



Original Research

## The Effects of Refrigeration Temperature and Storage Time on Apoptotic Markers in Equine Semen

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### ABSTRACT

Evaluation of the damage caused by the sperm preservation process is crucial to improving fertilization rates. The objective of this study was to evaluate the effects of refrigeration temperature (5°C and 15°C) and storage time (0, 12, 24, 48, and 72 hours) on apoptotic markers in equine semen. Membrane phosphatidylserine translocation index, caspase activation index, and DNA fragmentation index were analyzed using epifluorescence microscopy. Analysis of variance was used for statistical analysis, and Tukey test was used to compare means. The significance level was set at  $P < .05$ . The results demonstrated that for transport duration shorter than 24 hours, semen quality was maintained when stored at either 5°C or 15°C. A storage temperature of 5°C should be used when it is necessary to transport semen for longer than 24 hours. There was a significant decrease in semen quality after 48 hours of refrigeration.

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## 1. Introduction

Equine semen refrigeration techniques have been studied because there is great interest in maintaining the fertilization potential of equine semen over prolonged periods [1]. Refrigeration provides advantages such as reductions in animal transport and lodging expenses, as well as a lower risk of acquiring diseases [2].

Padilla and Foote [3] and Amann and Graham [4] reported that care must be taken during the refrigeration and transport of semen to maintain the fertilization capacity of spermatozoa. Any damage incurred during the production, collection, or storage of male gametes may affect the fertilization process. Thermal stress can result in direct structural damage, such as membrane rupture, or indirect damage, such as changes in cellular functions [5]. Recently, phenomena similar to apoptosis were identified by sperm

cell analysis [6–8]. These phenomena affect cell death and inflict different degrees of damage on spermatozoa structures that are important for longevity [9].

During the initiation of apoptosis, cells lose plasma membrane asymmetry. Phosphatidylserine, which is normally present inside the membrane of a healthy cell, is translocated and expressed on the exterior. This process can gradually lead to membrane damage [10]. It has been shown that phosphatidylserine translocation can be used as an early marker of membrane deterioration in frozen–thawed human [11], bovine [6], and equine spermatozoa [12,13].

A number of authors have demonstrated the existence of caspase-dependent factors in the apoptosis pathway in human ejaculate [14,15]. These enzymes are considered to be major transducers and effectors within different apoptosis signaling pathways in somatic cells. The enzymes belong to a highly specific family of proteases that contain the amino acid cysteine within their active sites [14]. From a functional point of view, the caspases involved in apoptosis can be classified as initiators (caspases 8, 9, and 10) or effectors (caspases 3, 6, and 7) [16].

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The semen analysis method, with the best ability to predict fertility, is an examination of sperm chromatin structure. This test evaluates the susceptibility of spermatozoan DNA to denaturation. The ability of spermatozoan DNA to maintain an intact double stranded configuration is determined by exposure to an acidic environment. This method can be used to evaluate DNA integrity in fresh, refrigerated or frozen semen [17]. Typically, sires that exhibit elevated numbers of cells with compromised DNA in fresh samples also show an accelerated decrease in DNA quality when their semen is refrigerated or stored long-term [18].

Modifications on sperm cell similar to those observed during apoptosis result in reduced sperm cell longevity in the female reproductive tract. This is especially problematic in equine species because they have long estrous cycles and require greater care in determining ovulation and the optimal timing for insemination [19]. The objective of this study was to analyze the effects of refrigeration temperature and storage time on the phosphatidylserine translocation index (PSTI), caspase activation index (CaspI), and DNA integrity in equine semen.

## 2. Materials and Methods

Three ejaculates from six different stallions were used in our experiments. The ejaculates were collected using an artificial vagina. After harvesting, the ejaculate was filtered to remove gel, diluted to a concentration of  $50 \times 10^6$  spermatozoa/mL in a milk-based medium (Botu-semen; Botupharma Botucatu, SP, Brazil), and refrigerated at 5°C or 15°C for evaluation at 0, 12, 24, 48, and 72 hours.

Analysis of the semen samples was conducted using epifluorescence microscopy (Leica, Germany) at 1,000× magnification. A total of 200 cells from each sample were counted.

To evaluate the PSTI, the Annexin V-FITC Apoptosis Detection Kit II (556570; BD Bioscience Pharmingen, San Jose, CA) was used according to the manufacturer's recommendations. Semen aliquots were diluted in an Annexin V buffer solution (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM CaCl<sub>2</sub>) to a concentration of  $1 \times 10^6$  spermatozoa/mL. Aliquots of 100 µL (final concentration of  $1 \times 10^5$  spermatozoa/mL) of the samples were placed in Eppendorf 1.5 mL cryotubes (Eppendorf, Hamburg, Germany), and 5 µL of Annexin V-FITC, 5 µL of propidium iodide (PI, 50 µg/mL), and 2 µL of Hoechst 33342 (H342, 40 µg/mL) were added. The samples were then homogenized and incubated for 15 minutes. The cells observed during the analysis were classified as viable cells (An−/PI−), viable cells exhibiting phosphatidylserine translocation (An+/PI−), injured cells with phosphatidylserine translocation (An+/PI+), or dead cells (An−/PI+). The PSTI was calculated based on the relationship between the number of An+/PI− and the total number of An−/PI− [20].

An FITC-VAD-FMK reagent (G7462, Promega) was used as an in situ marker of the CaspI, according the manufacturer's instructions. One milliliter of phosphate-buffered saline (PBS) and  $1 \times 10^6$  spermatozoa were added to 1 µL of 5 mM FITC-VAD-FMK, following which the samples were homogenized and incubated at room temperature for 20 minutes in the dark. After incubation, this solution was centrifuged ( $200 \times g/5$  min) and resuspended in PBS at the initial

concentration. Next, 5 µL of PI (50 µg/mL) and 2 µL of Hoechst 33342 (H342, 40 µg/mL) were added. The final reading was taken after 5 minutes. The different cell patterns observed during the analysis were classified as viable cells (Casp−/PI−), viable cells with activated caspase (Casp+/PI−), injured cells with activated caspase (Casp+/PI+), and dead cells (Casp−/PI+). The CaspI was calculated as the ratio of the number Casp+/PI− and the total number of Casp−/PI− [20].

To evaluate the DNA fragmentation index, the acridine orange test was used according to the method of Unanian [21]. A semen sample in 2 mL of TALP was centrifuged three times at  $700 \times g$  for 3 minutes. The pellet was resuspended in TALP at a concentration of  $50 \times 10^6$  spermatozoa/mL. A smear was prepared from this solution and allowed to dry at room temperature for 60 minutes. The smear was fixed in Carnoy's solution (three parts methanol to one part acetic acid) for 12 hours. Subsequently, the air-dried slide was incubated in 3 mL of acridine orange solution (10 mL of 1 µg/mL acridine orange solution, 40 mL of 0.1 M citric acid, and 2.5 mL of 0.3 M disodium phosphate; pH = 2–3) for 5 minutes at room temperature in the dark. Finally, the smear was carefully washed in distilled water, and a coverslip was applied before drying was complete.

### 2.1. Statistical Analysis

For statistical analysis, the mean and standard deviation were calculated for each evaluated parameter. An analysis of variance with a randomized block design and Tukey test with a  $P < .05$  significance level were used to compare the means.

## 3. Results

The association of annexin and propidium iodide staining (An/PI) was used to identify the different sperm populations, with or without PS translocation, using epifluorescence microscopy, and the data obtained are presented in Table 1. There was no significant difference in the percentage of viable cells that did not exhibit translocation of PS (An−/PI−) among the different temperatures and the storage times for fresh semen and semen cooled for up to 24 hours, but the percentage decreased over time ( $P > .05$ ) for both temperatures after 48 and 72 hours of cooling, especially compared with fresh semen and semen stored for 12 hours at 5°C. With respect to the percentage of cells with PS translocation

**Table 1**

Mean and standard deviation of the analysis by epifluorescence microscopy for assessing membrane translocation of phospholipids (An+), viable cells (An−/PI−), viable cells with translocation of phospholipids (An+/PI−), dead cells with translocation of phospholipids (An+/PI+), and dead cells (An−/PI+)

Treatments	An−/PI−	An+/PI−	An+/PI+	An−/PI+
Fresh semen	26.8 ± 9.3 <sup>a</sup>	17.4 ± 4.8 <sup>a</sup>	17.3 ± 8.3 <sup>b</sup>	37.7 ± 10.3 <sup>b</sup>
Stored semen				
12 hr, 15°C	21.2 ± 9.8 <sup>a,b</sup>	15.2 ± 4.2 <sup>a,b</sup>	17.9 ± 5.9 <sup>b</sup>	45.1 ± 9.5 <sup>b</sup>
12 hr, 5°C	22.5 ± 9.4 <sup>a</sup>	16.4 ± 4.3 <sup>a</sup>	16.4 ± 4.2 <sup>b</sup>	44.5 ± 9 <sup>b</sup>
24 hr, 15°C	18.3 ± 8.9 <sup>a,b</sup>	16.1 ± 2.5 <sup>a,b</sup>	19.7 ± 6.6 <sup>b</sup>	44.7 ± 8.6 <sup>b</sup>
24 hr, 5°C	20.3 ± 9.3 <sup>a,b</sup>	15.5 ± 3.1 <sup>a,b</sup>	20 ± 5.5 <sup>b</sup>	44.5 ± 9 <sup>b</sup>
48 hr, 15°C	8.5 ± 6.2 <sup>c</sup>	11.9 ± 5.4 <sup>b</sup>	17.5 ± 4.9 <sup>b</sup>	60.7 ± 8.9 <sup>a</sup>
48 hr, 5°C	12.7 ± 7.3 <sup>b,c</sup>	13.1 ± 4 <sup>a,b</sup>	16.4 ± 4 <sup>b</sup>	57.1 ± 10.4 <sup>a</sup>
72 hr, 15°C	4.9 ± 6.1 <sup>c</sup>	7.4 ± 4.2 <sup>c</sup>	23.8 ± 5 <sup>a</sup>	64.3 ± 6.1 <sup>a</sup>
72 hr, 5°C	8 ± 5.7 <sup>c</sup>	9.9 ± 4.6 <sup>b,c</sup>	21 ± 5.2 <sup>a,b</sup>	61 ± 6.9 <sup>a</sup>

<sup>a,b,c</sup>Different alphabets within the same column are different,  $P > .05$ .

(An+/PI–), fresh semen and semen refrigerated for 12 hours at 5°C presented the highest An+ rates, but there was no significant difference detected between these samples or between the different temperatures and cooling times up to 24 hours or for the samples stored at 5°C for 48 hours.

Assessment of Caspl also identified different populations, and these data are presented in Table 2. There was no difference in the percentage of viable cells without activation of caspase (Casp–/PI–) observed for the semen stored at either temperature for 12 hours compared with fresh semen. At 24 hours, there was no difference between the temperatures in percentage of Casp–/PI– cells between the two storage temperatures, but the semen samples stored at 15°C showed a lower percentage than fresh semen. The percentage decreased in semen stored for 48 hours compared with fresh semen ( $P > .05$ ). There was no difference in the percentage of cells with activated caspase (Casp+/PI–) between fresh semen and semen refrigerated for up to 24 hours at either temperature.

According to the data presented in Table 3, there were no significant differences in the DNA fragmentation index, PSTI, or Caspl associated with up to 24 hours of storage, independent of the refrigeration temperature. After 48 hours of storage, the best temperature with respect to maintaining viable sperm was 5°C according to the PSTI. There were large modifications in sperm quality observed after 48 hours.

#### 4. Discussion

This study evaluated the effects of refrigeration on phosphatidylserine translocation, Caspl, and DNA fragmentation in equine sperm under different storage periods and temperatures. It is the only study to date that has evaluated all of these parameters in refrigerated equine semen.

According to our results, the percentage of cells exhibiting PS translocation was not a good parameter for assessing sperm quality, as no significant differences in this parameter were detected among the experimental groups. Similar results have been found between samples in seminal quality analyses following cryopreservation of human [21] and swine [7] semen. Only research on cryopreservation of cattle semen has shown an increased rate of PS translocation [6]. This difference between species can be explained by the sensitivity of the spermatozoa of each species to refrigeration stress [22].

The functional significance of the cellular An+/PI– ratio remains unclear, but it is unlikely that these cells are

**Table 3**

Means and standard deviations of the DNA fragmentation index (DFI), membrane phospholipid translocation index (PSTI), and caspase activation index (Caspl) analyzed microscopically

	DFI	PSTI	Caspl
Fresh semen	6.6 ± 3.1 <sup>c</sup>	40.2 ± 9.4 <sup>c</sup>	39.6 ± 8.7 <sup>b</sup>
Stored semen			
12 hr, 15°C	9.9 ± 5 <sup>a,b,c</sup>	43.9 ± 14.7 <sup>b,c</sup>	44.3 ± 12.9 <sup>a,b</sup>
12 hr, 5°C	8.9 ± 4.9 <sup>b,c</sup>	43.9 ± 13.3 <sup>b,c</sup>	44 ± 13.8 <sup>a,b</sup>
24 hr, 15°C	12.9 ± 7.1 <sup>a,b,c</sup>	49.7 ± 15 <sup>a,b,c</sup>	49.6 ± 15.8 <sup>a,b</sup>
24 hr, 5°C	11.2 ± 5.9 <sup>a,b,c</sup>	45.7 ± 15 <sup>b,c</sup>	47.4 ± 14.8 <sup>a,b</sup>
48 hr, 15°C	13.2 ± 7.5 <sup>a,b,c</sup>	59.6 ± 23.1 <sup>a,b</sup>	54.7 ± 24.4 <sup>a,b</sup>
48 hr, 5°C	12 ± 7.2 <sup>a,b,c</sup>	53.5 ± 15.7 <sup>a,b,c</sup>	51.6 ± 14.9 <sup>a,b</sup>
72 hr, 15°C	15.9 ± 7.8 <sup>a</sup>	65.9 ± 26.5 <sup>a</sup>	58.2 ± 24.9 <sup>a</sup>
72 hr, 5°C	13.9 ± 7.6 <sup>a,b</sup>	57.9 ± 17.9 <sup>a,b</sup>	55.9 ± 14.4 <sup>a,b</sup>

<sup>a,b,c</sup>Values within the same column that are marked by different alphabets are significantly different from each other,  $P < .05$ .

completely viable [11]. This result reveals the presence of damaged cells in semen samples evaluated as normal [6,23] by conventional analysis.

With respect to the percentage of cells with activated caspase, there was no significant difference found between fresh semen and semen refrigerated for up to 24 hours at either of the experimental temperatures. Ortega-Ferrusola et al. [9] also found no difference between fresh semen and semen cooled to 4°C. Owing to these factors, we opted to use the percentage of viable cells not presenting markers of apoptosis as a parameter of semen quality.

The percentage of viable cells without apoptosis markers was found to be a good indicator of semen quality. The results revealed no differences in the studied indices between different temperatures and storage times for fresh semen and semen stored for 24 hours. This result agrees with the findings of other studies in which only motility and conventional viability were analyzed [24,25]. The lack of changes in sperm observed at storage times ≤24 hours can be explained by the system used to maintain the temperature at 5°C, which is based on a cooling curve originally suggested by Katila [26]. This curve, which consists of temperature decreases of 0.1°C/min for the first 30 minutes and 0.05°C/min thereafter, avoids exposing the spermatozoa to cold shock. The system used to maintain semen samples at 15°C avoids the plasma membrane phospholipid transition phase, in which spermatozoa pass from the fluid to the gel state, because this transition only occurs at temperatures below 15°C [24].

With respect to the percentage of viable cells without activated caspase, there was no difference found between the experimental temperatures after cooling for 12 hours or compared with fresh semen. At 24 hours, the percentage of these cells in semen stored at 5°C was similar to that in fresh semen, whereas semen stored at 15°C showed a lower percentage of viable cells. These data are consistent with the results of Marti et al. [27], who indicated that caspases are activated in the first phase of apoptosis, whereas the translocation of PS is restricted to a later stage of apoptosis. This change can cause damage to the plasma membrane. Therefore, the ideal storage method for semen is refrigeration and transportation of specimens for a period of up to 12 hours at a temperature of 15°C, as suggested by Squires et al. [5]. At longer storage times, the 48 h/5°C group was similar to groups with shorter storage times (12 and 24 hours) but was inferior to fresh semen. This result

**Table 2**

Mean and standard deviation of the analysis by epifluorescence microscopy to assess activation of caspases (Casp+), viable cells (Casp–/PI–), viable cells with translocation of phospholipids (Casp+/PI–), dead cells with translocation of phospholipids (Casp+/PI+), and dead cells (Casp–/PI+)

	Casp–/PI–	Casp+/PI–	Casp+/PI+	Casp–/PI+
Fresh semen	27.6 ± 9.1 <sup>a</sup>	17.4 ± 4.3 <sup>a</sup>	17.7 ± 6.3 <sup>ab</sup>	37.1 ± 9.9 <sup>d</sup>
Stored semen				
12 hr, 15°C	21.8 ± 8.3 <sup>a,b</sup>	16.4 ± 3.8 <sup>a</sup>	17.8 ± 5.6 <sup>a,b</sup>	44.1 ± 9.2 <sup>c,d</sup>
12 hr, 5°C	23.1 ± 9.3 <sup>a,b</sup>	17.1 ± 4.2 <sup>a</sup>	16.9 ± 5.7 <sup>b</sup>	44 ± 8.1 <sup>c,d</sup>
24 hr, 15°C	18.4 ± 9.4 <sup>b</sup>	16.3 ± 3.8 <sup>a</sup>	19.4 ± 6.1 <sup>a,b</sup>	45.4 ± 11.6 <sup>c,d</sup>
24 hr, 5°C	20.2 ± 9.7 <sup>a,b</sup>	16.6 ± 3.8 <sup>a</sup>	19.6 ± 6 <sup>a,b</sup>	43.3 ± 10.5 <sup>c,d</sup>
48 hr, 15°C	9.6 ± 7.1 <sup>c,d</sup>	11.2 ± 5 <sup>b,c</sup>	20.2 ± 7.2 <sup>a,b</sup>	57.9 ± 13.5 <sup>a,b</sup>
48 hr, 5°C	13.8 ± 7.9 <sup>b,c</sup>	13.3 ± 4.6 <sup>a,b</sup>	21.4 ± 5.8 <sup>a,b</sup>	52.1 ± 9.8 <sup>b,c</sup>
72 hr, 15°C	4.8 ± 4.6 <sup>d</sup>	7 ± 5 <sup>c</sup>	23.5 ± 4.7 <sup>a</sup>	64.4 ± 6.4 <sup>a</sup>
72 hr, 5°C	3.8 ± 5.2 <sup>c,d</sup>	9.9 ± 4.2 <sup>b,c</sup>	23.7 ± 7.1 <sup>a</sup>	57.2 ± 8.2 <sup>a,b</sup>

<sup>a,b,c,d</sup>Different alphabets within the same column are different,  $P > .05$ .

indicates that there could already be a decrease in fertility after 48 hours of storage, based on the observed semen quality. Zidani et al. [28] found differences in fertility rates, using semen that had been refrigerated for 48 hours at 5°C versus 15°C; the fertility rate was lower in the 15°C samples. Jasko et al. [29] reported that the gestation rates obtained with semen refrigerated for 24 hours were similar to those obtained with fresh semen; however, a reduction of approximately 50% occurred in the fertility rate when this period exceeded 48 hours.

The index of DNA fragmentation was evaluated by staining smears with acridine orange and examining them by fluorescent microscopy, which is a simple assessment method compared with flow cytometry, but its interpretation is more subjective. During microscopic examination, the smears proved to be irregular in length, such that early cells exhibited a higher concentration of green-yellow cells at the point of origin, whereas there was a higher concentration of red cells at the end, following the pattern analysis from the middle to the end of the blade as scored by Naves et al. (2004) [30]. Differences were only found after 72 hours of refrigeration, so we believe that the test lacked sufficient objectivity to allow more satisfactory conclusions to be drawn.

The examined indices of apoptosis (ITPS and ICasp) also showed no significant differences owing to the fact that starting at 48 hours, some samples presented apoptotic indices of 100%, in which all of the viable cells were undergoing apoptosis, and other samples showed no apoptotic cells. When the indices were averaged, they approached the values found in samples stored up to 24 hours. Therefore, in contrast to what has been found in other studies [6,22], these indices did not represent a suitable parameter for evaluating seminal quality.

## 5. Conclusion

This work offers tools for the evaluation of semen quality and techniques for the evaluation of apoptosis in semen. Our results showed that refrigeration at 15°C or 5°C maintains good semen quality for up to 24 hours of storage. If longer transport times are necessary, a temperature of 5°C should be chosen. After 48 hours, a significant decrease in semen quality can already be observed, which could be one of the factors contributing to the decreased fertility associated with refrigerated semen. Therefore, analysis of the parameters examined in this study is recommended as an additional test to better evaluate the quality of spermatozoa during the refrigeration period.

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