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Influence of post-thawing thermal environment on bovine sperm characteristics and in vitro fertility

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Abstract

Our aim was to evaluate the effects of three thermal environments over time on kinetics, functionality and in vitro fertility of cryopreserved bovine spermatozoa. Four ejaculates from five bulls (n = 20) were cryopreserved. After thawing, semen was evaluated (0 hr), incubated for 4 hr in T36.0 (36.0°C), T38.0 (38.0°C) and T39.5 (39.5°C), and analysed every hour (1 hr, 2 hr, 3 hr, 4 hr). In vitro production of embryos was performed at 0 hr and 4 hr. Sperm motility and cell kinetics (Computer-Assisted Sperm Analysis) were impaired after 2 hr at T38.0 and T39.5 ($p < 0.05$). Flow cytometry revealed an increase in the cells with injured plasma membrane to 39.5°C and a general reduction in the mitochondrial potential over time ($p < 0.05$). In vitro fertility was impaired in all temperatures after 4 hr, but there was no difference between 36.0°C and 38.0°C. Our results suggest that the ex situ resilience of semen at 36.0°C after thawing with no major damage to the quality is limited to 3 hr. In normothermia or in thermal stress, sperm cells present a gradual reduction of movement and functionality, which were more significant after 1 hr of incubation. The in vitro production of embryos is impaired when the semen is kept in a thermal environment ≥36.0°C for 4 hr.

KEYWORDS
bull, heat stress, in vitro fertilization, semen quality, thermotolerance

1 | INTRODUCTION

The success in using animal reproductive biotechniques, including artificial insemination and fixed-time artificial insemination, is dependent on physiological events that may be affected by changes in the thermal environment conditions (De Rensis, Lopez-Gatius, García-Ispierto, Morini, & Scaramuzzi, 2017; Tusell et al., 2011). It is known that the exposure of sperm cells to temperature variations, often different from physiological temperature, can take place in several circumstances, either during spermatogenesis (Zhang et al., 2015) or in seminal collection, manipulation and cryopreservation procedures (Leahy & Gadella, 2011; Lymberopoulos & Khalifa, 2010).

The use of fixed-time artificial insemination (FTAI) is recommended in the production systems of beef cattle, because it allows using genetic material of superior bulls, besides concentrating calving and facilitating reproductive management (Dalton, Ahmadzadeh, Shafii, Price, & Dejarnette, 2004). In large-herds farms that need to inseminate a large number of cows in short intervals, the practice of

fixed-time artificial insemination after the simultaneous thawing of multiple semen straws is common (Oliveira et al., 2012). In this case, the semen straws remain in the thermal environment of thawing for different periods, during the insemination procedures (Dalton et al., 2004). As a consequence, sperm movement (Muiño, Tamargo, Hidalgo, & Peña, 2008), semen viability and fertility can be affected (Brown, Senger, & Becker, 1991). Therefore, it is of great interest to study the physiology of spermatozoa at a temperature of 36.0°C, since it corresponds to the recommended temperature for the thawing of conventional (Oliveira et al., 2012) or sexed bovine semen (ABS Global, 2009).

In turn, bovine females exhibit internal body temperature of 38.0°C in normothermia conditions (McDowell, Lee, & Fohrman, 1958). Therefore, this temperature is also used in laboratorial protocols for embryo production (Holm, Booth, Schimidt, Greve, & Callesen, 1999). This is therefore also a thermal environment of interest since it corresponds to the temperature to which the spermatozoa are subjected during the in vivo or in vitro fertilisation processes. Additionally, under conditions of thermal imbalance, the internal body temperature can reach 39.5°C (Robinson, 2014). This is, therefore, a temperature that can be endured by spermatozoa when a cow presents a thermal unbalance (Gebremedhin, Hillman, Lee, Collier, & Willard, 2008), which is common in several bovine producing regions (Romanello et al., 2018).

Thus, the adoption of sperm care after thawing can be a way to increase semen fertility, increase reproductive efficiency of the herds, as well as production profitability. For this purpose, it is essential to have more detailed information about the thermal resilience of sperm cells when subjected to different thermal conditions after thawing. Currently, the effects of challenging temperatures on specific damages in spermatozoa and on their fertilising ability are not understood in depth. Knowing the thermoresistance of sperm cells, practical measures can be implemented for the more adequate manipulation and use of semen in reproductive biotechniques, either for field or laboratory use. Therefore, the objective of this study was to evaluate how bovine cryopreserved spermatozoa are affected when submitted to different thermal environments over time and to determine the effects of post-thaw temperature on their kinetics, functionality and in vitro fertility.

2 | MATERIAL AND METHODS

2.1 | Bioethics

The experimental procedures were previously approved by the Commission of Ethics in the Use of Experimental Animals of Embrapa Southeast Livestock (Declaration CEUA-CPPSE 0512_2014), considering legal and ethical aspects of the interventions carried out. The procedures conducted also met the precepts described by the Brazilian Guideline for the care and use of animals in education or scientific research activities (CONCEA, 2016). The results were reported according to The Animals in Research: Reporting in Vivo Experiments Guidelines—ARRIVE (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010).

2.2 | Cryopreserved semen

The experiment was executed using the cryopreserved semen of five selected Canchim bulls (⅝ Charolese × ⅜ Zebu), from a gene bank that included 17 breeding bulls and 237 semen batches. The semen was frozen in the Embrapa Semen Processing Laboratory, a unit authorised by the Brazilian Ministry of Agriculture, Livestock and Supply for cryopreservation and maintenance of bovine semen. The semen was packed in 0.5 ml polyethylene straws and cryopreserved in citrate-yolk extender according to a defined protocol (Abud et al., 2014; Shoae & Zamiri, 2008). Semen samples were kept stored in cryogenic tank in liquid nitrogen at −196°C at least 1 month before analysis. Prior to use, the semen was evaluated for quality control and had recommended characteristics for cryopreserved bovine spermatozoa, namely sperm progressive motility ≥30%, sperm vigour ≥3.0, minimum of 70% morphologically normal cells and maximum limit of 10% of major defects (CBRA, 2013). As an additional criterion, the semen batch should have at least fifteen straws available in the bank. Thus, four different semen batches of each bull were selected for use, which represented four replicates per animal, totalling twenty batches studied (n = 20).

2.3 | Thermal treatments

The semen was thawed at 36.0°C for 30 s (Oliveira et al., 2012). Three straws of the same batch were thawed, and the contents were immediately transferred to pre-heated 0.6 ml microtubes. After thawing, the semen was submitted to three thermal treatments, under different temperatures, in a thermoblock with digital microprocessor, a temperature control range of +5 to 150°C and a thermal increase of 0.1°C (K80-D01, Kasvi, Brazil). The first treatment (T36.0) consisted of incubating the semen at a controlled temperature of 36.0°C, the second treatment (T38.0) consisted of incubating the semen at 38.0°C, while the third treatment (T39.5) corresponded to the incubation at 39.5°C. The incubation period used for all treatments was 4 hr. Sperm characteristics were evaluated at predetermined incubation times, corresponding to the time immediately after thawing (0 hr), and at 1-hr interval (1 hr, 2 hr, 3 hr and 4 hr).

The thermal stability of the thermoblocks during the incubation was continuously monitored *in loco* using an infrared thermographic camera (Testo 890-2, Testo AG, Germany), in order to ensure the accuracy of treatments and ensure that the semen samples reached precisely the previously stipulated temperatures (Figure 1). The thermographic camera had a resolution of 640 × 480 pixels, a 42° × 32° lens, thermal sensitivity <40 mK (0.04°C) and a temperature range from −20 to +100°C.

At 0 hr, 1 hr, 2 hr, 3 hr and 4 hr, the characteristics of sperm movement and functionality of cellular compartments were evaluated. The fertility potential of the semen was evaluated by in vitro embryo production after thawing (0 hr) and after 4 hr (4 hr) of heat treatments. The details of these analyses are described in the following sections.

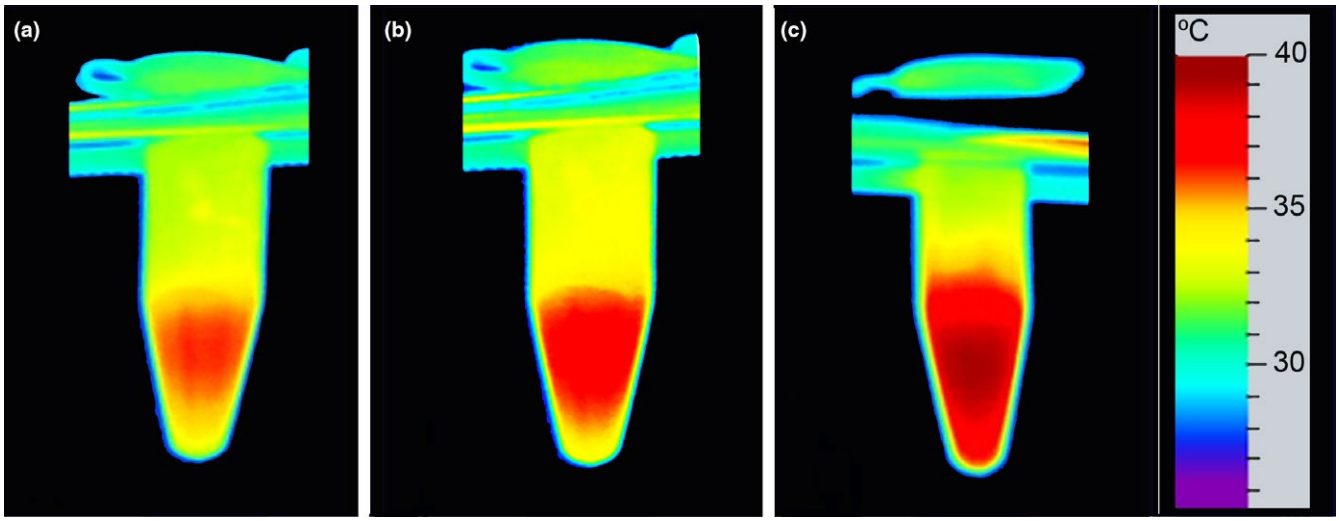

FIGURE 1 Illustrative images of thermograms of seminal samples deposited in 0.6 ml microtubes and subjected to thermal treatments at 36.0, 38.0 and 39.5°C. (a) Sample incubated at 36.0°C; (b) sample incubated at 38.0°C; (c) sample incubated at 39.5°C. Parameterised for rainbow colour palette and thermal scale from 25.0 to 40.0°C

2.4 | Computer-Assisted Sperm Analysis

The sperm kinetics was evaluated by computerised analysis (HTM-IVOS, version 12.3, Hamilton Thorne Bioscience, USA), with the equipment set up previously adjusted for bovine semen analysis. The semen was diluted in Talp medium (Bavister, Leibfried, & Lieberman, 1983) in order to adjust the maximum sperm concentration to 40 million cells/ml. The reading was then performed on 10 μl of sample deposited in a Makler's chamber (Sefi Medical Instruments Ltd, Israel). Total motility (%), progressive motility (%), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), beat-cross frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %) were analysed. Populations of fast movement cells (fast, %) and static cells (statics, %) were also quantified (Amann & Waberski, 2014). The set up used for the computer-assisted semen analysis is detailed in Supporting Information (Table S1).

2.5 | Evaluation of plasma membrane integrity, mitochondrial potential and oxidative stress of sperm cells

Functionality of sperm compartments was assessed by a compact flow cytometry (Muse® Cell Analyzer, Merck, Germany) with an optical design consisting of a green laser and three detectors, for forward scatter-FSC, as well as yellow and red fluorescence channels. Its fluidic system works with a flow cell that performs cell alignment by microcapillary. To evaluate membrane integrity, the sperm concentration was adjusted in Talp medium (1×10^6 sptz/ml) and then 50 μl of the sample was incubated with 450 μl of the count and viability assay kit (MCH100102, Merck, Germany) for 5 min at room temperature. The markers present in the kit are propidium iodide (viability marker) and LDS 751 (nuclear marker). These probes differentially stain viable and nonviable cells based on their permeability to the two DNA-binding dyes present in the reagent. Thus, the percentage of injured plasma membrane cells was determined. For determination of the mitochondrial potential, the sperm concentration was adjusted in Talp medium (1×10^5 sptz/ml), and a 100 μl aliquot of the sample was incubated with 95 μl of the working solution of the MitoPotential kit (MCH100110, Merck, Germany). This assay utilises the MitoPotential dye, a cationic, lipophilic dye to detect changes in the mitochondrial membrane potential and 7-AAD as an indicator of cell death. The result was expressed by the percentage of cells with low mitochondrial potential. To evaluate the cellular oxidative stress, a fluorescence probe based on dihydroethidium (DHE) was used. The reagent is cell permeable, and it has long been postulated that DHE upon reaction with superoxide anions undergoes oxidation to form the DNA-binding fluorophore ethidium bromide or a structurally similar product which intercalates with DNA resulting in red fluorescence. The sperm concentration was adjusted in Talp medium (1×10^6 sptz/ml), 10 μl of the sample incubated with 190 μl of working solution of the oxidative stress kit (MCH 100,111, Merck, Germany) at 37°C for 30 min. The results of cells in the population that presented injured plasma membrane, low mitochondrial potential and reactive oxygen species production were given in percentage (%).

2.6 | In vitro embryo production

Ovaries obtained at slaughterhouse were stored in a thermostatic container with saline solution heated to 36°C and immediately transported to the laboratory. Follicular aspiration was performed using a 10-ml syringe coupled to a 40 × 12 mm gauge needle, and the obtained contents were deposited in 50 ml graduated tubes. Then, the aspirated contents were filtered (WTA filter, WTA Tecnologia Aplicada, Brazil) and washed in PBS supplemented with 1% foetal

bovine serum (FBS). The oocytes were then transferred to a plate, where they were classified according to their quality (homogeneous cytoplasm and compact *cumulus* cells; Watanabe & Oliveira Filho, 2000). After selection, the *cumulus*-oocyte complexes were washed in TCM 199 solution (Gibco Life Technologies, USA) supplemented with 10% FBS (Gibco Life Technologies, USA) and washed once in maturation medium MIV-TCM 199 supplemented with 10% FBS, 5 μg/ml FSH, 50 μg/ml LH and 01 μg/ml estradiol). The oocytes were transferred to microtubes with 500 μl of MIV medium covered with 300 μl of mineral oil. The microtubes remained in a bench incubator (EVE WTA®, WTA Tecnologia Aplicada, Brazil) at a temperature of 38.5°C for 24 hr and low oxygen tension (6%CO_2 + 5%O_2 + 89%N_2). In this process, about 2000 oocytes were selected. After maturation, the oocytes were washed and transferred to plates with in vitro fertilisation medium.

For in vitro fertilisation, the same bulls and batches previously studied were used. The semen was submitted to three thermal treatments under the same previously studied conditions (T36.0, T38.0 and T39.5) and incubation time (0 hr and 4 hr). Before being used in the in vitro fertilisation, the semen was washed in two consecutive centrifugations (72 *g*), in TALP medium and in FIV medium for the removal of the diluent and seminal plasma. The concentration was adjusted to 1 × 10^6 sptz/ml, and the medium used in the in vitro fertilisation was the modified Tyrode's medium (Talp) plus penicillamine, hypotaurine and epinephrine (PHE) solutions and 10 μg/ml heparin. The gametes remained incubated in plates with 70 μl microdroplets covered with mineral oil, for 20–22 hr at 38.5°C with low oxygen tension. After fertilisation, the structures were washed and cultured in five-well plates with in vitro culture medium, modified CR4 medium plus 2.5% FBS and BSA (Watanabe, 1998). All procedures for the production of embryos were performed in incubator with controlled low oxygen atmosphere and temperature (EVE WTA®, WTA Tecnologia Aplicada, Brazil).

Semen fertility was evaluated considering the cleavage rates (Cleavage Rate = number of cleaved structures/number of viable oocytes, %), of embryo production after 7 days of cultivation (Embryos D7 = number of embryos produced up to D7/number of viable oocytes, %), of total embryo production (Total Embryos = total number of embryos produced/number of viable oocytes, %) and embryo hatching (Hatching Embryos = number of embryos hatched/total number of embryos produced, %; Supporting Information Figure S1).

2.7 | Statistical analysis

The variables of kinetics and functionality of the sperm cells were evaluated using a mixed linear model that included the fixed effects of thermal treatments (T36.0, T38.0 or T39.5), evaluation times (0 hr, 1 hr, 2 hr, 3 hr and 4 hr) and the interaction of the heat treatment with the evaluation time, besides the batches random effects in each bull and residue. Since bulls and batches were the same in all treatment combinations and evaluation time, a repeated measures structure of batches was used in each bull evaluated. These analyses used the PROC MIXED procedure of the Statistical Analysis System, version 9.4 (SAS Institute Inc., Cary, NC, USA). After the significant effects were verified in the *F* test of the analysis of variances, the Tukey test was used as a procedure to compare the averages.

The variables related to in vitro embryo production were evaluated using a generalised linear mixed model, considering the binomial distribution of the variables and using the logit link function. The model considered the heat treatment fixed effects (T36.0, T38.0 or T39.5) and the evaluation times (0 hr and 4 hr) and the interaction of the heat treatment with the evaluation time, besides

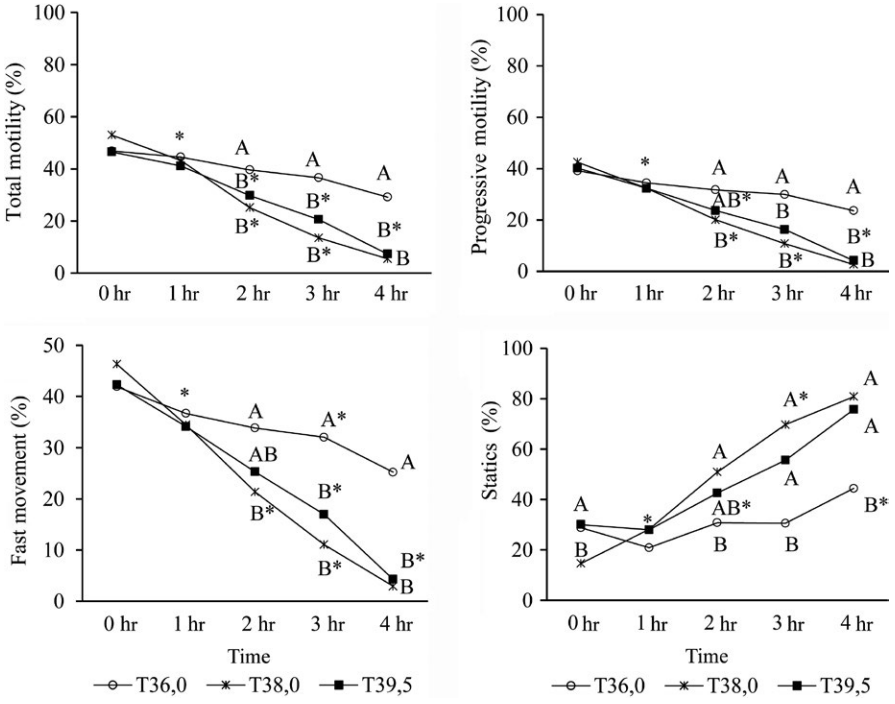

FIGURE 2 Mean values of total motility, progressive motility, fast-moving and static (%) populations in cryopreserved bovine semen subjected to heat treatments at 36.0 (T36.0), 38.0 (T38.0) and 39.5°C (T39.5) for 4 hr. A, B Indicate difference between treatments ($p < 0.05$). *Indicate difference between times within each treatment ($p < 0.05$; $n = 20$, five bulls; four replicates)

the batches random effects in each bull and residue. Since the bulls and batches were the same in all treatment and time combinations, a structure of repeated measures of batches in each bull evaluated was used. These analyses used the PROC GLIMMIX procedure of the aforementioned programme. After the significant effects were verified in the *F* test of the analysis of variances, the Tukey test was used as a procedure to compare the averages. A significance level of 5% was considered for all analyses.

3 | RESULTS

The total motility decreased over incubation time in all treatments, in which lower values in T38.0 and T39.5 were observed after the second hour ($p < 0.05$). Progressive motility behaved in a similar way, but with a more significant reduction in T38.0 and T39.5 after the third hour of incubation (Figure 2). The percentage of fast movement spermatozoa decreased over time for all treatments.

Up to the first hour, there was no difference between treatments regarding the percentage of fast movement spermatozoa, which presented a significantly lower reduction at T36.0 in relation to the other temperatures, after the third hour of incubation. The percentage of static spermatozoa increased with incubation time in all treatments, and a higher percentage was observed at T38.0 and T39.5 after the third hour ($p < 0.05$).

It was observed that the cells had movement characteristics that did not differ immediately after thawing (0 hr; Table 1). There was a reduction in VAP, VSL and VCL as a function of the incubation period, with significantly lower values after the second hour at T38.0 (46.67 μm/s, 41.02 μm/s, 73.88 μm/s) when compared to those observed at T36.0 (56.0 μm/s, 49.14 μm/s, 87.85 μm/s; $p < 0.05$). The ALH, STR and LIN behaved in a similar way among each other, with

TABLE 1 Mean (±standard error) of the bovine kinetic semen parameters after thawing and incubation at 36.0 (T36.0), 38.0 (T38.0) and 39.5°C (T39.5) for 4 hr ($n = 20$, five bulls; four replicates)

	0 hr	1 hr	2 hr	3 hr	4 hr
Average path velocity (VAP – μm/s)					
T36.0	61.89 ± 3.48^{a}	56.85 ± 3.48^{ab}	56.00 ± 3.48^{Aab}	52.39 ± 3.56^{Abc}	46.58 ± 3.65^{Ac}
T38.0	60.34 ± 3.33^{a}	53.69 ± 3.33^{ab}	46.67 ± 3.33^{Bb}	33.59 ± 3.65^{Bc}	21.76 ± 4.29^{Bd}
T39.5	61.47 ± 3.76^{a}	57.07 ± 3.76^{ab}	50.81 ± 3.76^{ABb}	50.81 ± 4.13^{Abc}	50.81 ± 4.29^{Ac}
Straight-line velocity (VSL – μm/s)					
T36.0	53.35 ± 3.08^{a}	50.57 ± 3.08^{ab}	49.14 ± 3.08^{Aab}	45.31 ± 3.15^{Abc}	40.28 ± 3.23^{Ac}
T38.0	51.41 ± 2.94^{a}	46.91 ± 2.94^{ab}	41.02 ± 2.94^{Bb}	29.72 ± 3.22^{Bc}	19.58 ± 3.79^{Bd}
T39.5	53.20 ± 3.32^{a}	50.69 ± 3.32^{ab}	44.28 ± 3.32^{ABb}	43.11 ± 3.65^{Abc}	34.99 ± 3.79^{Ac}
Curvilinear velocity (VCL – μm/s)					
T36.0	104.74 ± 5.51^{a}	86.68 ± 5.51^{b}	87.85 ± 5.51^{Ab}	84.66 ± 5.65^{Ab}	76.70 ± 5.80^{Ab}
T38.0	99.88 ± 5.26^{a}	83.99 ± 5.26^{b}	73.88 ± 5.26^{Bb}	52.18 ± 5.79^{Bc}	33.28 ± 6.84^{Bd}
T39.5	105.39 ± 5.97^{a}	88.15 ± 5.97^{b}	80.72 ± 5.97^{ABbc}	83.25 ± 6.58^{Abc}	68.36 ± 6.84^{Ac}
Amplitude of lateral head displacement (ALH – μm/s)					
T36.0	3.95 ± 0.24^{a}	3.15 ± 0.24^{b}	3.08 ± 0.24^{b}	3.40 ± 0.24^{Aab}	3.04 ± 0.25^{Ab}
T38.0	3.87 ± 0.23^{a}	3.40 ± 0.23^{a}	2.62 ± 0.23^{b}	2.30 ± 0.25^{Bb}	1.50 ± 0.30^{Bc}
T39.5	4.09 ± 0.26^{a}	3.25 ± 0.26^{b}	3.15 ± 0.26^{b}	3.57 ± 0.29^{Aab}	2.92 ± 0.30^{Ab}
Beat-cross frequency (BCF – Hz)					
T36.0	34.28 ± 2.12^{ab}	36.62 ± 2.12^{Aa}	33.67 ± 2.12^{Aabc}	30.63 ± 2.17^{Abc}	28.90 ± 2.22^{Ac}
T38.0	30.93 ± 2.03^{a}	30.17 ± 2.03^{Ba}	21.34 ± 2.03^{Bb}	17.42 ± 2.22^{Bb}	10.58 ± 2.60^{Bc}
T39.5	34.23 ± 2.28^{ab}	36.42 ± 2.28^{Aa}	29.82 ± 2.28^{Abc}	29.96 ± 2.51^{Abc}	23.89 ± 2.60^{Ac}
Straightness (STR – %)					
T36.0	86.05 ± 5.05^{a}	88.83 ± 5.05^{a}	87.44 ± 5.05^{a}	81.41 ± 5.20^{Aa}	75.81 ± 5.36^{Aa}
T38.0	84.95 ± 4.80^{a}	87.45 ± 4.80^{a}	79.25 ± 4.80^{a}	60.69 ± 5.36^{Bb}	48.64 ± 6.47^{Bb}
T39.5	86.07 ± 5.54^{ab}	88.60 ± 5.54^{a}	81.13 ± 5.54^{ab}	86.00 ± 6.19^{Aab}	70.45 ± 6.47^{Ab}
Linearity (LIN – %)					
T36.0	52.68 ± 3.39^{ab}	60.23 ± 3.39^{a}	57.84 ± 3.39^{a}	52.09 ± 3.49^{Aab}	47.78 ± 3.60^{Ab}
T38.0	53.05 ± 3.22^{a}	57.75 ± 3.22^{a}	50.90 ± 3.22^{a}	39.91 ± 3.60^{Bb}	31.94 ± 4.34^{Bb}
T39.5	51.87 ± 3.72^{ab}	59.07 ± 3.72^{a}	53.27 ± 3.72^{ab}	54.05 ± 4.15^{Aab}	42.90 ± 4.34^{ABb}

Note. ^{A,B}Different upper case letters indicate difference between treatments ($p < 0.05$).
^{a,b,c,d}Lowercase letters indicate difference between times ($p < 0.05$).

slight reduction over time, and this decrease was higher at T38.0 mainly after the third hour of incubation, when a difference was observed in relation to T36.0 and T39.5 ($p < 0.05$). The BCF decreased after the second hour in all treatments, with a difference observed between treatments at the first hour of incubation, with higher values at T36.0 and T39.5, a difference that remained until the final of incubation. There was a significant difference in all kinetic parameters at the third hour of incubation, with lower values observed at T38.0 and higher values at T36.0 and T39.5 ($p < 0.05$).

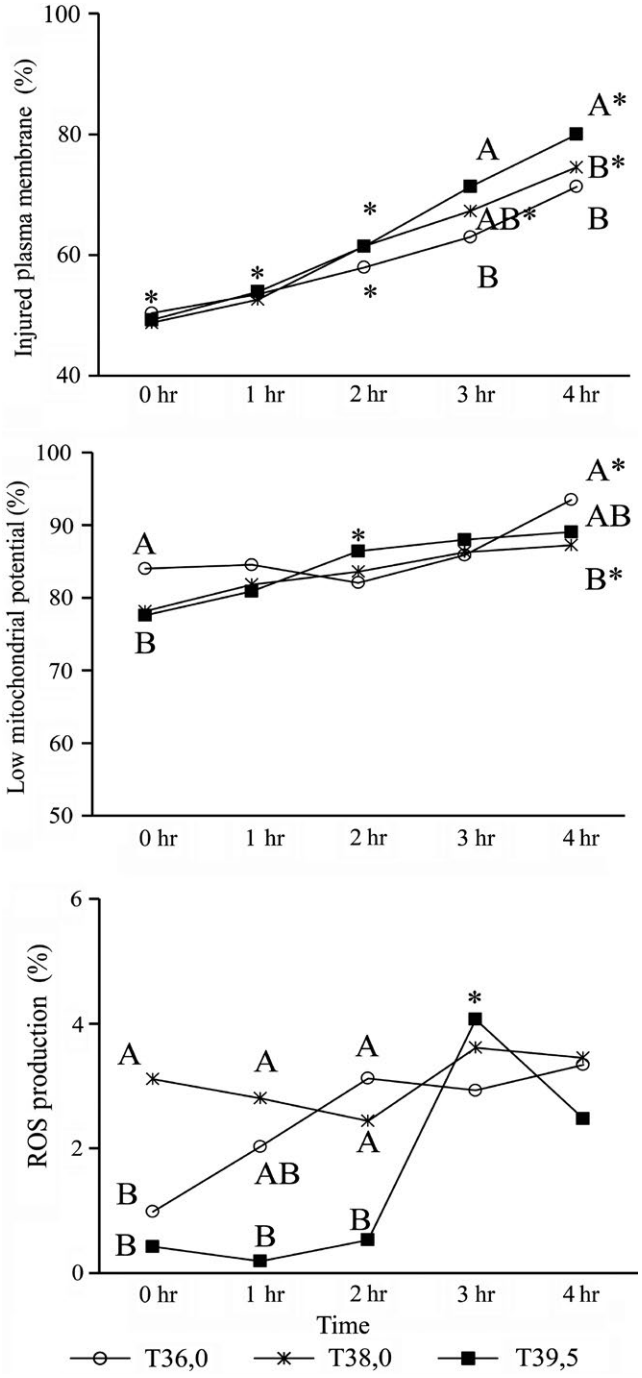

FIGURE 3 Percentage of cells with injured plasma membrane, low mitochondrial potential and reactive oxygen species production, in cryopreserved bovine semen after thawing and thermal treatments at 36.0 (T36.0), 38.0 (T38, 0) and 39.5°C (T39.5) for 4 hr. A, B Indicates difference between treatments ($p < 0.05$). *Indicates difference between times within each treatment ($p < 0.05$; $n = 20$, five bulls; four replicates)

The physical structure of the plasma membrane was impaired in all treatments (Figure 3), but a higher percentage of cells with damaged membrane (80%) was observed after 3 hr at T39.5 ($p < 0.05$). Over the incubation time, there was an increase in the percentage of cells with low mitochondrial potential in all the thermal treatments. At the last evaluation hour, the T38.0 samples presented a lower percentage of cells with low mitochondrial potential (87.24%, $p < 0.05$). There was higher production of reactive oxygen species in the semen at T36.0 and T38.0 until the second hour of incubation, with values ranging between 3 and 4 hr.

There was a statistically significant difference in the in vitro production of embryos when compared to the semen used immediately after thawing and after 4 hr of incubation, regardless of the incubation temperature (Figure 4). Spermatozoa incubated at T36.0 differed significantly from those maintained at T38.0 and T39.5 exhibiting a higher rate of cleavage and embryonic production on the seventh day of culture ($p < 0.05$). The total embryo production rate also differed, with higher values observed at T36.0 when compared to those obtained at T39.5 ($p < 0.05$). The embryonic hatching rate differed between treatments, higher at T36.0 (41%) followed by T38.0 (11%) and lower at T39.5 (6%; $p < 0.05$).

4 | DISCUSSION

Thermal energy is a physical agent capable of causing significant sperm motility reduction (Senger, Becker, & Hillers, 1975). Our results demonstrate that the thermal increment at T38.0 and T39.5 impaired total motility and progressive motility in a similar way, mainly after the second and third hours of incubation. This finding may have been mediated by damage to the mitochondrial function and consequent synthesis of ATP in the spermatozoa. Energy availability is essential for sperm motility and this is promoted by the action of the mitochondria located in the intermediate part of the spermatozoid. Hyperthermia may lead to dephosphorylation and activation of glycogen synthase kinase 3 (GSK_3), a protein kinase that binds to proteins of the outer mitochondrial membrane and regulates its permeability, reducing its ability to produce energy (Gong et al., 2017). Thus, any change in mitochondrial function may be reflected in altered sperm motility (Gravance, Garner, Baumber, & Ball, 2000).

In fact, the sperm mitochondria showed to be sensitive to the thermal increase, and the mitochondrial potential was also affected by the temperature and the incubation times. The samples kept at 36.0°C presented higher percentage of cells with low mitochondrial potential after 4 hr, when compared to samples submitted to 38.0°C. This can be explained by the fact that mitochondrial potential expression is detectable only in potentially mobile spermatozoa, whose values were higher at T36.0. Significant reduction of mitochondrial potential concomitant with decreased plasma membrane integrity

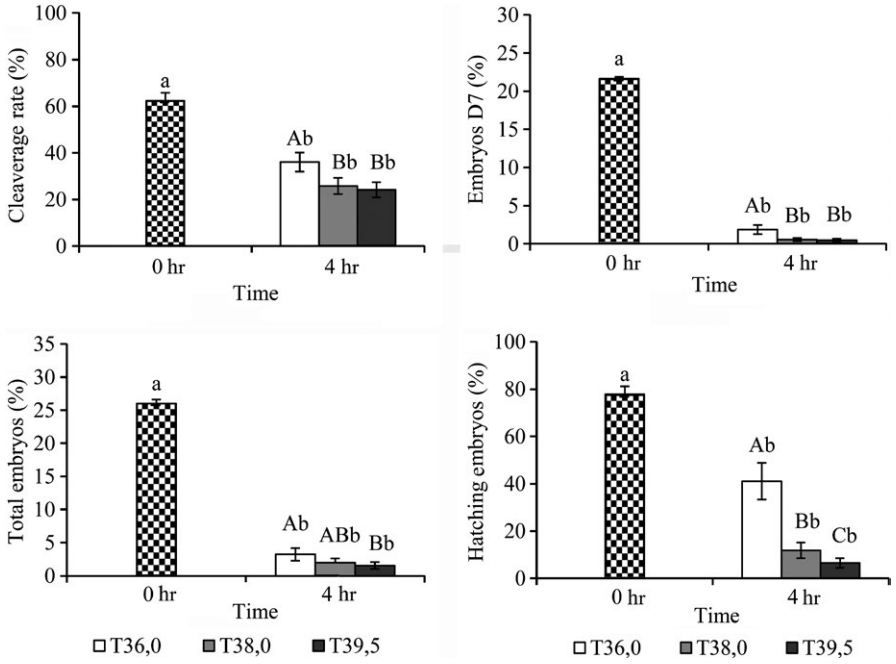

FIGURE 4 Cleavage, embryo production on D7, total embryo and embryo hatching rates (%) using bovine cryopreserved semen after thawing (0 hr) and after heat treatments at 36.0 (T36.0), 38.0 (T38.0) and 39.5°C (T39.5) for 4 hr. A, B Indicates difference between treatments ($p < 0.05$). a, b, c Indicates difference between times ($p < 0.05$; $n = 20$, five bulls; four replicates)

was previously reported when cryopreserved semen was subjected to caloric stress up to 41.0°C (Rahman et al., 2014).

When observing the spermatic subpopulations, about 25% of the spermatozoa were static after 3 hr of incubation when kept at 36.0°C, which shows that higher temperatures caused cell exhaustion in a more intense and precocious way. Considering the population of cells with motility, it was noticed that in the first hour there was a change in the pattern of cell movement, with reduction of cells with rapid movement. After the second hour, the fast-moving cells became static, regardless of the treatments, as the incubation time increased. This effect is presumed to have occurred as a function of the reduction over time in the frequency of head oscillations about the average path (BCF). In fact, the increase in thermal energy may lead to a decrease in total motility, progressive motility and speed of bovine spermatozoa (Rahman et al., 2014).

The other kinetics parameters (VAP, VSL, VCL, ALH, STR and LIN) were notably affected, both by treatment and by time. This demonstrated the harmful role of high temperatures on sperm movement, especially after 3 hr of incubation. The path velocities decreased over time possibly due to the reduction of lateral amplitude of the spermatic head during the movement and the number of beats per second. Since hyperactivation is characterised by increased VCL and ALH with decreased LIN for the computerised analysis of sperm motility (Verstegen, Iguer-Ouada, & Onclin, 2002), no characteristics of sperm hyperactivation were found in the samples studied. Additionally, maintaining a liquid medium under high temperature leads to the evaporation process, which may alter the viscosity of the medium, depending on its constituents. This effect was not monitored in the present study, but it is possible that some degree of evaporation may have occurred in the incubated samples up to 4 hr, causing an increase in the viscosity of the medium. The semen hyperviscosity is recognised as a condition that impairs normal sperm motility and progression, which may significantly impact its function (Elzanaty, Malm, & Giwercman, 2004). This effect has already been suggested in cryopreserved semen of buffalo bulls that received supplementation of unsaturated fatty acids in the diet, with noticeable sperm changes in BCF, STR and LIN after 90 days of treatment (Gonçalves et al., 2014).

The functional evaluation using flow cytometry demonstrated the negative effects of the thermal increase over time. The cause of the changes in the integrity and functionality of the cellular compartments studied is related to the biophysical and biochemical particularities of these organelles. Lipids are the main constituents of the plasma membrane and give this structure a certain fluidity, whose degree depends, among other factors, on the temperature to which the cells are exposed (Kumar & Atreja, 2012). Cells exposed to oxidative stress may undergo lipid peroxidation, a phenomenon that also alters membrane fluidity and impairs cell transport (Gutteridge & Halliwell, 2010). The effects of lipid peroxidation on sperm function can be observed mainly in the decrease of motility and alteration in membrane fluidity (Aitken, 2017). Damage to the spermatic membrane at increasing levels was observed throughout the incubation time, in all the thermal treatments, with a higher incidence of damaged membranes in the samples incubated at the highest temperature. Although the ROS production pattern was different among the three temperatures studied, they were quantitatively equivalent to the induction of oxidative stress after the third hour of incubation. The formation of reactive oxygen species

may have increased the sensitivity of the plasma membrane to lipid peroxidation, in a thermodependent manner, especially for cells maintained at 39.5°C.

Reactive oxygen species produced in spermatozoa and in infiltrating leucocytes can cause male infertility, primarily because they affect sperm motility (Guthrie, Welch, & Long, 2008). In the present work, oxidative stress was evaluated by the detection of superoxide anion, using a fluorescent probe based on dihydroethidium. Thermal stress increases the production of superoxide anion (Ahmad et al., 2017), which was not confirmed in the present study. A small percentage of cells was detected under an oxidative stress situation, but a peak of stress was observed at the third hour for spermatozoa incubated at 39.5°C. The low magnitude of values found for oxidative stress can be explained by the fact that the superoxide radical has, in aqueous solution, a very short half-life of several milliseconds, which makes it difficult to detect it even during the rapid acquisition performed in flow cytometry. In addition, the superoxide anion can be converted to other reactive oxygen species, such as hydrogen peroxide, through the dismutation reaction, if the antioxidant superoxide dismutase is not present in the medium (Aitken, 2017), making it difficult to detect it.

The reduction observed in all the points used as reference indicators for in vitro fertility demonstrates that the thermal challenge, regardless of the temperature used, was detrimental to the fertilising capacity of spermatozoa. This demonstrates that, from the laboratory point of view, the shorter the time elapsed between thawing and using in the in vitro fertilisation, the greater the chance of fertilisation and satisfactory embryonic development. Considering the differences between the in vivo and in vitro fertilisation processes, in the latter the gametes are artificially approximated by means of laboratory manipulation. Thus, it is possible that some parameters of sperm motility have a reduced influence on the in vitro fertilisation results. Studies have reported the absence of difference in total embryo production, when considering total and progressive motility (Suzuki, Geshi, Yamauchi, & Nagai, 2003), as well as the other parameters of sperm movement evaluated by a computerised system (Alomar et al., 2008). However, in the present study, the results of total and progressive motility were related to the results obtained in the in vitro embryo production, which suggests a direct correlation between these characteristics. Recently, the study of sperm subpopulations with rapid motility has shown that this characteristic has a positive correlation with in vitro fertility (Ferraz et al., 2014).

Competence for embryonic development in mammals is dependent on the genetic and nongenetic contributions of their progenitors (Warner et al., 1998). The losses in the movement capacity, in the plasma membrane and the mitochondrial potential of spermatozoa were reflected in the in vitro production of embryos. The cleavage rate demonstrated that the semen was able to fertilise the oocytes, even after setting the thermal challenge for 4 hr. However, the percentage of embryos that reached the blastocyst stage was low in all treatments. The spermatozoa maintained at 36.0 or 38.0°C had similar embryonic production, and the lowest observed rates were with the higher temperature increase. The embryonic hatching rate was also affected by the treatments, lower as the temperature increased. In the bovine species, sperm chromatin damage does not block in vitro fertilisation and, therefore, no change in the cleavage rate is early observed. However, after the first cleavage occurs, chromatin damage causes apoptosis, which reduces developmental capacity up to the blastocyst stage (Fatehi et al., 2006). Compared to the in vivo processes, bulls experiencing scrotal thermal stress produce spermatozoa that, when used in fertilisation, generate embryos with delayed or reduced pronuclear formation, with low developmental capacity up to the blastocyst stage and with increased incidence of apoptosis (Walters, Saacke, Pearson, & Gwazdauskas, 2006).

Based on the observed results, it can be inferred that the simultaneous thawing of bovine semen straws, as what occurs during large scale FTAI procedures, can be considered a safe procedure, provided that the thermal stability of the semen at 36.0°C and the residence time under this condition is a maximum of 3 hr. Under physiological temperature and at the temperature of thermal stress, the thawed spermatozoa should be used as close to ovulation as possible, so as to maintain characteristics of greater fertilising potential. Irrespective of the thermal environments studied, the in vitro production of embryos is impaired when the thawed semen is kept under incubation for a period of 4 hr.

In conclusion, the sperm characteristics of total motility, progressive motility and fast movement were directly related to the in vitro fertilisation capacity of the cell. Simultaneous thawing of straws during the FTAI procedure can be carried out provided they remain for a maximum period of 3 hr under the thawing conditions. In conditions of normothermia or thermal stress, the sperm cells present a gradual reduction in their movement capacity and the functionality of their compartments, more significant after 1 hr of incubation. The in vitro production of embryos is impaired when the thawed semen is maintained under the effect of thermal environment equal to or higher than 36.0°C for a period of 4 hr.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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