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COMPARATIVE ANALYSIS OF CRYOPRESERVATION OF MALE GAMETES WITH AND WITHOUT SEMINAL PLASMA FROM *CANIS LUPUS FAMILIARIS*

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Summary

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Based on the controversial data about removal of seminal plasma (SP) on semen parameters, especially on semen cryotolerance, three different ways of plasma separation were applied. The first one included SP removal after centrifugation at 100×g for 5 minutes. the second - SP removal by centrifugation at 650×g for three minutes while the third – SP removal by centrifugation at 1000×g for 1 minute. All semen samples with SP – control and without SP – experimental were extended with standard Rota Cryoprotective medium to final sperm concentration of spermatozoa 100–200 $\times 10^6$/mL (i.e., 50–100 $\times 10^6$ cells in a volume of 0.5 mL) and freezed at –150 ^oC. After thawing, the results from the experiment revealed that removal of seminal plasma did not improve statistically significantly the semen quality parameters, analysed by CASA of male gametes during cryopreservation.

Key words: dog semen, seminal plasma removal, cryopreservation

INTRODUCTION

The composition and function of seminal plasma has been studied in many species. Especially in dogs this fluid has mainly prostatic origin. This is due to the fact that, unlike other species, the prostate is the only additional sexual gland in the dog's reproductive system (Christensen, 1979) At this stage, the role of SP in the process of cryopreservation of male gametes and their fertility is still debatable and too controversial. There are a lot of data that prostate fluid (seminal plasma) is not relevant to the fertile characteristics of male gametes (Iguer-Ouada, & Verstegen, 1997.) In contrast, it was experimentally confirmed that the addition of prostate fluid to thawed dog semen resulted in an increased fertility after artificial intravaginal insemination. (Nöthling & Volkmann, 1993). In *in vitro* studies of dog semen stored at room temperature, the addition of SP to the ejaculates worsened their quality. The reported disturbances affected the survival and progressive mobility parameters (England & Allen, 1992). The study has been conducted over a pe-

riod of six hours with semen analyses performed every second hour. Another study confirms these results and the authors claim that after the addition of SP to the tested ejaculates in the first few minutes their speed parameters were significantly better than those of untreated samples, but afterwards rapid deterioration of motility has been recorded. The authors noted a dramatic decrease in the volume of the ejaculate, which correlated positively with a much higher sperm density in volume units. From the experiment it can be concluded that the strong decrease in the volume of the seminal plasma did not have a practical negative impact on fertility.

Questions about the role of canine SP concern also protocols related to cryopreservation of male gametes. Many protocols for cryopreservation of canine semen could be found in the literature. In some of them, the removal of seminal plasma is described as a compulsory technological step that needs to be accomplished. Such recommendations are given by various authors (Rota, 1995; Sirivaidyapong & Ursem, 2001; England & Allen, 1992). In protocols with removal of seminal plasma as a technological step, it is recommended to do this by centrifugation at 600–700 ×g for 4.5 minutes. (Rijsselaere, 2002; Schafer-Somi, 2006). According to Koderle (2009), removal of seminal plasma by centrifugation prior to cryopreservation not only disrupts all parameters of the CASA spermogram, but also leads to denaturation of chromatin in male gametes. Based on the above-mentioned controversies regarding the role of SP, we tested the effect of three different ways of seminal plasma elimination on sperm motility and velocity parameters after cryopreservation.

MATERIAL AND METHODS

Semen preparation and processing

Ejaculates (n=8) were obtained from clinically healthy dogs of different breeds aged between two and six years. A method of manual semen collection was used. Each ejaculate was divided into two parts – control and test samples. Control sample was semen with seminal plasma. The test samples were divided in 3 parts and the SP was removed by three different centrifugation modes: for the first part seminal plasma was removed after centrifugation at 100×g for 5 minutes. In the second part SP was removed by centrifugation at 650×g for three minutes and for the third part SP was removed by centrifugation at 1000×g for 1 minute. The Hermple Z 326K centrifuge was used for SP removal. After centrifugation, the SP was removed by pipette to 1 mm from the top of the pellet. All samples, both the experimental and the control ones, were diluted by standard Rota Cryoprotective medium. The control was diluted as soon as it was received. Test samples were diluted after separation of the seminal plasma and its removal by centrifugation. Dilution of all samples was performed in a ratio so as to provide a final sperm concentration of 100–200 $\times 10^6$ spermatozoa/mL (i.e., 50–100 $\times 10^6$ cells in a volume of 0.5 mL).

Evaluation of semen

The sperm motility of each ejaculate was evaluated using a Computer Assisted Sperm Analyzer (CASA) system (Microptic, Spain), an analytical module for motility and concentration. Cover slides 20×20 mm were used with 10 μL semen volume. Control samples were evaluated after dilution with the cryoprotective medium. A test sample was evaluated after centrifuga-

tion and resuspension with the cryoprotective medium. Computer-assisted sperm analysis was performed on all samples after the cryopreservation process. Motility of the sperm was evaluated by progression (static, progressive, non-progressive, fast, medium, slow) of at least 1,000 sperm in at least 5 visual fields of each sample.

Cryopreservation and thawing

The test and control samples were displaced in cryotubes in a volume of 0.5 mL. The equilibration was performed over a period of 3 hours at 4 °C. The cryopreservation of the samples was performed using an accelerated freezing mode at temperature –150 °C in a biofreezer. A minimum of 72 h was provided prior to samples' thawing. The thawing was performed in a water bath at 65 °C with an exposure time of 60–80 s (until the solid phase disappeared). The tubes were then placed in a water bath at 37 °C for 3–5 minutes and the motility of the frozen-thawed sperm was evaluated by CASA.

RESULTS AND DISCUSSION

A statistically significant difference in the percentage of sperm with progressive movement was found between the test and control samples before the freezing. Controls had the highest percentage of progressive sperm compared to all experimental groups (Fig. 1 and 2). From the test samples, these that were centrifuged at 1000×g for 1 minute had the highest percentage of progressive motile sperm, followed by samples centrifuged at 650×g for 3 minutes. The lowest percentage of progressive sperm had the samples centrifuged at 100×g for 5 minutes.

Regarding the other two assessment factors for motility – non-progressive and static sperm, controls continued to demonstrate resistance versus all experimental groups. Among the test groups, the best sperm parameters had the samples processed at 1000×g for 1 minute, and the worst parameters were found in the test group subjected to centrifugation at 100×g for 5 minutes. After removal of seminal

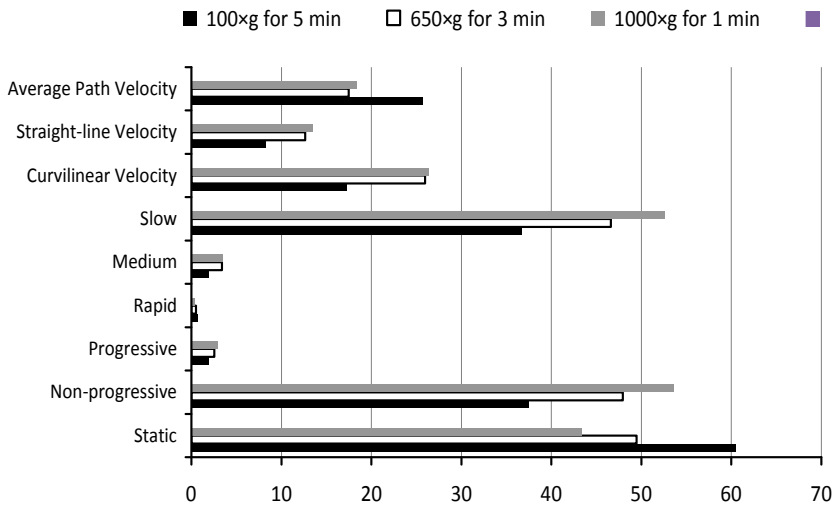

Fig. 1. CASA data on motility, progression and speed parameters of sperm from test and control samples prior to the cryopreservation process.

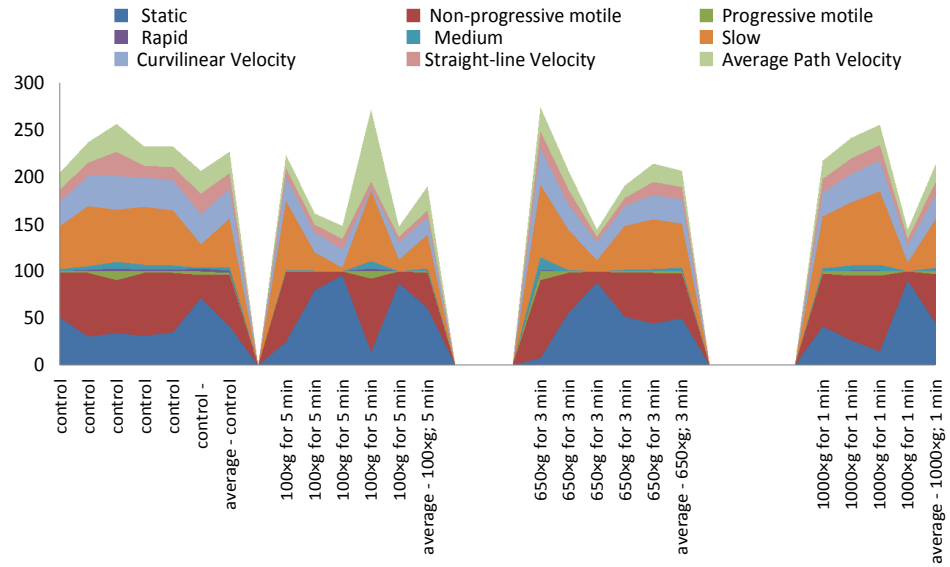

Fig. 2. CASA results after the cryopreservation.

plasma and sperm subject to centrifugation, there was a general decrease in sperm with a rapid and moderate motility, compared to the control, with slow-moving gametes being the highest in a control group treated at 1000×g for 1 min. In terms of speed parameters, the tendency for stability of the controls as compared to the test groups was still observed.

The rotational speed parameters are distributed as follows and calculated in mm/s. Curvilinear velocity (VCL) had the highest value in the control followed by the samples with removed sperm plasma at 1000×g mode for one minute. The lowest value was again in the 100×g mode for five minutes. After removal of seminal plasma and sperm subject to centrifugation, there was a general decrease in sperm with a rapid and moderate degree of movement compared to the control, with slow-moving gametes being the highest in a control group treated at 1000×g for 1 minute. In terms of speed parameters, a tendency for stability of the controls as compared to the test groups was still observed. The rotational speed parameters were distributed as follows and calculated in mm/s . Curvilinear velocity (VCL) has the highest value in the control followed by the samples with removed sperm plasma at the 1000×g mode for one minute. The lowest value was again in the 100×g mode for five minutes. For the other speed parameter – straight line velocity (VSL), the trend remained the same. There were significant differences in the only speed parameter average path velocity (VAP), which was the fastest in the 100×g test group for five minutes. Up to this stage of the experiment, it can be concluded that removal of sperm plasma did not improve the CASA parameters of male gametes in the cryopreservation process.

Studies conducted before sperm exposure to ultralow temperature ranges indicate that worsening of parameters occurs immediately after centrifugation. These disorders are likely to be of a general na-

ture and are due to the following effects. With longer sperm processing associated with centrifugation, sperm count and resuspension of precipitated sperm with a cryoprotective diluent may lead to shock on gametes, metabolic disturbance and increased oxidative stress. In the treatment of sperm, there are also thermal influences that increase the risk of temperature shock as the temperature in the centrifuge can practically never be equalised with the temperature of the seminal fluid, the cryoprotector and the conventional utensils used in the processing of sperm. Another factor are the purely mechanical effects on the gametes during the centrifugation process. In our opinion, the most favourable mode was at 1000×g for one minute. Too high speeds as 1000×g are likely to cause greater mechanical damage to the gametes, despite less exposure time. Also, the long exposure time of five minutes for 100×g is traumatic, as the metabolic disturbances that occur in the longer gametes treatment are likely to be leading. Deep low temperature storage have substantially altered the parameters of spermograms compared to pre-freezing as controls continued to demonstrate better viability, followed by test groups at 650×g for three minutes and 1000×g for one minute. Regarding the progressive mobility, all samples together with the control showed degraded characteristics, which is a normal phenomenon after exposure to ultralow temperature regimes. For sperm with non-progressive mobility the control had significantly better parameters than the test groups. Among the test groups, the highest percentage of non-progressive movable sperm was observed in the 650×g centrifugation mode for three minutes and the worst in the groups centrifuged at 1000×g per minute. For static sperm, the tendency was maintained, with the lowest percentage being the control, and the highest at 1000 ×g for one minute. It is evident from the figure that as a result of the low-temperature effects, the rate of fast sperm and medium-velocity sperm decreased sharply, with no significant difference in any of the samples. In slow-moving spermatozoids, the percentage was the highest in the controls followed by test groups 650×g for three minutes and 1000×g for one minute.

From the results presented, it can be concluded that the removal of sperm plasma did not improve the CASA parameters of male gametes during cryopreservation. Similar results are obtained and by Jennie Risopatrón (2012). Studies conducted before sperm exposure to ultra-low temperature indicated worsening of parameters immediately after centrifugation. These disturbances are likely to be of a general nature and are due to the following impacts. With longer sperm processing associated with centrifugation, sperm count and resuspension of sediment precipitates with cryopreservation dilution, it is likely to cause sperm shock, metabolic disturbance and increased oxidative stress. During treatment of ejaculates, there were also thermal influences increasing the risk of temperature shock as the temperature in the centrifuge can practically never be equalised with the temperature of the cryoprotector and the remaining instrumentation used in the processing of the sperm. Except this, there is information, that some seminal plasma proteins (14, 24, 70 and 82 kDa) had a positive correlation with semen characteristics (Cheema *et al.,* 2011).

CONCLUSION

As a result of the data obtained, we can conclude that the cryopreservation of dog sperm without SP did not achieve satisfac-

tory results compared to the control that was frozen with sperm plasma. Semen subjected to cryopreservation is not suitable for a marker study. Here the data overlap with a parameter from the curvilinear velocity sperm, where the control and test group 3 had almost the same value. The results and conclusions confirmed the potential significance of our CASA analyses as sperm quality markers.

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