



# Fertility comparison of frozen bull semen stored in cryogenic deep freezer ( $-152\text{ }^{\circ}\text{C}$ ) and $\text{LN}_2$ container

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## ABSTRACT

The present study aimed to use cryogenic deep freezers that could be a feasible alternative for cryopreserved semen storage. A total of 284 straws from three Simmental bulls and 272 Simmental cows were used. The experimental group consisted of 151 semen straws that were stored at  $-152\text{ }^{\circ}\text{C}$  for a week. Moreover, the control group consisted of 133 semen straws that were stored at  $-196\text{ }^{\circ}\text{C}$ . Firstly, two samples per bull ( $n = 6$ ) were examined in terms of sperm kinetic parameters by CASA. Furthermore, plasma membrane, acrosome integrity (PMAI) and high mitochondrial membrane potential (HMMP) were analyzed by flow cytometry. Then, artificial inseminations were performed on Simmental cows with 272 straws belonging to two groups. Then, 56th-day Non-return Rate (NRR56) was determined. All spermatological data were subjected to a linear mixed model. Chi-Square test was performed to NRR56 between storage temperature groups. Also, logistic regression analysis was used to examine the effect of bull, storage temperature and age of cows on pregnancy status. While age of cows was included in the final logistic regression model, effect of bull  $\times$  storage temperature was not included because it was found as non-significant. The post-thaw PMOT and STR of cryopreserved bull semen, which was stored at  $-152\text{ }^{\circ}\text{C}$ , had lower and statistically significant values ( $p < 0.05$ ). However, frozen bull semen, which were stored at  $-152\text{ }^{\circ}\text{C}$ , kept its fertility ability as which stored at  $-196\text{ }^{\circ}\text{C}$ . Besides, NRR56 of semen stored at  $-152\text{ }^{\circ}\text{C}$  and  $-196\text{ }^{\circ}\text{C}$  were detected as 57.24% (83/145) and 55.91% (71/127), respectively ( $p > 0.05$ ). Nevertheless, these results should be supplemented with more pre-freezing and post-thaw sperm quality analyses and more fertility data for increasing the accuracy of the method.

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## 1. Introduction

Artificial insemination (AI) is a basic and common assisted reproductive technology for breeding farm animals. Fresh, chilled and cryopreserved semen with their relative success rates has been used from the first AI application until now. Especially, cryopreserved bull semen comprises 95% of all kinds of semen that is used for artificial insemination in the dairy industry [1]. Using cryopreserved semen brings storage problems and responsibilities. Storage of semen is a kind of storage of organisms in various

environments for years. Until to date, lots of methods have been developed for storing cryopreserved semen, and  $\text{LN}_2$  has been determined as the most suitable for using [2–4]. Nevertheless,  $\text{LN}_2$  has some disadvantages like contaminations, affected by climatic and seasonal temperature changes, costs, labor force and human initiative [5,6]. The aim of the present study was to investigate a feasible alternative method for cryopreserved bull semen storage and its success in terms of fertility.

Effective storage techniques bring effective reproductive success and effective genetic improvement. Thus,  $\text{LN}_2$  based cryopreserved semen storage has been used in the livestock industry with its pros and cons for decades. In this method,  $\text{LN}_2$  supply is the determinant factor of a successful storage process. However, this necessity brings some disadvantages such as risks of microbial pathogen contaminations, supplying, labor force, maintenance and security

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problems for vets and farmers [7,8]. Therefore, various studies aimed to avoid the use of LN<sub>2</sub> in the storage of semen [9–11]. In various mammalian species such as caprine, Medrano et al. [9] and Batista et al. [10] used cryogenic deep freezers (CDF) and revealed their success for both freezing and storage the goat semen at  $-152^{\circ}\text{C}$ . In another study, Alamo et al. [11] showed the semen characteristics of dogs were not affected by CDF conditions after 240 days of storage.

Today, semen could be frozen at temperatures between  $-80$  and  $-140^{\circ}\text{C}$  [12,13]. Therefore, it can be expected that storing frozen semen at lower temperatures than the freezing range does not harm semen. Because  $-152^{\circ}\text{C}$  is below the semen freezing temperature range, CDF could be suitable for cryopreserved bull semen preservation. Although CDFs have some disadvantages such as electricity dependency, mechanical complexity and higher temperature than liquid nitrogen, they are considered to be a viable alternative to liquid nitrogen in semen storage due to its properties such as easy maintenance, regular temperature and low cost of use [14,15]. On the other hand, CDFs are considered to be very advantageous semen storage tools in terms of capacity. For example, considering the maximum number of frozen bull semen straw, a 35 L liquid nitrogen container with six canisters can hold 12 goblets with a capacity of 1500 straws. Containers with 10 canisters, which can hold smaller volume goblets, can accommodate a maximum of 20 goblets with a capacity of 800 straws. In contrast, 128 L CDF can fit around 100 goblets when it is fully loaded with goblets with a capacity of 1500 straws. Cryogenic freezers have the capacity to hold approximately 200 pieces of the 800 straws goblets. These calculations show that a 128 L CDF has the capacity of almost 10 large and bulky 35 L nitrogen container. This will save farmers and companies who store frozen semen from the loss of space, labor, expense and time required for the efficient storage of 10 nitrogen tanks and the valuable frozen bull semen stored in them.

In the present study, the fertility success of the cryopreserved bull semen, stored at  $-152^{\circ}\text{C}$  in the CDF for a week, was aimed to determine. For this purpose, a number of fertility trials were done as the main objective of the study (a totally of 272 inseminations were done). Since the bull and ejaculate differences do not represent the fertility success completely [8,9], few straws were used to evaluate the spermatological characteristics of the groups to only give a view and information about frozen bull semen that has been used for artificial inseminations. Considering the low number of studies on the storage of semen at CDFs, this study was aimed to present that using CDFs could be a feasible alternative for cryopreserved semen storage as a new perception. Besides, the determination of Non-return Rate 56 (NRR56) was performed to confirm the semen fertility after storage at  $-152^{\circ}\text{C}$ .

## 2. Materials and methods

### 2.1. Study design

A total of 284 cryopreserved bull semen straws that were produced from three different Simmental bulls (Bull 1, Bull 2 and Bull 3) on the same date were bought from an enterprise. They were received in the LN<sub>2</sub> container. Immediately after the samples were received, they were divided into two groups. Moreover, 151 semen straws, which were determined as the experimental group (CDF), were transferred to the CDF. The control group (LN<sub>2</sub>), which consisted of 133 semen straws, were stored at  $-196^{\circ}\text{C}$  in LN<sub>2</sub> container.

A total of 272 Simmental cows were enrolled in the study. Cows, which were from the same enterprise, were housed together under the same nutrition and care conditions. They were fed with TMR twice daily for ad libitum intake and free access to water. The barns were naturally ventilated and had artificial lighting. Cows were

subjected to systematic examinations. In the study, 272 Simmental cows aged two to six were included. The cows had no disorders in terms of general and reproductive health and their body condition score was between 2.5 and 3. To provide uniformity along with the cows, G6G/Ovysynch synchronization procedure was applied according to Sonat et al. [16]. This study was approved by the Ankara University Animal Ethics Committee (2019-3-26) and all experiments were conducted according to ethical principles and laws.

A total of six semen samples (two semen samples per bull) for each storage temperature were thawed and examined in terms of sperm kinetic parameters (total motility, progressive motility, other movement traits) by CASA. Additionally, plasma membrane, acrosome integrity (PMAI) and high mitochondrial membrane potential (HMMP) status were examined by using flow cytometry. For field trials as the main objective of this study, 272 Simmental cows were inseminated with straws belonging to different groups (145 for CDF; 127 for LN<sub>2</sub>). Besides, 56th-day Non-return Rates (NRR56) were determined.

### 2.2. Computer-assisted sperm analysis

Sperm motility characteristics of post-thawed bull semen samples were evaluated with a CASA system (Sperm Class Analyzer v.4.2.; Barcelona, Spain). Sperm kinematics were driven and processed by SCA v.3.2.0 software. The CASA system was based on the analysis of consecutive and digitalized photographic images that were taken in a time-lapse of 60 frame rate/sec. A 5  $\mu\text{L}$  of post-thawed semen sample was put onto a pre-warmed slide covered with a coverslip (22 mm  $\times$  22 mm). Total sperm motility (TMOT), progressive motility (PMOT), VAP, VSL, VCL, LIN and STR were determined with 10 $\times$  magnification objective, at  $37^{\circ}\text{C}$  [17].

### 2.3. Flow cytometry analysis

Sperm plasma membrane and acrosome integrity (PMAI) status were determined by CytoFlex flow cytometer (CytoFlex; Beckman Coulter, USA). All sperm samples were evaluated using argon laser beams generated by 488 nm (50 mW laser output). FITC-PNA (Fluorescein isothiocyanate-conjugated pea-nut agglutinin)/PI (Propidium iodide) dual-staining method was used for determining of PMAI. For this purpose, 2.5  $\mu\text{L}$  of FITC-PNA (100  $\mu\text{g}/\text{mL}$ ) and 1.5  $\mu\text{L}$  PI (2.99 mM) were added into 5  $\mu\text{L}$  of semen, that was previously diluted in 246  $\mu\text{L}$  of Phosphate-Buffered Saline (PBS) solution [18]. Then, diluted semen samples with dyes and the solution were assessed flow cytometrically after a 20-min incubation at  $37^{\circ}\text{C}$ . Apart from non-cellular events, spermatozoa was evaluated in 4 subgroups: a) Cells with intact plasma membranes and acrosomes (PI and FITC-PNA negative cells were accepted as viable spermatozoon; PMAI%), b) PI-positive and FITC-PNA negative cells with the defective plasma membrane and intact acrosome, c) PI negative and FITC-PNA positive cells with the intact plasma membrane and defective acrosome, d) PI and FITC-PNA positive cells with the defective plasma membrane and acrosome (Fig. 1).

The high mitochondrial membrane potential (HMMP) status of frozen-thawed semen was determined with the 5,5',6,6'-tetrachloro-1,1',3,3'-tetramethyl benzimidazolyl-carbocyanine iodide JC-1 staining method. Straws were thawed ( $37^{\circ}\text{C}$  for 30 s) and  $5 \times 10^6$  spermatozoa (5  $\mu\text{L}$ ) added to 245  $\mu\text{L}$  of PBS which include 2.5  $\mu\text{L}$  JC-1 and incubated in a dark room for 30 min in a water bath at  $37^{\circ}\text{C}$  [18]. After incubation, debris was gated out and analyses of HMMP and LMMP were done with CytExpert 2.2 software (Fig. 1).

### 2.4. 56th-day non-return rate

For the 56th-day Non-return Rate, a total of 272 Simmental

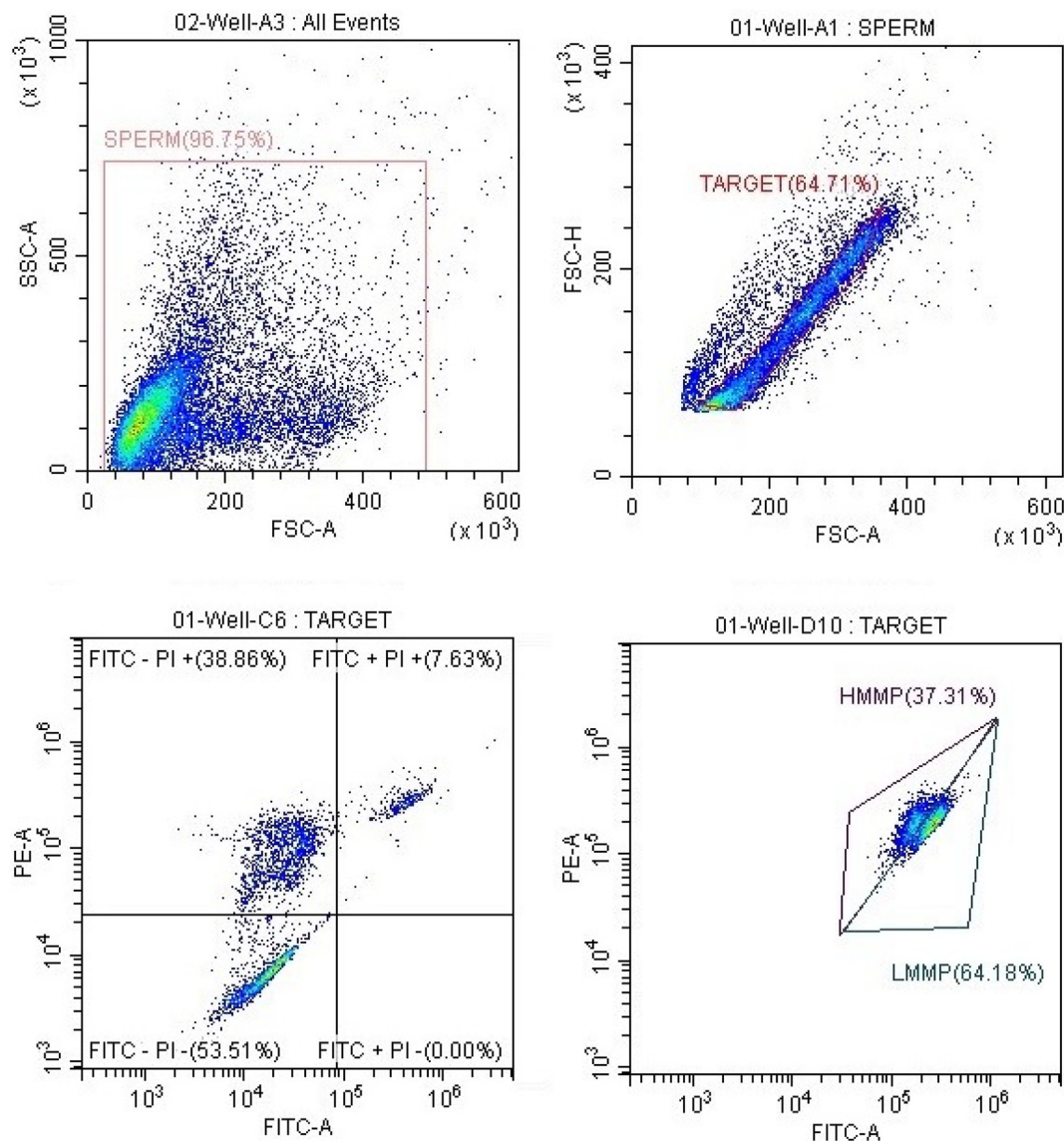


**Fig. 1.** Flow cytometric examination of frozen bull semen that used for field trials. (Gating process).

**SPERM:** Sperm population determined by gating the intersection of forward scatter area (FSC-A) on the x-axis and side scatter area (SSC-A) on the y-axis; **TARGET:** Single sperm was detected by gating the main plot of x-axis (FSC-A) and y-axis (forward scatter height; FSC-H). The same gating was also done for SSC-A (x-axis) and SSC-H (y-axis); **PMAI:** Plasma membrane and acrosome integrity, determined by gating of the percentage of non-stained cells (FITC - PI-); **FITC:** Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA). **PI:** Propidium iodide; **HMMP:** High mitochondrial membrane potential, determined by gating of the percentage of orange-red fluorescence; **LMMP:** Low mitochondrial membrane potential, determined by gating of the percentage of green fluorescence.

cows were inseminated by recto-vaginal method. Artificial inseminations were done as fixed time according to the protocol [16] and completed between February and June. Moreover, 145 (Bull 1: 48; Bull 2: 46; Bull 3: 51) cows were inseminated with the CDF group semen, and 127 (Bull 1: 42; Bull 2: 39; Bull 3: 46) cows were inseminated with the LN<sub>2</sub> group semen. Non-return Rates of the inseminated cows were calculated as the proportion of cows not re-inseminated within 56 days after first artificial insemination. Ultrasound examinations were also done on all of the cows to confirm pregnancies.

## 2.5. Statistical analysis

For statistical analysis, all spermatological data (CASA and Flow cytometry parameters) were subjected to a linear mixed model. Bulls (Bull 1, Bull 2 and Bull 3) were included as a random effect

while storage temperature (−152 °C and −196 °C) was included as a fixed effect in the model. Chi-Square test was performed to 56th-day Non-return Rate between storage temperature groups. Then, logistic regression analysis was used to examine the effect of bull, storage temperature and age of cows on pregnancy status. The interaction term “effect of bull x storage temperature” and “effect of bull x storage temperature x cow age” were not included in the final logistic regression model because they were found non-significant. Odds ratios and 95% confidence intervals were calculated. For all analyses, SPSS® 22.0 for Windows was used. Besides,  $p < 0,05$  was considered significant. Data were reported as mean value (X) ± standard error of the mean (SEM).

## 3. Results

In the results of the study, it was observed that PMOT and STR

parameters were statistically significant and had higher values in LN<sub>2</sub> group (67,21%; 79,24%) than CDF (56,48%; 70,61%) ( $p < 0,05$ ). Although all of the spermatological data from frozen semen stored in the CDF at  $-152^{\circ}\text{C}$  had numerically lower values than stored in the LN<sub>2</sub> at  $-196^{\circ}\text{C}$  (Table 1); the other spermatological parameters except PMOT and STR were found similar and differences among groups were not statistically significant ( $p > 0,05$ ).

Non-return rate values (Table 2) were also obtained statistically similar between groups ( $p > 0,05$ ). Moreover, results of the examinations of the effect of bull, storage temperature and age of cows on pregnancy status (Table 3) were not found statistically significant ( $p > 0,05$ ).

## 4. Discussion

Improvements in animal reproduction necessitate useful alternatives in frozen semen storage that avoid complications of traditional methods. In the study, CDF was examined in frozen bull semen storing as an alternative to LN<sub>2</sub> based systems. The present study evaluated the influences of storage temperature for storing bovine frozen semen on the post-thawed sperm quality and fertility. It was found that there were statistically differences in PMOT and STR parameters. Post-thawed PMOT and STR values of frozen bull semen stored in CDF were lower than LN<sub>2</sub> container (Table 1). Even though the other parameter values in CDF group were found numerically lower than LN<sub>2</sub>, there was no statistical significance.

In a previous study, frozen bovine semen was stored for 6 months in CDF and concluded that storing frozen bull semen at  $-152^{\circ}\text{C}$  is successful as well as LN<sub>2</sub> in terms of post-thawed motility, viability and abnormality [8]. Alamo et al. [11] tried to keep frozen canine sperm in LN<sub>2</sub> and CDF for up to 4 months. At the end of the 4 months, they obtained similar results in terms of motility, viability and acrosome status of post-thawed dog sperm. Batista et al. [10] and Medrano et al. [9] cryopreserved goat semen and stored them in CDF up to 1 year, and similar results have been obtained in terms of post-thawed spermatological parameters. In this research, on the contrary to the previous studies, it was represented that PMOT and STR values of CDF group decreased according to semen stored in LN<sub>2</sub>. While differences in PMOT and STR parameters between CDF and LN<sub>2</sub> storage methods were statistically significant, the rest of the spermatological traits were not. Interestingly, structural traits like PMAI and HMMP were obtained similar, although some movement traits were affected by higher

**Table 1**  
Results of spermatological examination of semen stored at different temperatures.

| Parameters | Groups             |                        | SEM   | P     |
|------------|--------------------|------------------------|-------|-------|
|            | CDF (n: 6)         | LN <sub>2</sub> (n: 6) |       |       |
| TMOT (%)   | 75,00              | 79,35                  | 6,96  | 0,170 |
| PMOT (%)   | 56,48 <sup>a</sup> | 67,21 <sup>b</sup>     | 6,57  | 0,009 |
| VCL (μm/s) | 84,08              | 85,47                  | 10,84 | 0,651 |
| VAP (μm/s) | 52,59              | 58,07                  | 7,97  | 0,238 |
| VSL (μm/s) | 40,66              | 50,27                  | 5,58  | 0,082 |
| STR (%)    | 70,61 <sup>a</sup> | 79,24 <sup>b</sup>     | 8,01  | 0,039 |
| LIN (%)    | 45,88              | 55,99                  | 6,35  | 0,145 |
| PMAI (%)   | 61,74              | 67,93                  | 8,88  | 0,181 |
| HMMP (%)   | 40,80              | 41,88                  | 8,06  | 0,765 |

a, b: Differences between the means in the same row with different letters are statistically significant ( $p < 0,05$ ).

CDF: Cryogenic Deep Freezer, LN<sub>2</sub>: Liquid Nitrogen, TMOT: Total motile sperm, PMOT: Progressive motile sperm, VAP: Average path velocity, VCL: Curve-linear velocity, VSL: Straight line velocity, LIN: Linearity, STR: Straightness, PMAI: Plasma membrane and acrosome integrity, HMMP: High mitochondrial membrane potential.

**Table 2**  
Means of NRR56 values of semen stored at different temperatures.

| Groups          | n   | NRR56  | NRR56 (%) |
|-----------------|-----|--------|-----------|
| CDF             | 145 | 83/145 | 57.24     |
| Bull 1          | 48  | 31/48  |           |
| Bull 2          | 46  | 24/46  |           |
| Bull 3          | 51  | 28/51  |           |
| LN <sub>2</sub> | 127 | 71/127 | 55.91     |
| Bull 1          | 42  | 23/42  |           |
| Bull 2          | 39  | 21/39  |           |
| Bull 3          | 46  | 27/46  |           |

CDF: Cryogenic Deep Freezer, LN<sub>2</sub>: Liquid Nitrogen, NRR56: Non-return rate 56.

storing temperatures. Some researchers defend the opinion that the fertilization ability of the spermatozoa depends highly on *in-vitro* quality [19–22]. On the other hand, Christensen et al. [23] and Stalhammar et al. [24] said that the bull and ejaculate differences affect the fertility success only less than 1%. Because of this information, few straws were used to evaluate the spermatological characteristics of the groups, to only give a view and information about frozen bull semen that was used for artificial inseminations. Nevertheless, it was concluded that storing frozen semen in CDF for a week might affect the post-thawed *in-vitro* bull semen quality.

There are very few fertility results about alternative frozen semen storing method trials. These studies were conducted on goats and dogs. In a study that was conducted on goats, it was concluded that storing frozen goat semen at  $-152^{\circ}\text{C}$  did not affect post-thaw fertility rates. On the other hand, in a study that was concluded with dogs, 40% pregnancy success was obtained by inseminations done with frozen dog semen stored at  $-152^{\circ}\text{C}$  [10,11]. According to our knowledge, there is not any study about the fertility of frozen bull semen after storage in CDFs. The present study gives important preliminary results in this aspect. In the present study, artificial inseminations were done with frozen bull semen stored in LN<sub>2</sub> and CDF and no difference was found between groups. On the other hand, it was observed that pregnancy status was not affected by bulls, storage conditions or age of cows. Although storing frozen bull semen at  $-152^{\circ}\text{C}$  was detected as harmful for *in-vitro* semen quality, it did not affect the fertilization ability. The study in which lots of artificial insemination trials were done (145 inseminations for CDF, 127 inseminations for LN<sub>2</sub>), revealed important fertility results in terms of frozen bull semen stored at  $-152^{\circ}\text{C}$  in CDF. It might be concluded that CDFs could be an alternative storage method for frozen bull semen without losing their fertility properties.

Although semen freezing has been made by different methods (alcohol, dry ice, nitrogen vapour or automatic) at various subzero temperatures (from  $-80$  to  $-140^{\circ}\text{C}$ ) until to date, they have been stored into the LN<sub>2</sub> ( $-196^{\circ}\text{C}$ ). Temperature differences between freezing and storing, provide reliable prevention on frozen semen from environmental conditions. However, the disadvantages, that LN<sub>2</sub> have, necessitates the investigation of new methods for storing frozen semen. Cryogenic deep freezers, thanks to their low

**Table 3**  
Result of logistic regression analysis on pregnancy status.

| Variable                   | Odds Ratio | %95 CI        | P-Value |
|----------------------------|------------|---------------|---------|
| <b>Bull</b>                |            |               | 0,527   |
| Bull (1 vs 2)              | 1,475      | 0,750 - 2,904 | 0,260   |
| Bull (1 vs 3)              | 1,172      | 0,611 - 2,249 | 0,633   |
| <b>Storage temperature</b> | 0,920      | 0,536 - 1,579 | 0,763   |
| <b>Age of cows</b>         | 1,089      | 0,908 - 1,306 | 0,358   |

Bull (reference- Bull 1, 1- Bull 2, 2- Bull 3), Storage temperature (reference-  $-152^{\circ}\text{C}$ , 1 to  $-196^{\circ}\text{C}$ ).

temperatures, put forward the thesis that semen can be stored safely with a temperature value of  $-152^{\circ}\text{C}$  that is below the freezing temperature of semen. Post-thawed CASA (motility and other kinetic parameters) and flow cytometric analyses showed that post-thawed semen quality could be affected at  $-152^{\circ}\text{C}$  with a decrease in some movement traits. However, fertility results obtained in the field showed the semen stored in CDF for a week could be successful as much as conventionally stored frozen semen. The spectacular fertility results obtained in the presented study verify the accuracy of this thesis.

## 5. Conclusion

The study attempted to reveal the uncertainty in the frozen bull semen storage without  $\text{LN}_2$  dependency. Although the low number of post-thawed spermatological analyses have been done, a huge number of the fertility trials could be enough to reveal the effects of CDF storage for 1-week. According to the data obtained in this study, it was observed that CDF had harmful effects on spermatological properties. On the contrary, similar fertility rates between storage methods provided to keep our optimistic look on this subject. In conclusion, it might be said that CDFs can take their place among the alternatives of liquid nitrogen in frozen bull semen storage. Nevertheless, the detrimental effect of the 1-week storage period on spermatological characteristics indicates that there will be limitations to the use of this alternative. Further studies should be performed to investigate and fully understand the extent to which the long-term storage of frozen bull semen in CDFs would achieve in terms of spermatological parameters and how these extensions would affect the fertility.

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## CRediT authorship contribution statement

**Mehmet Borga Tirpan:** Conceptualization, Methodology, Resources, Writing – review & editing. **Kemal Tuna Olğaç:** Methodology, Validation, Formal analysis, Data curation, Writing – original draft, Visualization. **Firat Korkmaz:** Writing – original draft, Visualization. **Ali Sonat:** Investigation, Data curation, Resources. **Ufuk Kaya:** Formal analysis, Investigation, Data curation. **Ergun Akçay:** Methodology, Writing – review & editing, Supervision.

## Declaration of competing interest

The authors declare no conflict of interest.

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