cygnoides). The diluents of Glucose (G), Tris-Glucose (T), Lactated Ringer's-Glucose (LG), and Lactated Ringer's (L), all of which contained 6% DMF, were used as cryoprotectants. The researchers collected semen samples from four geese, twice a week over a four-week period, by means of abdominal massage; they then calculated how much sperm each goose ejaculated. Next, the semen samples were pooled and their spermatological parameters were determined. Their volume (4x (mL)), concentration (×108/mL), pH, motility (%), and vitality (%) rates were 0.31±0.01, 3.49±0.32, 7.13±1.06, 67.75±1.28, and 70.00±2.03, respectively. Then, these pooled semen samples were equally divided into four groups. Once they were frozen and thawed, the researchers discovered that the diluent L had the highest motility rate: 40.12% ± 1.35. The motility rates of the other diluents were as follows: LG (28.25%± 1.48), G (21.50% ± 1.41), and T (5.12% ± 0.83). Likewise, the vitality rates (%) of the diluents were as follows: L (41.93% ±1.87), LG (31.50%±1.88), G (29.43% ±1.45), and T (10.56%±1.34), respectively. Freezing and thawing appeared to lower each diluent's vitality and motility rates. However, for the Lactated Ringer's (L), this decrease was predictable. Therefore, Lactated Ringer's diluent containing 6% DMF can be used in cryo-preservation of goose semen.

INTRODUCTION

People breed geese for their meat, livers, and feathers (this changes from region to region) (Taskin *et al.*, 2013; Onder *et al.,* 2017; Boz *et al.,* 2019; Sengul & Yeter, 2020). Geese are important for poultry farming because of their high adaptation abilities and high feed conversion rates (Wang, 2009; Taskin *et al.*, 2017; Sari & Saatci, 2020). However, their reproductive properties are low. Female geese have a low egg yield and a high tendency to incubate. Male geese, likewise, generally have a low sperm quality (Gee, 1995; Xu *et al.*, 2013; Boz *et al.*, 2014).

Improvement of breeding characteristics of geese is possible with artificial insemination and freezing of semen. Both techniques conserve gene resources and gene transfer without transporting animal materials. They also reduce the risk of disease and pathogen transmission during mating and make more efficient use of male breeder geese (Reedy *et al.*, 1995; Blesbois *et al.*, 2005). Freezing the semen allows researchers to preserve the goose's high genetic potential and reuse it later (Blesbois *et al.*, 2007).

Breeders do not tend commonly to use artificial insemination to breed geese. This method should be used in herds including low number of male geese (Chrzanowska & Chełmońska, 1997). Moreover, geese

lose much of their fertility during the final two months of their six-month breeding period (Lukaszewicz *et al.*, 2003) due to physiological reasons. Therefore, geese require assisted reproductive techniques during those last two months. During periods of high fertility, the semen can be frozen and stored. During the last two months (low fertility), breeding is supported by artificial insemination (Partyka *et al.*, 2011).

In vitro storage conditions dictate how fertile avian sperm can be (Sarkar, 2020). Furthermore, the number of studies that focus on freezing goose semen is very limited. If semen is to be frozen, it needs to be well diluted and of good quality (Ameen *et al.*, 2014; Łukaszewicz *et al.,* 2020). So far, in various studies, researchers have used glycerine (glycerol), DMSO (dimethylsulfoxide), DMA (dimethylacetamide), and DMF (dimethylformamide) as cryoprotectant for cryo-preservation of goose semen. Glycerin (glycerol) is not preferred so much because it can be toxic for insemination. Recently, researchers have used 6% DMF as the cryoprotectant for freezing goose semen (Lukaszewicz, 2001). DMF protects goose spermatozoa well during the cryo-preservation process and does not act as a contraceptive in the female egg canal (Santiago-Moreno *et al.*, 2011).

The aim of this study was to freeze goose semen by using different diluents containing 6% DMF for long-term storage. Artificial insemination and semen freezing techniques are not widely used in geese today. However, the results of this study may contribute to the solution of important reproductive problems in goose production. In addition, it is thought that different applications of the techniques applied in this study may contribute positively to the protection of bird species in danger of extinction.

MATERIAL AND METHODS

Animal Material and Semen Collection

An approval (December 12, 2015-4/9) was obtained from Animal Experiments Local Ethics Committee of Kirsehir Ahi Evran University (Kırşehir, Turkey) before the study. This study was conducted between February and April 2019 in a henhouse belonging to Kirsehir Ahi Evran University, Faculty of Agriculture Animal Science Department. Four 2-year old male geese (*Anser cygnoides*) were used. No restriction was applied to the geese except for sexual abstinence. They were fed commercial feed (18% crude protein, 2600 kcal ME / kg) ad libitum, as well as subjected to a natural photoperiod. In order to prevent fecal contamination, they were prevented from accessing feed and water twelve hours before the researchers collected their semen. They were also washed in artificial ponds to reduce contamination and enhance the quality of their semen. The researchers applied abdominal massage to the geese so that they could extract and collect their semen (Burrows & Quinn 1937). They were given a three-week period beforehand so that they would adjust to giving semen. All necessary measures were taken to protect them from cold shock and contamination. The researchers collected semen early in the morning with wide-mouthed glass tubes which had been heated to +37.5 °C and sterilized. This process lasted for four weeks (twice a week, eight samples per goose, four geese = thirty-two samples in total). Semen collected and liquefied were observed separately and care was taken to avoid contamination. The researchers used 0.01 ml precision injectors to determine how much semen each goose produced (Ayset 70570). Last, all of the samples were pooled to eliminate individual differences and were kept at + 37.5 °C until the researchers diluted it.

Semen traits

Spermatozoa concentration: The samples' spermatozoa concentrations (ml) were counted using hemocytometry, and expressed as $x10^8$ sp/ml. The researchers diluted (1/500) 0.01 ml of sperm with 5 ml of Hayem solution, and then spread it over a Thoma slide in order to count the sperm. The concentration was calculated using the following hemocytometer count equation:

$$Concentration\ (\mu l) = \frac{Number\ of\ Spermatozoa\ Counted}{Large\ Square\ Area\ x\ Large\ Square\ Height\ x\ Reconstitution\ Rate}$$

pH Value: The pH values of the samples were determined using a universal indicator.

Motility (%): The 5 µl of semen was taken over a slide (heated at +37.5 °C) and then closed with a coverslip at the same temperature. It was then placed on a heating plate (Type D, Leica Mats). Next, two observers examined the samples on at least three different microscope fields and expressed their findings in terms of %. A phase-contrast microscope (Leica DM750) (equipped with a heating table) was used in the study.

Vitality (%): The researchers calculated the samples' vitality rates using Eosin nigrosin sperm staining technique. Dead spermatozoa were red or purple, while living spermatozoa were either white or colorless/ clear (Lemoine *et al.*, 2011). Vitality rate expressed in terms of %.

Table 1 – Chemical composition of the diluents.

Diluents	Glucose (G)	Tris-Glucose (T)	Lactated Ringer's-Glucose (LG)	Lactated Ringer's (L)
Distilled water	100 ml	100 ml	100 ml	100 ml
DMF	%6 (v/v)	%6 (v/v)	%6 (v/v)	%6 (v/v)
Glucose-monohydrate	0.37 M D(+)	300 mM/L D(+)	1.00 g	-
Tris (hydroxymethyl) aminomethane	-	30mM/L	-	-
Potassium chloride	-	-	0.03 g	0.03 g
Calcium chloride	-	-	0.02 g	0.02 g
Sodium lactate	-	-	0.31 g	0.31 g
Sodium chloride	-	-	0.60 g	0.60 g

Diluents: Table 1 shows the diluents used in the study.

Freezing & Thawing Procedure

Diluting and equilibrating semen: After the pooled semen samples' spermatological properties were analyzed, it was split into four groups and placed into graded sterile plastic tubes (with mouths). The researchers then added G, T, LG and L – one per tube – at a ratio of 1/2 ratio (1 part semen, 2 parts diluent). During this procedure, care was taken to ensure that the diluents and the semen samples were at the same temperature (+37.5 °C) and the diluent was gradually added to the semen sample. After diluting, the vitality and motility of the semen samples were assessed and then recorded. They were later drawn to straws (Minitube 0.25 ml) and equilibrated for 90 minutes at + 5 °C (Straws of concentration 0.29±0.80 ($x10^8$ / ml). After equilibrating, the vitality and motility of the semen samples were assessed and then recorded.

Freezing & thawing: Semen in straws was frozen in liquid nitrogen vapor at -80 °C for five minutes (using a polystyrene floating raft). They were stored at -196 °C liquid nitrogen. Later, they were removed and placed in a water bath (+37 ° C) for five seconds so that they could thaw them out. Last, their vitality and motility rates were evaluated again and recorded (Sexton, 1981).

Statistical Analysis

First, the researchers analyzed the data using one-way ANOVA. Next, they carried out the significance test ($p<0.05$) and assessed the results, accordingly. Analysis of variance was used to study the differences between the groups. Then, they used the DUNCAN test to analyze other important properties. Finally, all of the data was analyzed through SPSS 15.0 statistical software.

RESULTS AND DISCUSSION

Geese have lower semen quality than other poultry. Furthermore, their semen quality and fertility characteristics differ according to species and races. This makes it difficult to compare data from other studies involving geese (Chelmonska & Lukaszewicz, 1995). It is required to find out the ejaculate volume, pH, sperm concentration, motility and vitality rates in order to determine the quality of the collected semen (Mocé & Graham, 2008).

Ejaculate volume

Male geese averagely produce between 0.2 and 0.3 ml of ejaculate. Similar studies have reported that the amount of ejaculate was reported to be 0.22±0.04 ml in turkey, 0.30±0.10 ml in rooster and 0.16±004 ml in duck (Nahak *et al.*, 2015; Kuzlu & Taskin, 2017; Aro, 2019). In this study, each goose produced 0.31 ± 0.01 ml (Table 2) of ejaculate per day. In contrast, the amount of ejaculate has been reported as 0.05-0.5 ml (Lukaszewicz, 1997) in Italian white geese, 0.4-1.3 ml (Kurbatov *et al.*, 1976) in Kubanskaya geese and 0.05-0.38 ml in Slovak white geese (Svoradová *et al.*, 2019). How much ejaculate a goose produces depends on its age – the older the goose, the less it yields (Lukaszewicz *et al.*, 2003).

pH

The pH value plays an important role when it comes to motility rate. For example, among turkeys, there

Table 2 – Ejaculate volumes (ml) of the fresh semen collected (eight times) individually from goose.

Goose number	1	2	3	4	General average
Ejaculate volume	0.30 ± 0.01^{b}	0.31 ± 0.02^{ab}	0.33 ± 0.01^{a}	0.32 ± 0.01^{ab}	0.31±0.01

Values with different superscripts within row differ significantly ($p<0.05$). ($\bar{X}\pm Sx$).

is an opposite relationship between motility and pH levels (Akcay *et al.*,1997) Likewise, semen collection technique can also affect pH level (Zawadzka *et al.*, 2015). In this study, the pooled semen samples had a pH value of 7.13±1.06 (Table 3). This is similar to the pH value of 7.3 reported by Dubos *et al.* (2006) for the sperm of Canadian geese (Branta canadensis).

Table 3 – Spermatological values of mixed geese semen ($\bar{X}$± Sx).

Evaluated traits			
pH	Density (x10^8 /ml)	Motility (%)	Vitality (%)
7.13±1.06	3.49±0.32	67.75±1.28	70.00±2.03

Sperm concentration

Other studies on various breeds of geese have revealed that the sperm concentrations were 651x10^6/ml (Mialon-Richard, 2004) and 110-1015 x10^6/ml (Lukaszewicz, 2006) for Landes geese (Anser anser), 0.8 x10^6/ml for Vladimir geese (Vladimirs kie garden), 0.8x10^6/ml for Kholmogors geese (Kholmogorskie garden), 1.15x10^6/ml for China geese (Chinese garden) and 0.3-1.0x10^6/ml for Kubanski geese (Kubanskie garden) (Davtian, 1986). In this study, the sperm concentration value of the samples was 3.49 ± 0.32x10^8/ml (Table 3). The older geese get, the more concentrated their sperm tends to be (Lukaszewicz *et al.*, 2003).

Motility and Vitality

In this study, the pooled semen samples had a motility rate of 67.75%±1.28 and a vitality rate of 70.00%±2.03 (Table 3). Another study reported that the motility and vitality rates of goose sperm were 92.2% - 68.4% and 50% - 60%, respectively (Lukaszewicz *et al.*, 2002). If blood, feces, and lymph fluid occur during sperm removal, they can also adversely affect the motility and vitality rates of the semen (Váradi *et al.*, 2019; Tai *et al.*, 2001).

The researchers diluted the semen samples with G, T, LG, and L and then analyzed their vitality and motility rates (Figure 1). The differences between the groups in terms of vitality were significant (p<0.05). While group L had the highest vitality rate (63.00%±3.11) group T had the lowest vitality rate (40.93%± 3.61). When it came to motility rates, groups LG and L had similar results and the differences between the other groups were statistically significant (p<0.05). Group L had the highest motility rate (61.37% ± 2.32), group T had the lowest motility rate(34.37% ± 3.50).

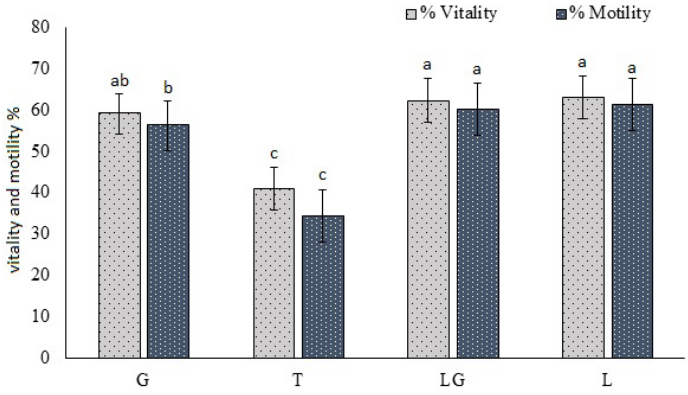

Figure 1 – Vitality and motility values (%) after dilution. Glucose (G), Tris-Glucose (T), Lactated Ringer's-Glucose (LG) and Lactated Ringer's (L). a-c means in a column with no common superscript differ significantly at p<0.05. ($\bar{X}$± Sx).

Figure 2 shows the motility rates of the samples, which were equilibrated at + 5 ° C for 90 minutes.

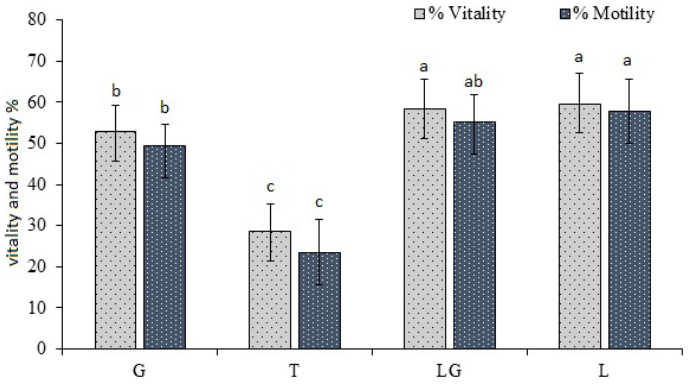

Figure 2 – Vitality and motility values (%) after 90 minutes of equilibration at +5°C. Glucose (G), Tris-Glucose (T), Lactated Ringer's-Glucose (LG) and Lactated Ringer's (L). a-c means in a column with no common superscript differ significantly at p<0.05. ($\bar{X}$± Sx).

Groups LG and L had similar results when it came to vitality rates and the differences between the other groups were significant (p<0.05). Group L had the highest vitality rate (59.75% ± 3.58) while group T had the lowest vitality rate (28.75% ± 2.81). Similarly, groups LG and L had similar motility results and the differences between the other groups were significant (p<0.05). Group L had the highest motility rate (57.87%±2.58); whereas, group T had the lowest motility rate (23.50%±1.92).

Figure 3 shows the vitality and motility rates of the samples after they were frozen and thawed.

The differences among the groups in terms of their vitality and motility rates at the end of freezing/thawing were significant (p<0.05). Group L had both the highest vitality and motility rates: 41.93%±1.87 and 40.12%±1.35. Likewise, group T had the lowest vitality and motility rates: 10.56%±1.34 and 5.12%±0.83. A similar study reported that vitality and motility rates of white Italian geese after their semen samples were frozen/thawed were 34.7% and 14.1% (Lukaszewicz, 2002).

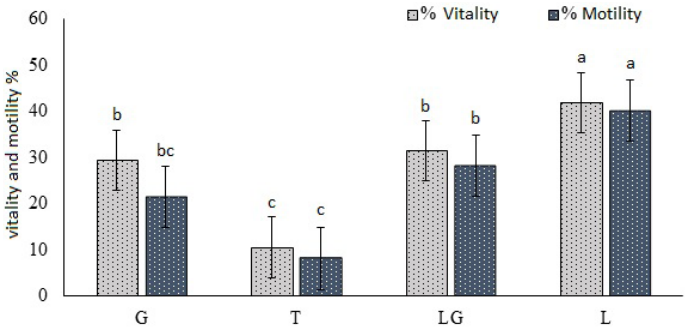

Figure 3 – Vitality and motility values (%) after freezing/thawing. Glucose (G), Tris--Glucose (T), Lactated Ringer's-Glucose (LG) and Lactated Ringer's (L). a-c means in a column with no common superscript differ significantly at $p<0.05$. ($\bar{X} \pm Sx$).

Vitality rates of semen freezing procedure steps (Fresh pooled semen, Equilibrated semen, Post-thaw semen) were determined (Figure 4). The decrease in vitality rate (%) was determined as T > G > LG >L, respectively. At the end of the procedure, the decrease in groups L and LG was at an acceptable level, while the vitality rate in groups T and G was lower.

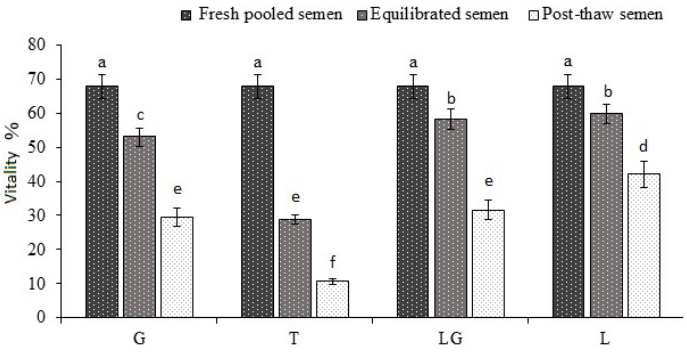

Figure 4 – Vitality % (fresh pooled semen, equilibrated semen, post-thaw semen). a-f means in a column with no common superscript differ significantly at $p<0.05$. ($\bar{X} \pm Sx$).

Moreover, the motility rates of Fresh pooled semen, Equilibrated semen and Post-thaw semen were determined (Figure 5). The decrease in motility rate (%) was determined as T > G > LG >L, respectively. At the end of the procedure, the motility rate of L was at an acceptable level, while the motility rates of LG, T and G diluents were low. This difference was thought to be associated with the chemical structures of the diluents. Furthermore, the result of the present study supports the idea that it is more advantageous to use tris-based diluents in ruminants instead of poultry (Tarig *et al.,* 2017; Kuzlu & Taskin, 2017).

The diluents and live material both are believed to have caused the differences between findings of this study (featuring DMF) and other studies. This indicates the importance of finding different freezing diluents for different poultry species (Lukaszewicz, 2001; Lukaszewicz, 2002; Váradi *et al.*, 2019). A similar study that diluted the sperm samples of Chinese Brown Geese with diluent that contained 4% DMSO (dimethylsulfoxide) yielded motility and vitality rates of 2.0 %± 2.5 and 7.3 %± 4.7, respectively. The same researchers also diluted a separate sample with DMA (dimethylacetamide) and yielded motility and vitality rates of 1.9 %± 2.5 and 27.0% ± 3.2, respectively (Tai *et al.*, 2001). In contrast, the findings of the present study are higher. This proves that DMF has a high protective property for goose spermatozoa during the freezing process (Santiago-Moreno *et al.*, 2011). Researchers have used DMF at various concentrations as cryoprotectants: 6 % in goats (Bezerra *et al.*, 2011), 6% in dogs (Lopes *et al.*, 2009), 8 % in forest indian chickens (Indian red jungle fowl) (Rakhaa *et al.*, 2020), 6% in rabbits (Domingo *et al.*, 2018), 5% in horses (Soni *et al.*, 2019), 15% in zebrafish (Diogoa *et al.*, 2019), 4, 6, 8, and 10% in ducks (Han *et al.*, 2005), and 7.5% in chickens (Miranda *et al.*, 2018).

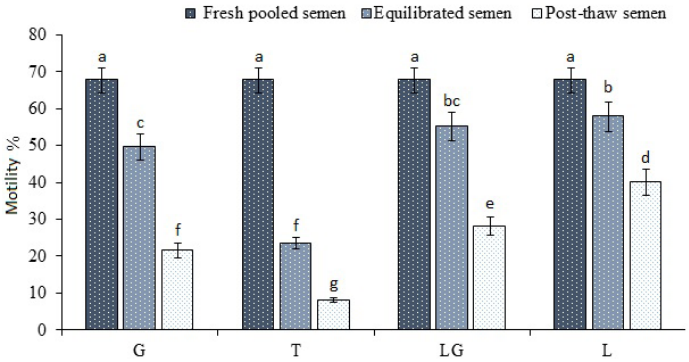

Figure 5 – Motility % (fresh pooled semen, equilibrated semen, post-thaw semen). a-g means in a column with no common superscript differ significantly at $p<0.05$. ($\bar{X} \pm Sx$).

The rate of fertile eggs in naturally mating goose herds ranges from 48% to 79% (Grunder & Pawluczuk., 1991). Geese need relatively less spermatozoa for fertile eggs than chicken and turkey. By inseminating geese once a week (Semen concentration of 14 million), fertile eggs of 54% can be obtained (Davtian & Pimenov., 1970; Grunder & Pawluczuk., 1991). In geese inseminated with fresh semen weekly, the rate of fertile eggs was reported to be 89% for semen concentration of 9 million and 95.5% for semen concentration of 20 million (Łukaszewicz., 2002). In addition, 37.5% of fertile eggs were obtained in insemination with Post-thaw semen (Łukaszewicz *et al.*, 2004). In the present study, it is thought that the inseminations to be made with the straws we prepared using L diluent ((Straws of concentration 0.29±0.80 x108 /ml, 40.12% motility and 41.93% Vitality) with 7-day intervals will create a fertility at the desired level.

CONCLUSIONS

Researchers need to find ways to eliminate the problems in breeding of geese with low reproductive

ability. Hence, further studies need to be conducted to investigate cryo-preservation of goose semen via artificial insemination. In this study, DMF was used as a cryo-protectant. It was determined that Lactated Ringer's Diluent containing 6% DMF yielded the highest vitality and motility rates. In short, we think that Lactated Ringer's Diluent containing 6% DMF can be used in cryo-preservation of goose semen.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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