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INFLUENCE OF COOLING SYSTEMS ON THE QUALITY OF OVINE SEMEN CRYOPRESERVED IN STRAWS

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ABSTRACT

Different types of cooling systems of ram semen were evaluated by the measurement and comparison of cooling rates and their effects on the post-thawing quality. Motility, vigor, morphology damages, viability and acrosomal status were tested at the end of 90 minutes of cooling. Regarding post-thawed semen, the same parameters were used, in addition to the assessment of the integrity of the plasmatic membrane, and the resistance test by the incubation of semen at +37ºC for four hours. Semen refrigeration was carried out in a domestic refrigerator and in a horizontal refrigerator. To control the temperature drop of semen in both equipments, the straws were disposed between plastic bags containing water at +32ºC, constituting four combinations of cooling procedures: RS (refrigerator without bag), RC (refrigerator with bag), BS (counter refrigerator without bag) and BC (counter refrigerator with bag), resulting in four cooling rates: -1.4ºC/min, -0.4ºC/min, -2.9°C/min and -0.45ºC/min, for RS, RC, BS and BC, respectively. At the end of the cooling period, BS treatment showed the lowest percentage of sperm motility ($P<0.05$), the lowest mean of live spermatozoa with intact acrosome, and the highest mean of dead spermatozoa with intact acrosome ($P<0.05$). As for morphological defects, BS treatment had the highest mean whereas the systems RC and BC resulted in the lowest means. There was no significant difference among treatments in relation to frozen-thawed semen at the end of the resistance test. It was concluded that the different cooling rates affected ram semen at the end of cooling stage but not at post-thawing.

KEYWORDS: Cooling protocols, cryopreservation, ovine, semen.

INFLUÊNCIA DE SISTEMAS DE REFRIGERAÇÃO SOBRE A QUALIDADE DO SÊMEN OVINO CRIOPRESERVADO EM PALHETAS

RESUMO

Avaliaram-se diferentes sistemas de refrigeração do sêmen ovino, através da aferição e comparação das curvas obtidas e seus efeitos sobre a qualidade do sêmen criopreservado. Ao final da refrigeração, os parâmetros espermáticos motilidade, vigor, defeitos morfológicos, viabilidade e estado acrossomal foram analisados. Para a avaliação pós-descongelação mais dois testes foram acrescentados: avaliação da integridade de membrana plasmática (IMP) e teste de exaustão com quatro horas de incubação a +37ºC. A refrigeração foi realizada em refrigerador doméstico e num balcão horizontal. Para controlar a queda de temperatura desses equipamentos, colocaram-se as palhetas entre bolsas plásticas contendo água aquecida a +32ºC, constituindo quatro sistemas: RS (refrigerador sem bolsa), RC (refrigerador com bolsa), BS (balcão sem bolsa) e BC (balcão com bolsa), os quais resultaram em quatro taxas de refrigeração: -1,4ºC/min, -0,4ºC/min, -2,9ºC/min e -0,45ºC/min, para RS, RC, BS e BC, respectivamente. Após a refrigeração, observou-se diferença na motilidade espermática ($P<0,05$), em que BS

apresentou menor média. O sistema BS obteve a menor média de vivos íntegros e também a maior de mortos íntegros ao final da refrigeração, diferindo de RC e BC. Quanto aos defeitos morfológicos pós-refrigeração, BS apresentou maior média (P<0,05), ao passo que RC e BC apresentaram as menores médias. Não foram observadas diferenças significativas entre os tratamentos na descongelação e ao final do teste de exaustão. Concluiu-se que as diferentes taxas de refrigeração afetaram o sêmen no final da fase de refrigeração, mas não após a descongelação.

PALAVRAS-CHAVE: Congelação, ovino, protocolos-refrigeração, sêmen.

INTRODUCTION

Artificial insemination (AI) along with the use of frozen semen is a kind of biotechnology which offers substantial benefits to animal production systems, especially for reproduction programs aiming at multiplying high genetic merit animals, thus the ones with high commercial value. Although frozen semen has been being used for 50 years by bovine industry, its use in sheep remains limited (CURRY, 2000; HOLT, 2000).

Currently, ewes´ highest fertility rates with frozen semen are obtained after laparoscopic AI with semen deposition directly into the uterus. This technique is considered responsible for expanding the use of semen in this species (EPPLESTON & Maxwell, 1993). However, this technique has limited application in the field routine because it requires expensive devices and skilled workers. The cervical AI technique is an alternative because its low cost and easy implementation would allow a more extensive use of frozen ram semen. Nevertheless, cervical application also has its limitations, as the difficult transposition of the ewe´s cervix with its curved rings, and the inability of cryopreserved sperm in traversing the cervix, due to the reduced motility and viability in the female genital tract and the excessive maturation of sperm membranes derived from the cryopreservation process, which promotes an increase in the population of capacitated and acrosome reacted spermatozoa (Maxwell & Watson, 1996; SALAMON & MAXWELL, 2000).

Cryopreservation process requires the exposure of spermatozoa to various stressors, such as temperature reduction, cellular dehydration, freezing, thawing and rehydration. The methods utilized in the field for sperm cryopreservation require simple materials such as styrofoam boxes with ice or coolers for refrigeration and styrofoam boxes with liquid nitrogen for freezing, which, although viable, show variations in the refrigeration and freezing curves. Automatic devices whose main purpose is to provide programmable and homogeneous temperature curves (GONZALEZ, 2004) have been tested for semen cryopreservation in the last years. A homogeneous and steady rate is important not only to avoid thermal shock on sperm, but also to standardize techniques and obtain a more homogeneous lot. Rodella (2006) used water bags during the cooling of semen in the refrigerator and obtained sperm quality and temperature drop (-0.5ºC / min) similar to those achieved in the automated system.

Freezing process is a sequence of events, such as dilution, cooling, freezing and thawing with different factors that can damage the semen. In other words, cooling causes a specific alteration related to the phase change in the plasma membrane lipidis (WATSON, 2000), which is different from the alterations that occur during freezing, for instance, osmotic, chemical and mechanical stresses on the cells (HAMMERSTEDT et al., 1990).

The purpose of this research was to evaluate different cooling systems of ram semen placed in straws and stored in a domestic refrigerator and a counter refrigerator. The effects of the use of an isolation system with water bags were compared, avoiding the occurrence of thermal shock and preserving, thus, sperm viability. Furthermore, the cooling rates obtained in the systems were characterized aiming at the best results at the end of the cryopreservation process.

MATERIALS AND METHODS

Semen collection and evaluation

Semen collection and cryopreservation were carried out at the Animal Reproduction Laboratory of Embrapa Recursos Genéticos e Biotecnologia

(Embrapa Genetic Resources and Biotechnology), at the experimental farm Sucupira, Brasilia, DF, Brazil. Three Santa Ines rams at adult age and showing good body condition were used. Each ram was submitted to a total of ten collections via an artificial vagina heated at +42ºC. The ejaculates were analyzed considering the following parameters: volume, aspect, gross motility, motility, vigor, concentration and sperm morphology. The ejaculates used in the cryopreservation process were those that showed the following minimum parameters: 0.5 mL volume, 70% motility, vigor 3 and concentration of $3x10^9$ sptz/mL.

Semen processing

After the first assessments of the ejaculates, pre-dilution was performed at the proportion of 1:1 (semen/extender) in a glycine-egg yolk-milk (GEM) freezing medium (GONZALEZ, 1999) at a temperature of +32ºC. Then the ejaculates were mixed, forming a pool of the samples of the three rams. The semen was rediluted and adjusted to a final concentration of $100x10^6$ total spermatozoa/doses and encased in straws (0.25 mL). The straws were refrigerated and distributed according to the following cooling techniques: refrigerator without water bag – RS; refrigerator with water bag – RC; counter refrigerator without water bag – BS; and counter refrigerator with water bag – BC.

Cooling systems

To perform the semen cooling in a domestic refrigerator, a compact Consul device (CRC12A model), with 120 liters capacity, was used. The systems were set up in the refrigerator. The first one was consistent with the traditional way of cooling semen, in which the support with the straws is directly placed in the refrigerator. In the second system, the straws were disposed among the water bags which were used as thermal isolation. The water bags were manually made with plastic bags, and they had the same measures as the straw holder (Nutricell® – Campinas/SP), 37 cm long, 15 cm wide. For the cooling process, one bag with 400 mL of volume of water and two bags with 200 mL were used. The bags were heated at +32ºC, then the 400 mL bag was placed under the holder and the other two were on the holder. Then they were withdrawn as follows: after 35 min, the first 200 mL bag, at 50 min the second 200 mL bag and at 60 min the 400 mL bag, totalizing 90 min of cooling. The widrawn order followed the methodology by RODELLO (2006), in which a similar isolation system is used for cooling ovine semen in a Minitub® 518C refrigerator. For the cooling process, a counter refrigerator with forced air circulation and temperature monitor (ELO800/SPE) was used. Two refrigeration systems with and without water bags were used in a 90-min-interval. Considering the model of the straw holder used in the counter refrigerator, the water bags measures were different from the ones used in the refrigerator, being 18 cm long and 15 cm wide. Two bags of 250 mL were placed on the holder and one of 400 mL was placed under it. After 35 min of refrigeration the first 250 mL bag was withdrawn, at 57 min the second one, and at 60 min the 400 mL one.

Monitoring the refrigeration curves

The temperatures of the refrigeration curves obtained in the systems refrigerator, refrigerator with bag, counter refrigerator, and counter refrigerator with bag were measured in all ten lots with a digital thermometer HD 8802 model IT18 (Delta OHM) with type-K sensor. The sensor was inserted in a straw with semen and the freezing medium, GEM (glycine-egg yolk-milk) (Figure 1).

Evaluations of the refrigerated semen

At the end of the semen refrigeration, three straws from each treatment (RS, RC, BS and BC) were heated again at +37ºC for 37 seconds and subjectively evaluated as for motility (0-100%) and vigor (0-5) by light microscopy. One aliquot of 20 microliters of semen from each straw was diluted in 1 mL of formol-saline solution for the analysis of sperm morphology, and then stored in 1.5 mL Eppendorf tubes at +5ºC for later reading in phase-contrast microscopy.

The evaluation of the acrosome viability and integrity was determined by light field microscopy through double staining (Trypan-blue + Giemsa), according to DIDION (1989). In an Eppendorf tube, 20 μL of semen were added to 20 μL of Trypan-blue for 12 min. This sample was used to make a smear,

which was fixed in methanol for 5 min and, after dried, immersed in Giemsa for 8 to 24 hours.

A total of 200 cells per slide were counted and the spermatozoa found were classified according to the following description:

- live acrosome-intact spermatozoon – pinkish head and dark pink acrosome;
- live acrosome-reacted spermatozoon– pink head and pinkish acrosome (discoloured);
- dead spermatozoon with intact acrosome – blue head and dark pink acrosome;
- dead acrosome-intact spermatozoon – blue head and blue acrosome (discoloured).

Cryopreservation process

The trays with the straws were transferred to a polyethylene box with liquid nitrogen at a distance of 5 cm above the nitrogen level, remaining there for 20 min, and then they were dipped in liquid nitrogen, and stored in a cryogenic cylinder at -196ºC.

Semen thawing

The samples were thawed at +37ºC for 30 seconds and deposited in 5 mL glass tubes and kept heated at +37ºC. The same parameters considered for refrigerated semen were evaluated besides two more tests: plasmatic membranes integrity (PMI) and the resistance test. Three straws per lot of each refrigeration/freezing system were evaluated.

Plasmatic membrane integrity

The integrity of the plasmatic membrane was assessed using a combination of carboxyfluorescein diacetate (CFDA) fluorescent probes and propidium iodide (PI) as described by HARRISON & VICKERS (1990). An amount of 10 uL of thawed semen was added to 40 mL of a solution of 10 mL of buffered saline formalin, 10 mL of CFDA and 5µL of PI combined with 480µL of sodium citrate at 2.94%. After incubation for 15 minutes at room temperature, an aliquot of 10 mL of this suspension was deposited between slide and cover slip. Two hundred cells were counted through an epifluorescence microscope, and the sperm showed intact membranes when stained in green and damaged membrane when stained in red or green and red.

Thermo-resistance Test (TRT)

The thawed straws were submitted to resistance test at +37ºC for 240 minutes (PAGANINI FILHO, 1997). No extender was added at any moment. The analyses at the time of thawing (time 0) and at 240 minutes (four hours) of incubation were carried out.

Experimental design

The collected data were analyzed using SAS software (Statistical Analysis System) (2004) and using the GLM procedure (analysis of variance), in a 2x2 factorial arrangement, considering the effects of the refrigeration systems, the presence or absence of the water bags and the interactions between the systems. Whereas any effect was observed, Duncan's average was used, considering significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Figure 1 presents the cooling curves obtained in the four systems. The system set up in the domestic refrigerator without water bags (RS), control group, showed a marked drop in temperature, dropping -18°C at the first minutes, probably because the blades remained in direct contact with the refrigerator environment. A similar temperature drop was reported by RODELLO (2006) who observed a decrease of -19.3ºC at the first minute of ram semen cooling in the refrigerator. RS had an average decrease of -1.4ºC/min, after 19 minutes it reached +5°C, temperature at which the blades remained stabilizing for more than 71 minutes, until 90 minutes of cooling and stabilization were completed.

The rhythm obtained in the refrigerator with water bag (RC) provided an average drop of -0.4° C/min, reaching +5°C after 65 minutes. The semen remained stabilized for 25 minutes. This rate is similar to that found by RODELLO (2006) in the refrigerator (Minitub ® 518C) with water bags, achieving an average rhythm of -0.5°C /min.

The cooling system in the counter refrigerator without water bags (BS) presented an even faster temperature drop, -21ºC at the first minute, with an average of -2.9ºC/min, reaching +5°C after 9 minutes and stabilizing within 81

minutes. The average rate achieved in the system in counter refrigerator with water bag was -0.45ºC/min, reaching +5°C after 59 minutes. The straws stabilized for 31 minutes.

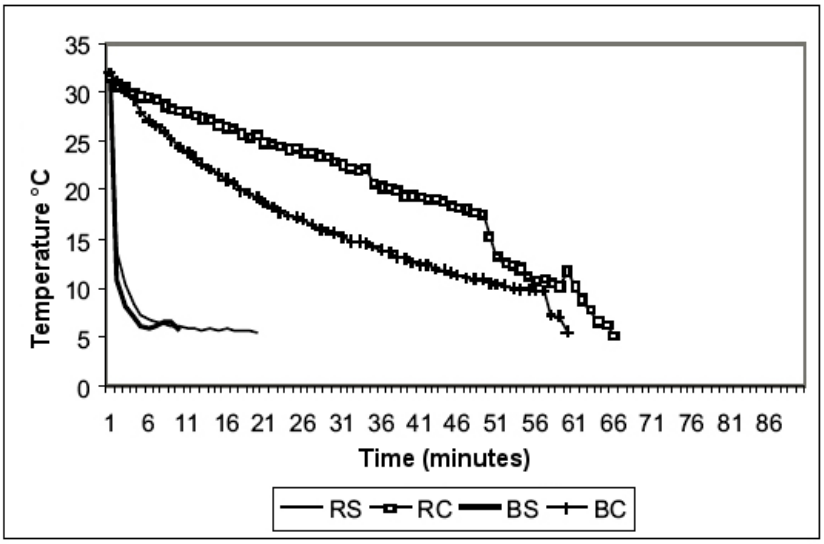

FIGURE 1. Cooling curves of ram semen (ºC/min) determined for cooling in the refrigerator without water bag (RS), refrigerator with water bag (RC), counter without water bag (BS) and counter without water bag (BC).

Regarding the evaluated parameters, no significant interactions between the cooling system (refrigerator and counter) and the isolation system (with and without water bag) were verified.

By comparing the four systems at the end of the semen refrigeration process, sperm motility showed a significant difference (P <0.05) (Table 1). The control group RS and the RC and BC treatments did not differ regarding motility (63.8%, 67.5% and 67.8%, respectively). However, BS treatment had the lowest average -58.1% (Table 1). This difference in motility was due to the water bags used (P <0.05), not the refrigeration system (refrigerator or counter). BS system presented the fastest temperature drop; hence, it is likely that the semen refrigerated in this period has been damaged. It is known that fast semen refrigeration from +30ºC to 0ºC (WATSON, 2000), or even from +37 ºC to 5ºC (LÓPEZ, 1999), induces a lethal stress in some cells, proportional to the refrigeration rate, the temperature interval and the temperature limit, known as heat shock. Heat shock can cause disruptions in the plasma membrane which allow cations and enzymes loss from the sperm, irreversibly reducing their motility and metabolic activity (WHITE, 1993). The changes also include a decrease in glycolysis and fructolysis and, consequently, in cellular respiration, and an increase of degeneration of deoxyribonucleic acid (RODELLO, 2006). Such data are consistent with those reported by OLLERO et al. (1998). These authors used a refrigeration rate of -0.25°C / min in the study of refrigeration, freezing and thawing effects and the cryoprotective ability of four extenders on sheep sperm, obtaining a 62% subjective motility average post-refrigeration in Triladyl-yolk medium.

As for spermatic vigor, there was no significant difference among treatments after refrigeration (Table 1).

The percentage of live acrosome-intact spermatozoa (VI) after 90 minutes of refrigeration showed a significant difference among treatments (P <0.05). BS method (85%) had the lowest live intact spermatozoa average, besides the highest mean of dead spermatozoa with intact acrosome (14.1%) (Table 1). The percentage of live intact spermatozoa

followed a tendency in motility, reinforcing the idea that BS system may have decreased sperm quality, due to the accentuated temperature drop. The difference regarding averages of VI and MI sperm is due to the use of water bags (P <0.05). The four treatments showed no significant differences in the percentage of live acrosome-reacted spermatozoa (VR) and percentage of dead spermatozoa with reacted acrosome (MR) (Table 1).

The percentage of morphology defects in fresh semen was 9.4 ± 2.2%, and after refrigeration, an increase of defects was observed in the four systems, with a significant difference (P <0.05). BS treatment had the highest mean of defects after refrigeration (15.4%), whereas RC and BC treatments, which used water bags, had the lowest averages of morphological defects, 11.5% and 11.1%, respectively. Control group (RS, 13.6%) was similar to the other three treatments (Table 1). Phase transition of the plasmatic membrane resulting from fast refrigeration is responsible for increasing the changes of acrosomal and tail defects (PARKS & GRAHAM, 1992). According to BATEMAN (2001), who evaluated the effects of diluents and refrigeration methods in function of canine semen, semen refrigeration results in an increase of morphological defects mainly in the spermatozoa tail.

TABLE 1. Motility means (± SD) (%), vigor (0-5), total percentage of sperm morphological defects (MD), percentage of live acrosome-intact spermatozoa (VI), dead spermatozoa with intact acrosome (MI), live acrosome-reacted spermatozoa (VR), dead spermatozoa with reacted acrosome (MR) from ram semen refrigerated in different systems (RS - refrigerator without water bag, RC – refrigerator with water bag, BS - counter without water bag and BC - counter with water bag)

	Motility (%)	Vigor (0-5)	MD (%)	VI (%)	MI (%)	VR (%)	MR (%)
RS	63.8 ± 2.7[a]	3 ± 1.6	13.6 ± 3.3[a,b]	87.3 ± 4.0[a,b]	11.7 ± 4.1[a,b]	0.1 ± 0.2	0.9 ± 0.4
RC	67.5 ± 4.9[a]	3.1 ± 1.6	11.5 ± 2.6[b]	90.4 ± 2.4[a]	9.7 ± 3.6[b]	0.1 ± 0.1	0.8 ± 0.5
BS	58.1 ± 9.5[b]	3 ± 1.6	15.4 ± 3.8[a]	85.3 ± 5.6[b]	14.1 ± 5.5[a]	0.1 ± 0.1	1.2 ± 2.1
BC	67.8 ± 4.9[a]	3.9 ± 0.3	11.1 ± 2.3[b]	90 ± 3.5[a]	9.3 ± 3.4[b]	0.02 ± 0.1	1.3 ± 2.0

[a,b] Means in the same column followed by different letters differ from each other (p <0.05).

After semen thawing, there was no significant difference in sperm motility and vigor among the four treatments. The result suggests that the rate used during cooling had no effect on semen after the freezing process. This corroborates the findings by JANUSKAUSKAS et al. (1999), who observed no differences between two rates for bovine semen cooling, slow (-0.1°C / min) and fast (-4.2ºC / min) on subjective motility after automated freezing . It also agrees with the results reported by Bittencourt et al. (2006), who observed no significant difference in total and progressive motility parameters of thawed goat semen, after using the combination of two cooling rates (-0.46°C / min and -1.07°C / min) and two balance times +5°C (one and two hours). However, these findings differ from the findings of Rovay (2006), who evaluated the effect of two cooling protocols for goat semen, using two rates of decline (--0.12ºC / min and -0.4°C / min), and had a higher total post-thawing motility (54%) with the curve -0.4 ° C / min.

Most assessments of sperm viability verify the integrity of the plasma membrane, because an intact and competent membrane is essential for the sperm to be capable of fertilizing the oocyte. When the sperm is submitted to a decrease in temperature during the cryopreservation steps, there is a destabilization or even rupture of the membrane due to this phase transition, which cause changes from a fluid state to the gel. A consequence of membrane disruption is the loss of intracellular components, such as metabolic enzymes and ATP, leading to cell death (JANUSKASUSKAS et al., 1999). The results of IMP evaluation after thawing, showed no significant difference between treatments RS, CR, BS and BC (16.8 ± 5.1%, 13.4 ± 4.9%, 13.8 ± 4 2%,

13.6 ± 4.9%, respectively) (Table 2). According to SALAMON & MAXWELL (2000), after cryopreservation sperm motility can range between 40% and 60%, but only about 20% to 30% of the spermatozoa membranes remain intact. Rodella (2006), in the study of percentage of motile (50.1%, 42.1%, 46.8% and 50.3%) and intact cells (27.2%, 23.3%, 25.5% and 27.4%) after thawing, found similar values to those by the previously mentioned authors. In this work, however, significant difference was observed in relation to these authors, because the percentage of cells with intact membranes was less than half the average of motile cells at the time of thawing. ALMEIDA (2006) suggests that, during cryopreservation, the sperm is submitted to many adverse conditions that hinder their membranes long before affecting their motility.

Regarding the percentage of morphological defects of semen after thawing, there was no significant difference among treatments. However, an increase in the percentage of defects in treatments with a water bag (RC and BC, 14.9% and 15.3%, respectively) was verified when compared with values after cooling. According to Bateman (2001), the percentage of spermatozoa with normal morphology decreases slightly after thawing, with a concomitant increase in tail defects, suggesting that freezing and thawing make defects generated by refrigeration more apparent, especially the ones in the tail.

It is known that the processes of cooling, freezing and thawing accelerate the maturation of sperm membranes, thus they increase the proportion of capacitated and acrosome-reacted spermatozoa (Salamon & Maxwell, 2000). This leads to a decrease in longevity of sperm cells in the female genital tract, which may impair fertility. GILLAN et al. (1997) reported similar findings, with 61% F standard (not qualified), 18% B standard (capacitated) and 21% AR standard (acrosome-reacted) for fresh ram semen, compared with 7.2% (F), 66% (B) and 26% (AR) for frozen-thawed semen. In this study, there was no significant difference among treatments in the percentage of sperm with intact acrosome after thawing, and the treatments RS, CR, BS and BC presented 51.9 ± 7.6%, 46.4 ± 8 1%, 54% ± 7.7 and 49.7 ± 8.7%, respectively (Table 2).

A decrease in the number of live intact spermatozoa was verified in thawed semen when compared to refrigerated semen. Even with the considerable increase of dead spermatozoa with intact acrosomes in the four treatments after freezing, there was no significant difference among treatments in the percentage of MI sperm (Table 2). As for the percentages of live acrosome-reacted and dead acrosome-reacted spermatozoa after thawing, no significant differences among treatments (Table 2) were found. These results are consistent with the study by GILLAN et al. (1997), which suggests that the steps of freezing and thawing increase the number of capacitated sperm, but produce less effect on the number of reacted acrosomes.

TABLE 2. Mean (± SD) of motility (%), vigor (0-5), total percentage of sperm morphological defects (MD), percentage of live spermatozoa with intact acrosome (VI) and with reacted acrosome (VR), of dead spermatozoa with intact acrosome (IM), and with reacted acrosome (MR) and percentage of cells with intact membranes (IMP), of ram semen refrigerated in different systems (RS - refrigerator without water bag, RC - refrigerator with water bag, BS - counter without water bag, and BC - counter with water bag) and frozen in N2 vapor immediately after thawing

	Motility (%)	Vigor (0-5)	MD (%)	VI (%)	VR (%)	MI (%)	MR (%)	IMP (%)
RS	41.2 ± 6.2	3 ± 0	13.5 ± 1.5	51.9 ± 7.6	0.1 ± 0.2	44.9 ± 7.7	3.2 ± 0.9	16.8 ± 5.1
RC	35.3 ± 9.9	2.9 ± 0.1	14.9 ± 2.4	46.4 ± 8.1	0.1 ± 0.2	50.1 ± 8	3.4 ± 0.7	13.4 ± 4.9
BS	40.2 ± 5.7	3 ± 0.1	15.3 ± 2	54 ± 7.7	0.3 + 0.4	41.9 ± 7.9	3.7 ± 1.7	13.8 ± 4.2
BC	38.2 ± 7.7	3 ± 0.1	15.3 ± 2.6	49.7 ± 8.7	0.1 ± 0.2	47.6 ± 9.2	2.6 ± 1.4	13.6 ± 4.9

The degree of damage that occurs in sperm during cryopreservation becomes apparent in the incubation. The greatest the damage, the lowest is the spermatozoon life span in the female genital tract (BAG et al., 2004).

Regarding the resistance test, there were no significant differences in motility and vigor percentage among the four treatments at the end of four hours of incubation (Table 3). However, very low sperm motility was observed for the treatments, which is probably due to the fact that sperm membranes were very damaged at thawing. These results differ from Rodella´s (2006), who reported 32.1% for motility, at the end of the resistance test of semen cooled in the refrigerator and frozen in liquid N2 vapor. After ram semen thawing, the membrane integrity was drastically reduced, whereas the effect on motility was not as evident. The simultaneous analysis of membrane integrity and motility revealed a large population of spermatozoa with membrane damages that were mobile immediately after thawing. Sperm with damaged membrane, although viable, quickly lose motility within a few hours of incubation at +37°C (VALCÁRCEL et al., 1994). The decrease in motility during incubation may also be connected to the reduction of the sperm ability to generate ATP due to mitochondrial damage or to the toxic effect of aromatic amino acid oxidase enzyme, released by dead spermatozoa (JOSHI et al., 2005).

At the end of the incubation period there was no significant difference in the percentage of cells with intact membranes among treatments (Table 3). The production of free radicals during semen storage has been identified as the main cause of reduced sperm motility and integrity (VISWANATH & SHANNON, 1997), reduction of energetic metabolism and denaturation of sperm DNA (BAUMBER et al., 2000). It is known that within an aerobic or partially aerobic system, the production of reactive oxygen species (ROS) is inevitable. The free radical superoxide anion (O_2^-), peroxide (H_2O_2) and hydroxyl (OH) are the most harmful and the reactions that generate these radicals appear to be more active at higher temperatures (BAG et al., 2004). The temperature used in the resistance test is quite favorable. In the present study, semen incubation at 37^{o}C was performed after thawing a 0.25 mL palette in a 5 mL glass tube. It is possible that the space between the cap and the surface of the semen provided conditions for the establishment of an aerobic system, which may have contributed to lower results at the end of incubation.

The percentage of morphology defects at the end of the resistance test also showed no significant difference among the four treatments (Table 3). However, a slight increase of defects was similarly observed for the four treatments, RS, CR, BS and BC (15.6%, 16.3%, 17.3%, 16.5%, respectively) compared to the moment of semen thawing.

TABLE 3. Means (± SD) of motility (%) (0-5), total percentage of morphological defects of spermatozoa and percentage of cells with intact membranes (IMP) at the end of four hours of resistance test

	Motility (%)	Vigor (0-5)	DM (%)	IMP (%)
RS	0.8 ± 1	0.4 ± 0.5	15.6 ± 2.6	10.6 ± 3.5
RC	1 ± 1	0.5 ± 0.4	16.3 ± 2.8	9.3 ± 3.4
BS	5.1 ± 7.6	1 ± 1.3	17.3 ± 2.2	8.9 ± 3
BC	1.3 ± 1.7	0.5 ± 0.6	16.5 ± 2.8	9.3 ± 3.3

CONCLUSIONS

The use of plastic bags with water during the cooling process of ram semen is effective in controlling the temperature drop in both the domestic refrigerator and the counter. The different cooling rates affect the semen after the cooling period, but its quality after thawing was not affected by different cooling protocols.

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