



Effects of Storage Tanks and Ambient Temperature on the Liquid Nitrogen Evaporation and Semen Quality of Murrah Buffalo Bulls (*Bubalus bubalis*)

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ABSTRACT

Background: The integrity of storage tanks is essential in maintaining the liquid nitrogen used to preserve buffalo semen straws later utilized for artificial insemination. Faulty semen handling procedures, and equipment maintenance damage the spermatozoa leading to low-quality semen and low conception rates.

Methods: The study used two brands of liquid nitrogen tanks and four purebred mature Bulgarian Murrah buffaloes. The semen samples were collected twice a week and immediately processed by the center's laboratory technician following a slow freezing technique. After processing, the semen straws were stored in LN₂ tanks at -196°C.

Result: Results revealed that tank 2 was more efficient in maintaining the weekly amount of LN₂ and the frequent opening of tank covers significantly ($P < 0.05$) decreased the quantity of LN₂ after four days. Moreover, the different brands of LN₂ tanks did not affect sperm motility and livability, however, the frequency of exposing the semen straws to ambient temperature for 5 and 10 seconds lowered the sperm quality. In conclusion, semen straws should not be exposed to ambient temperature beyond the recommended time due to its deleterious effect on the semen quality compromising the success of insemination.

Key words: Evaporation loss, Evaporation rate, Liquid nitrogen, Murrah buffalo, Semen, Sperm motility.

INTRODUCTION

Artificial insemination (AI) has become one of the most important techniques for genetic improvement for farm animals including buffaloes. Successful conservation of semen for a longer period is obtained by maintaining the viability of spermatozoa from its collection until its use for insemination (Meena *et al.*, 2017). Likewise, dewars, LN₂ field tanks, and LN₂ are essential tools for preserving and storing semen for an extended time. The temperature in semen storage tanks is crucial in maintaining the cells, tissues, and viability of semen stored within the tanks (Ahmadzadeh, 2022; Bustani *et al.*, 2021; Thomas *et al.*, 2021). During the storage, the sperm are cooled to a very low temperature below zero at -196°C where all biological activity ceases (Getreu and Fuller, 2019). Hence, frozen semen straws that are properly stored and frozen last for a long time given that the temperature is maintained correctly (Bahmid *et al.*, 2022). However, since the straws' surface area to volume ratio is high, they are susceptible to thermal insults and become vulnerable to ambient temperature once lifted out of the LN₂ tanks. Moreover, the exposure of semen straws above -130°C and subsequent re-cooling cause irreversible damage.

Faulty semen handling procedures, improper semen handling techniques, and equipment maintenance damage the spermatozoa leading to lower conception rates (Sudheer *et al.*, 2017; Du Ponte, 2007). Therefore, special consideration should be given to semen storage and care and maintenance of the LN₂ tanks to ensure the fertility of

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frozen semen straws since the success of AI depends on the availability of good-quality semen (Ahmadzadeh, 2022; Nirmal *et al.*, 2020). Poor handling habits by veterinarians and technicians result in cumulative damage that lowers the sperm's fertilizing capabilities, and in some instances causes total infertility (Stroud, 2013). Since AI technicians normally open, and lift the canisters several times a week to draw semen straws and conduct AI services in various places, it is important to ascertain the effects of using different brands of LN₂ tanks and exposing the semen to ambient temperature at various times as this could

influence sperm motility and livability which are vital parameters in the success of artificial insemination. Hence this study is conducted.

MATERIALS AND METHODS

The study was carried out at the institutional farm of the DA-PCC at UPLB from February 05, 2024 - March 07, 2024. The experimental frozen semen straws were provided by the semen laboratory of the center which came from four purebred, mature Bulgarian Murrah buffalo bulls having good genetic traits ranging from 3-5 years, apparently healthy, and regularly used as semen donors. The semen samples came from the first or second ejaculate which was collected twice a week from 5:00 to 6:00 in the morning and was immediately processed by the laboratory technician of the center following a slow freezing technique. After processing, the semen straws were stored in the LN₂ tanks at -196°C.

The experiment used six new LN₂ field tanks with different brands provided by DA-PCC, Headquarters and Genepool, Nueva Ecija. Before the experiment, the empty tanks were inspected for any defects to maintain the amount of LN₂, and capably store frozen semen straws throughout the experiment. Before the study, the tanks were individually weighed, consistently filled with the required amount of LN₂, and observed daily for one week for any reduction until it finally stabilized. During the experiment, the tanks were placed outside the semen laboratory office and exposed to an ambient temperature of about 26-28°C. This was done to mimic the environmental condition to which the LN₂ field tanks of the AI technicians were exposed. The amount of LN₂ was monitored and filled with LN₂ weekly. The same procedure was done for one month.

The frozen semen straws were randomly assigned into three treatments replicated four times with 3 replicates per treatment. The treatments used in the experiment were: T₁ - Control (undisturbed); T₂ - 3 times a week of opening the tank covers and semen exposure to ambient temperature for 5 seconds; and T₃ - 5 times a week of opening the tank covers and semen exposure to ambient temperature for 10 seconds.

The amount of LN₂ in each tank was consistently measured once a week by getting the initial weight (every Monday), and the final weight (every Friday) using a calibrated weighing scale. The individual weights of the LN₂ field tanks were recorded and deducted to get the actual weights of the amount of LN₂ left in the tanks. The evaporation rate was computed using the derived formula:

$$\text{Evaporation rate} = \frac{\text{Initial weight (kg)} - \text{Final weight}}{\text{Initial weight}} / \text{Days in between}$$

while LN₂% loss was computed by using this formula:

$$\text{LN}_2 \% \text{ Loss} = \frac{\text{Initial weight (kg)} - \text{Final weight}}{\text{Initial weight}} \times 100$$

A total of 72 experimental frozen semen straws packed in 0.5 ml capacity were used in this study. Twelve semen straws for each treatment were drawn and evaluated for their post-thaw sperm motility characteristics. The semen samples in T₁ were thawed at 39-42°C for 15 to 20 seconds while treatments 2 and 3 were exposed to ambient temperature for 5 and 10 seconds using the same thawing temperature and were immediately examined with the assistance of an experienced laboratory technician of the semen laboratory using Computer-Assisted Sperm Analysis (CASA) Androvision. The averages from the 12 values per treatment were computed and subjected to statistical analysis.

The samples used in the livability evaluation were obtained from semen straws from each treatment used in the post-thaw motility testing. The stain used in the livability analysis contained eosin (0.2 g) and nigrosine (1.0 g) dissolved in 20 ml of 0.03% sodium citrate solution. The solution was mixed thoroughly using a magnetic stirrer at 300 rpm. Two drops of semen were placed in a clean glass slide then 1-2 drops of stain were added, dried, and evaluated within 24 hrs. A digital microscope (Optika®, B-293) objectives E-plan 40×/0.65 was connected to a laptop to determine the livability of post-thawed semen. Fully stained and half-stained spermatozoa were considered dead while live sperm were those that did not absorb the stain. The percentage of live sperm was computed using the formula lifted from Mamuad *et al.* (2005).

Per cent live sperm =

$$\frac{\text{Total number of live sperm counted}}{\text{Total number of sperms counted in the field}} \times 100$$

The averages from the 12 values per treatment were computed and subjected to statistical analysis.

Data analysis

All data were analyzed using ANOVA in Completely Randomized Design (SAS® OnDemand Academics Software). Comparison of treatment means was analyzed using the Least Significant Difference (LSD) at a 5% level of significance. Results were presented as mean±standard deviation.

RESULTS AND DISCUSSION

Liquid nitrogen plays a vital role in effectively storing and keeping the quality of frozen semen for prolonged periods. Hence, it should be stored away from direct sunlight and placed in a cool, clean, dry, and well-ventilated environment (Dalton, 2022; Thomas *et al.*, 2021; Duponte *et al.*, 2007). However, Llanto (2017) observed that all LN₂ tanks in provincial and field stations (e.g. center or field tanks) were stored in vacant spaces inside the offices and placed directly on the floor in the Bicol region. The same set-up was done in this experiment where LN₂ field tanks were stored outside the semen laboratory room and placed directly on the floor. The tanks were exposed to an

environmental temperature that ranges from 26-28°C for one month to simulate the environmental temperature to which the LN₂ field tanks of the AI technicians were exposed when rendering AI services in the municipalities.

Evaporation loss (%)

The evaporation loss of LN₂ stored in different LN₂ field tanks and the frequency of opening the field tank cover is presented in Table 1. A significantly higher ($P<0.05$) evaporation loss ($49.79\pm4.47\%$) was obtained in tank 2 compared to tank 1 ($45.60\pm5.37\%$). This suggests that tank 2 is more efficient in maintaining the weekly amount of LN₂. Pomeroy *et al.* (2019) reported that the designs of LN₂ tanks (e.g. size, shape, construction materials, welds, and sealants) do not prevent the loss of the tank's structural integrity. Moreover, the gradual loss of vacuum caused by metal fatigue and structural stress also increases the use of LN₂. Although there were no tests done to measure the evaporation loss of different LN₂ tanks used by the AI technicians in the service areas, higher evaporation loss is expected due to the frequent opening of tank covers and lifting of canisters for some time, especially those AI technicians who conduct several AI services in a week. Furthermore, mishandling the tanks during transport may cause damage resulting in premature loss of LN₂ (Thomas *et al.*, 2021).

Similarly, the evaporation loss significantly differed ($P<0.05$) across treatments. The highest percent evaporation loss was observed in T₃ ($51.94\pm3.31\%$) followed by T₂ ($47.57\pm4.50\%$) and T₁ ($43.57\pm4.52\%$) respectively. This suggests that the frequent opening of tank covers (T₂ and T₃) can significantly ($P<0.05$) decrease the quantity of LN₂ within 4 days respectively. The nitrogen evaporation rate depends on how the tank is opened, the type of tank, and the integrity of the tank (Thomas *et al.*, 2021). Therefore, weekly monitoring of LN₂ levels is important to ensure the quality of stored frozen semen.

Evaporation rate (L/day)

The evaporation rate of LN₂ tanks and the frequency of opening the tank covers are presented in Table 2. The evaporation rate of LN₂ in tank 2 (0.25 ± 0.02 L/day) is significantly ($P<0.05$) higher than in tank 1 (0.20 L/day) which is consistent with the results obtained in evaporation loss. However, the evaporation rates of T₁ (0.20 ± 0.02 L/day) and T₂ (0.25 ± 0.02 L/day) were higher than that stated in the company's manual which is 0.14 L/day for tank 1 and 0.10 L/day for tank 2. The difference in evaporation rates can be explained by how the end-user handled the LN₂ field tanks.

The experimental LN₂ field tanks were exposed to higher environmental temperatures at 26-28°C for one month. This shows that LN₂ tanks with higher evaporation loss resulted in a higher evaporation rate. Likewise, evaporation rates were affected ($P<0.05$) by the frequency of opening the tank covers at various times. The highest evaporation rate was found in T₃ (0.25 ± 0.02 L/day) while T₂ (0.22 ± 0.03 L/day) and T₁ (0.21 ± 0.02 L/day) did not differ ($P>0.05$) from each other. This means that opening the tank covers more than five (5) times a week significantly ($P<0.05$) increases the evaporation rates which is unavoidable since AI technicians may conduct several AI services in a week. Moreover, the field tanks are exposed to different environmental conditions and are transported via motor vehicles.

Although gradual changes in liquid nitrogen levels are normal and expected, monitoring the amount of LN₂ weekly is necessary to maintain its capacity to store frozen semen. Regular maintenance (e.g. monitoring of tank weights and liquid gas levels) varies from laboratory to laboratory and may involve daily or weekly inspections (Pomeroy *et al.*, 2019). With proper handling, most tanks will last for years but all liquid nitrogen tanks will eventually fail due to aging and loss of vacuum (DuPonte, 2007).

Table 1: Evaporation Loss (%) of LN₂ in different LN₂ field tank models and frequency of lifting the canisters.

LN ₂ field tanks	T ₁	T ₂	T ₃	Overall Mean $\pm$ SEM
1	41.39 $\pm$ 4.29	45.28 $\pm$ 5.06	50.14 $\pm$ 3.28	45.60 $\pm$ 5.37 ^b
2	45.75 $\pm$ 4.09	49.88 $\pm$ 2.78	53.75 $\pm$ 2.50	49.79 $\pm$ 4.47 ^a
Overall Mean $\pm$ SEM	43.57 $\pm$ 4.52 ^c	47.57 $\pm$ 4.50 ^b	51.94 $\pm$ 3.31 ^a	

Means in the column and row followed by different letters are significantly different at ($P<0.05$).

T₁ - No opening of the tank cover; T₂ - 3 times a week of opening the tank cover for 5 seconds, T₃ - 5 times a week of opening the tank covers.

Table 2: Evaporation rate (L/day) of LN₂ used in storing Murrah buffalo semen.

LN ₂ Tanks	T ₁	T ₂	T ₃	Overall Mean $\pm$ SEM
1	0.19 $\pm$ 0.02	0.20 $\pm$ 0.02	0.23 $\pm$ 0.01	0.20 $\pm$ 0.02 ^b
2	0.23 $\pm$ 0.02	0.25 $\pm$ 0.01	0.27 $\pm$ 0.01	0.25 $\pm$ 0.02 ^a
Overall Mean $\pm$ SEM	0.21 $\pm$ 0.02 ^b	0.22 $\pm$ 0.03 ^b	0.25 $\pm$ 0.02 ^a	

Means in the column and row as followed by a different letter are significantly different at ($P<0.05$).

T₁ - No opening of the tank cover; T₂ - 3 times a week of opening the tank cover for 5 seconds, T₃ - 5 times a week of opening the tank covers.

P-values of the main effects

Table 3 presents the p-values of the main effects of LN₂ field tank brands and semen exposure to ambient temperature and their interaction effect on the buffalo semen parameters. The utilization of different LN₂ field tank brands did not significantly ($P>0.05$) affect sperm motility and livability. Although the evaporation loss and rates differed ($P<0.05$) from each other (Tables 1 and 2) both tanks are efficient in maintaining LN₂ levels. However, both parameters were significantly ($P<0.05$) affected by the frequent semen exposure to ambient temperature at various times. This indicates that different brands of LN₂ tanks can be used in storing semen but weekly monitoring of the quantity of LN₂ is needed due to some evaporation loss. Moreover, frequent semen exposure to ambient temperature lowers semen quality.

Post-thaw sperm motility (%)

The post-thaw sperm motility of Bulgarian Murrah buffalo bulls stored in different LN₂ field tank brands and exposed to ambient temperature at various times is presented in Table 4. The highest average sperm motility was noted in tank 1 ($86.15\pm1.12\%$) followed by tank 2 ($84.38\pm1.35\%$) but did not ($P>0.05$) differ from each other which means that using LN₂ field tank brands does not affect sperm motility especially when the amount of LN₂ is regularly monitored. This conforms with the study of Llanto (2017), who stated that storage did not affect the post-thaw motility of the frozen semen. However, 70% post-thaw motility was noted in their study which was lower than the values obtained in this study with 86.15-84.38% respectively. The higher sperm motility obtained in this study can be due to the fewer times the semen straws were exposed to ambient temperature. In contrast, sperm motility varies ($P<0.05$)

across treatments. The lowest sperm motility was found in T₃ ($79.92\pm1.40\%$) followed by T₂ ($85.46\pm1.37\%$) and T₁ ($90.43\pm0.95\%$), respectively. The frequency of exposing the frozen semen straws to ambient temperature for at least 5 and 10 seconds resulted in lower sperm motility (T₃ - 79.92 and T₂ - 85.46% vs T₁ - 90.43%). This is in agreement with the study of Mamuad *et al.* (2005), who observed that too long and frequent exposure of frozen semen in the air damages the quality of frozen semen. Recommendably, the transfer of frozen semen should be done at least 3 seconds while exposing and all other actions for transferring should be conducted within 5 seconds. Moreover, several handling activities if improperly performed expose frozen semen to temperatures that lead to cell damage or even death which occurs when the internal temperature of the semen straws increases above -130°C (Stroud, 2013). To stabilize the crystalline formation of frozen semen it should be maintained at -120°C (Mamuad *et al.*, 2005). The damage from each exposure is cumulative and straws inside the tank can be damaged especially when semen straw lifted out of the LN₂ tank is re-introduced to below those temperatures (Stroud, 2013). It is interesting to note, that the experimental frozen semen straws were not re-introduced to LN₂ and were immediately evaluated, however, a significant decrease in sperm motility was observed in treatments (T₂-85.46% and T₃-79.92%) after exposure to ambient temperature for a certain time. Although frozen semen properly stored in LN₂ at -196°C stays viable for at least a thousand years, damage to sperm occurs in sperm when the transformed large crystals invade the cell membranes and cellular organelles. The severity of damage to cells depends on how high the temperature gets above -130°C and the duration of exposure above -130°C. Moreover, since the temperature in the necks of standard

Table 3: P-values of the main effects of LN₂ field tank brands and semen exposure to ambient temperature and its interaction effect on the buffalo semen parameters.

Semen parameter	P-values		Interaction
	LN ₂ tanks	Semen exposure	LN ₂ tanks × Semen exposure
Sperm motility (%)	0.2271	0.0001	0.6948
Sperm livability (%)	0.1343	0.0001	0.7847

Table 4: Post-thaw sperm motility (%) of Bulgarian Murrah buffalo bulls stored in different liquid nitrogen tanks (LN₂), and exposed to various environmental conditions.

LN ₂ tanks	T ₁	T ₂	T ₃	Overall Mean±SEM ^{ns}
	No semen exposure	3 times lifting; 5 secs exposure	5 times lifting; 10 secs exposure	
1	90.76±1.66	86.07±1.44	81.63±1.82	86.15±1.12
2	90.09±0.99	84.85±2.39	78.20±2.09	84.38±1.35
Overall Mean±SEM	90.43±0.95 ^a	85.46±1.37 ^b	79.92±1.40 ^c	

Means in the column followed by a different letter are significantly different at ($P<0.05$).

T₁ - No semen exposure to ambient temperature; T₂ - 3 times a week semen exposure to ambient temperature for 5 seconds, T₃ - 5 times a week semen exposure to ambient temperature for 10 seconds.

dewars is about -75°C and the room temperature is expected to have a higher temperature, it is common for frozen semen to be exposed and damaged (Sudheer *et al.*, 2017; Stroud, 2013).

The increased duration of semen straw to room temperature at about $28-30^{\circ}\text{C}$ significantly decreases the sperm motility of Simmental cattle which corroborates with the results of this study. The lowest sperm motility (P_4 -44.88%; P_5 -35.88% and P_6 -20.50%) was found in samples with the highest lifting time of about 25, and 30 seconds, respectively which are unfit for AI use because their motility was $< 40\%$ (Hoesni *et al.*, 2022). On the contrary, higher sperm motility was obtained in this study due to the faster time of exposing the semen to ambient temperature. Furthermore, the progressive motility decreased when the semen doses were subjected to higher temperatures. The magnitude of the damaging effects increases with longer exposure time (Sudheer *et al.*, 2017). On the other hand, the temperature and other climatic factors (e.g. humidity, photoperiod) directly affect the animals (Singh *et al.*, 2023). One of the most significant characteristics influencing sperm's ability to fertilize is motility (Broekhuijse, 2015; Feitsma, 2009). It indicates the progressive movement of sperm in the female reproductive tract to reach the egg and initiate fertilization (Bahmid *et al.*, 2022). Sperm motility is crucial in determining semen quality both before and after thawing since it naturally controls the sperm's capacity to fertilize oocytes (Puglisi *et al.*, 2012). Moreover, the estimation of post-thaw motility is increasingly being utilized to evaluate the success of freezability of buffalo bull semen (Parmar *et al.*, 2021).

Post-thaw live sperm (%)

The post-thaw live sperm of Bulgarian Murrah buffalo bulls stored in different LN_2 tanks and exposed to ambient temperature is presented in Table 5. The per cent livability of frozen semen straws was comparable and not affected ($P>0.05$) by using different LN_2 field tanks which coincides with the observations of Llanto (2017), who obtained a good, fertile, and relatively high percentage (83.69-93.05%) of live sperm in all experimental stations irrespective of storage. The percentage of live spermatozoa is important because it determines the ejaculate quality (Singh *et al.*, 2018). Although a lower percentage of live sperm was recorded in

this study, frozen semen with $>70\%$ live sperm is considered good (RCA, 2004). Therefore, it can be used for artificial insemination while sperm with questionable fertility has $<50\%$ live sperm (Vale, 2004). Conversely, there was a significant ($P<0.05$) reduction in percent sperm livability across treatments. The number of times the semen straws are exposed to ambient temperature for a longer time led to a decrease in sperm livability which corroborates with the study of Mishra *et al.* (2013) who observed a lower percent live sperm count when the ambient temperature increases to $25-35^{\circ}\text{C}$. Additionally, a drastic reduction in the percentage of live cells was observed with successive exposure of straws to room temperature. Notably, 25% of live cells in the straw were lost after a 10-second exposure while a higher percentage of live cells is expected to be lost given continued exposure at room temperature (Wells *et al.*, 1973). Furthermore, semen straws should not be exposed above the LN_2 even for a short time (10 seconds) as they become warm faster, and any exposure leads to irreversible damage to sperm viability (Minimum Standards for Production of Semen 2022). Hence, a decreased number of semen exposure to warmer temperatures eliminates fluctuations in semen quality (Amundson *et al.*, 2023). The length of lifting semen straws at room temperature ($28-30^{\circ}\text{C}$) affected the sperm livability of Simmental cattle. The treatments with longer lifting times (25 and 30 seconds) led to 35.13% and 21.01% sperm livability which is not recommended for AI use (Hoesni *et al.*, 2022). Lower sperm livability may lead to lower conception rates. Therefore, a sperm with high livability should be considered.

On the other hand, trained users might assume that exposing frozen semen to ambient conditions does not cause any damage as long as the contents are in a frozen state. However, thermal damage occurs after very short ambient exposures (Lieberman *et al.*, 2016). The frost line serves as a landmark and commonly recommended area for semen straw removal (Ahmadzadeh *et al.*, 2022) while the neck of dewars is the working area where samples are handled and consequently exposed to potentially damaging temperatures. When semen is held above the frost line, a decrease in fertility occurs, as slight warming can cause sperm cells to die. If the desired straw cannot be located within 7-10 seconds of lifting the canister, it should be placed back into liquid nitrogen for 10-15 seconds. Furthermore,

Table 5: Post-thaw live sperm (%) of Bulgarian Murrah buffalo bulls stored in different LN_2 tank models and exposed to various environmental conditions.

LN_2 tanks	T_1	T_2	T_3	Overall Mean $\pm$ SEMns
	No lifting; No exposure	3 times lifting; 5 secs exposure	5 times lifting; 10 secs exposure	
1	90.38 $\pm$ 1.31	86.39 $\pm$ 1.35	81.34 $\pm$ 1.38	86.04 $\pm$ 0.98
2	89.09 $\pm$ 0.86	85.08 $\pm$ 2.19	78.33 $\pm$ 1.73	84.17 $\pm$ 1.21
Overall mean $\pm$ SEM	89.74 $\pm$ 0.78 ^a	85.74 $\pm$ 1.27 ^b	79.84 $\pm$ 1.13 ^c	

Means in the column followed by a different letter are significantly different at ($P<0.05$).

T_1 - No semen exposure to ambient temperature; T_2 - 3 times a week semen exposure to ambient temperature for 5 seconds, T_3 - 5 times a week semen exposure to ambient temperature for 10 seconds.

avoid using fingers to grab a straw of semen, as the heat of the hands can cause the straw to begin to thaw and again decrease the fertility of that semen (Amundson *et al.*, 2023).

CONCLUSION

The amount of LN₂ was significantly affected by using different brands of LN₂ field tanks. LN₂ field tank 2 was more efficient in terms of maintaining the weekly amount of LN₂. Likewise, the frequent opening of tank covers and leaving them open for 5 to 10 seconds lowered the amount of LN₂ within 4 days. There were no interaction effects ($P>0.05$) between the LN₂ field tank brands and the semen exposure to ambient temperature at various times.

The use of different LN₂ brands did not affect sperm motility and livability while the frequency of exposing the frozen semen straws to ambient temperature lowered the sperm motility and livability especially those samples exposed for a longer time.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All the semen samples were donated by the semen laboratory of the DA-PCC at UPLB. The researchers did not directly handle the animals. The semen collection was a regular activity of the center.

Conflict of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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