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MANAGEMENT OF THE STORAGE OF CRYOPRESERVED SPERM ON DAIRY CATTLE FARMS

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Abstract: 26 liquid nitrogen tanks were selected from different dairy cattle farms. Three sperm doses were introduced in a frequently used canister, while another three straws were deposited in another canister that did not contain any sperm doses, to determine whether the refilling with liquid nitrogen had been done appropriately. Then, 10 sperm doses belonging to the same freezing lot were stored in our laboratory under ideal conditions to be used as control doses. After certain time period, the doses were collected from the farms and were analysed to obtain data about their total sperm motility and the individual kinetic parameters of each sperm. Four sperm subpopulations (SP) with different patterns of motility were identified using a cluster multivariate analysis. The results show that the mean total sperm motility has hardly decreased for the doses stored in the frequently used canister (45.2 ± 6.9%) in comparison with the doses stored in the rarely used canister (46.9 ± 59.0). However, the decrease in total motility was greater when compared with the control doses (59.0%). As for the sperm SP, (SP4 rapid and progressive sperm) which contained 31% of the total of sperm (control doses), differed the most when control doses were compared to straw stored in farm tanks. The percentage of the latter was reduced to 10 % after being stored in the tanks of the farms for 7 mo.

Such damage in SP 4 is progressive and cumulative and would probably reduce drastically compromising the fertility of the aforementioned sperm doses.

Keywords: motility, sperm subpopulations, management

Introduction

In the last 25 years, we have succeeded in improving the genetics and the management of the dairy cattle, which has resulted in an increase in milk

production. However, this improvement has triggered a decrease in the reproductive efficiency in the livestock farms *(Lucy, 2001; López-Gatius, 2003)*. During the last decade the pregnancy rate has been declining year after year and nowadays it stands at about 35% *(Quintela et al., 1997)* in the northwest of Spain (Galicia). In the beginning, the reasons for the decrease in fertility were attributed only to the genetic selection of the animals in favour of milk production *(Quintela et al., 1997).* However, the cause may be multifactioral and not only due to genetic selection *(Lucy, 2003; García-Ispierto, 2007*). Several factors could be influencing the reproductive efficiency of the dairy herds. For instance, stress due to heat (*Lucy, 2003; García-Ispierto, 2007)*, nutritional causes *(Roche, 2000)*, the comfort of the cow *(Hansen et al., 1999)* and incorrect practices in the management of the sperm doses in the farm, which could lead to a reproductive decline. The last factor has been widely revised as it could be one of the main reasons for the decrease in fertility *(Larson and Graham, 1958; Pickett and Berndtson, 1974; Senger et al., 1980; Saacke, 1983; Barth, 1993).*Correct management of the storage of cryopreserved sperm is fundamental in achieving suitable pregnancy rates when artificially inseminating dairy cattle. On several occasions, artificial insemination (AI) organisations have published recommendations with the intention of helping farmers and AI technicians in the handling of the doses of frozen-thawed semen. Despite these attempts, incorrect management has been confirmed in several studies in which not only professional inseminators *(Pickett, 1971)* are implicated, but also farmer-inseminators *(López Gatius, 2003)* under the supervision of vets and AI organisations. It seems that the problem is more significant with the farm owner/inseminator who have minimum training and whose technique has not been supervised. As time goes by, even professional inseminators may develop bad habits and a lax attitude towards the management of semen and insemination technique, which means a decrease in fertility.

Table 1. Mean values (±SD) of the kinematic parameters for the four subpopulations identified in frozen-thawed semen of dairy farms

Kinematic Parameters	SP 1	SP 2	SP 3	SP 4
Nº spz (%)	5996 (35.8)	2446 (14.6)	2977 (17.8)	5291 (31.6)
VCL (µm/s)	126.3±20.a	167.9±26.8b	64.3±24.5c	185.6±23.3d
VSL (µm/s)	100.1±17.8a	64.8±24.5b	37.8±20.4c	148.5±23.9d
VAP (µm/s)	111.5±17.2a	129.7±25.1b	48.1±21.7c	166.1±21.3d
LIN (%)	79.9±12.0a	38.8± 14.0b	57.4±23.1c	80.6±12.1d
STR (%)	89.8±8.6a	50.9±19.3b	75.2±21.7c	89.6±9.5d
WOB (%)	88.9±9.1a	77.7±11.6b	73.6±16.9c	89.7±7.4d
ALH (µm)	3.1±1.3a	5.2±1.5b	2.4±1.0c	4.3±1.5d
BCF (Hz)	8.7±3.1a	9.2±3.5b	5.6±3.4c	9.0±2.8d

a, b, c, d: different letters indicate significant differences between subpopulations ($P<0.05$)

So far, the experiments carried out to evaluate the decline in membrane integrity and motility of frozen-thawed semen were based on subjective microscopic assessment. Nowadays, much more precise and objective techniques are available, Computer-assisted semen analyses (CASA), for evaluating the functional characteristics of the sperm. Moreover, they allow us to quantify the different sperm subpopulations *(Muiño et al., 2008)*. The identification and quantification of sperm subpopulations with different patterns of movement can be vital to help estimate the fertilizing capability of the sperm doses of bovine frozen-thawed semen. It is likely that the inappropriate management of the sperm doses has a specific influence on the percentage of sperm subpopulations capable of reaching the female oviducts. Therefore, if the number of sperm is insufficient, the sperm will not be able to cross the barriers of the reproductive tract of the female and fertilize the oocyte *(DeJarnette et al., 1992; Nadir et al., 1993)*.

The main aims of the present study were:

1. To evaluate whether the farmer-inseminators have acquired bad habits and neglected their attitude towards the management of the semen, and consequently, whether they have contributed, together with other factors, to the decline in the fertility of the dairy cattle.
2. To investigate the influence of the management of the sperm doses during the storage on the frequency of distribution of the spermatozoa inside the different sperm subpopulations.

Material and Methods

Experimental design

In this study, 26 herds of Holstein-Friesian high-yielding dairy cows (26 liquid nitrogen tanks) were analysed in Northwest Spain. All the dairy farms were classified into two groups in order to evaluate the sperm stored in their tanks. One of the groups was made up of 17 small farms with an average of 36 cows and 23 heifers. The other group was made up of 9 collaborating farms with an average of 75 cows and 46 heifers. The herds were selected because they represented the largest dairy area in Galicia. Several parameters such as, tank condition and location, were written down in each farm.

An ejaculate from a *toro Asturiano de los Valles*[1] was selected and processed to be cryopreserved. Of the whole lot of frozen-thawed straws, 156 were selected to be deposited in the 26 cryogenic storage tanks (with a capacity of 18-20 L of liquid nitrogen). Three straws from the lot were placed in a canister with

[1] *Asturian Bull from the Valleys*. A breed of bull typical of Asturias that can also be found surrounding areas such as the east of Galicia.

semen which was routinely used by the cattle farmer. Thus, it was subjected to different types of manipulation such as raising and lowering the canister. Another three doses from the lot were placed in a canister without semen to know whether the frequency of refill with nitrogen was correct. The rest of the straws from the lot were stored in our laboratory and were used as control doses. They were also assessed every so often to get a standard of comparison as they were stored under ideal conditions. The liquid nitrogen in the control tank was kept at less than 5 cm below the neck of the tank.

When the doses were deposited, some characteristics related to the management of the tank, such as the state of the tank and its insulation, were investigated.

17 tanks from collaborating farms were classified as tanks in good conditions, with a lack of damage on the surface of the tank and its neck. However, nine tanks were in poor condition due to denting (Table 2).

Table 2. Characteristics related to the management of the tank

Parameters studied			**% Sperm total motility**	**% sperm inside SP**
Condition of the tank	Good Condition N=17	Poor Condition N=9	N.S	N.S
Frequency of refill with liquid nitrogen	30 dias N=18	45 dias N=7	N.S	N.S
Size of the catlle farms	Small farms(36 cows) N=17	Higher farms(75 cows) N=9	N.S	N.S

N.S indicate non-significant differences between the parameters studied and % sperm total motility and % sperm inside SP.

As for tank insulation in the farms, they were classified into 3 categories: on the floor and not protected (9 farms), on the floor but protected inside a box (11 farms) and kept upright, ideal conditions (7 farms) (Figure 2.a and 2.b).

After 7 mo, the doses stored in the tanks of the collaborating farms were collected (6 sperm doses). These were labelled with the farm´s origin. And the doses were transferred to our laboratory in order to evaluate the effects of the management on their sperm quality.

Collection and freezing of semen.

An ejaculate from an *Asturian bull from the Valleys* was collected by using an artificial vagina (internal temperature at 45º C). Then ejaculate volume and sperm concentration were evaluated (Accucell; IMV, L'Aigle, France). The morphology and sperm motility were subjectively assessed. After the initial evaluation, each ejaculate was diluted to a concentration $92x10^6$ sperm/ml using a

commercial extender Bioxcell (IMV, L'Aigle, France). This diluted semen was cooled from 22º C to 5º C for 1 ½ h (the cooling rate was of 0.22ºC/min approximately) and stored at 5ºC for a period of 2½ h. Thus total equilibration time was of 4 h. After dilution, it was packaged in 0.25 ml straws (23x106 sperm/ml) and frozen in vapours of liquid nitrogen inside a programmable freezer. We followed the standard freezing curve IMV Digit-cool, L'Aigle, France) for bovine semen (-5ºC/min from +4ºC to -10ºC; 40ºC/min from -10ºC to -100ºC; and -20ºC/min from -100ºC to -140ºC). Subsequently, the doses were deposited in liquid nitrogen (-196ºC) for a month and an initial evaluation of the motility (59%) was carried out in the Artificial Insemination Centre of Somio (SERIDA), Gijón, Asturias. After this period, the straws were delivered to the collaborating farms.

The experiment

Six straws from each farm were thawed simultaneously in a water bath at 37ºC for 40 s and their content was deposited in 5 ml Falcon tubes. The frozen-thawed semen was incubated at 37ºC for 2 h. One aliquot was evaluated immediately after warming (0 h) and another 2 hours later in order to assess the number of sperm per 5 µL. Sperm kinetic parameters were also assessed using the CASA system (ISAS, Valencia, Spain) in order to determine the existence of sperm subpopulations in the samples of bovine frozen-thawed semen. The analyses were also done to determine if cryogenic storage of the sperm on the farms damaged the structural integrity of the aforementioned sperm subpopulations. The sperm motility parameters of each cryopreserved sample were examined immediately after thawing (0 h) and after 2 h incubation at 37ºC using CASA.

The CASA system used captures 16 consecutive digital photographic images in a fraction of 0.64 s. This implies an image capture rate of one photographic image every 40 milliseconds. The images are taken from a sperm aliquot of 5 µL which is placed on slides and covered with 20 x 20 mm coverslips. Three microscopic fields were analysed in each sample using a phase contrast microscope supplied with a pre-warmed stage at 37ºC and a 100x magnification (Olympus BH2, Olympus Optical Co, U.K.). The total number of the spermatozoa analysed in each sample varied from 100 to 200. Non sperm particles were manually eliminated from each analysis. The total motility was defined as the percentage of spermatozoa with a mean velocity (VAP) of 10 µm/s approximately.
The kinetic parameters analysed for each sperm were the ones described by *Mortimer (1997, 2000)*: curvilinear velocity (VCL, µm/s), which is the distance covered by the spermatozoid along its trajectory according to time; rectilinear velocity (VSL, µm/s), which is the distance covered by the spermatozoid between the first and the last point of its trajectory per unit time; mean velocity (VAP, µm/s) which is, the distance covered by the spermatozoid along its mean trajectory per unit time; linear coefficient (LIN, %), defined as the percentage relation between VSL and VCL; straightness coefficient (STR, %) which is the percentage

relation between VSL and VAP; Wobble coefficient (WOB, %) defined as the percentage relation between VAP and VCL; mean lateral head displacement (ALH, μm), defined as the movement that the head of the sperm makes in its curvilinear trajectory from one point to another of the mean or linear trajectory; frequency of head displacement (BCF, Hz), which is the frequency with curvilinear trajectory that crosses the mean or linear trajectory according to time.

Statistical analysis.

The data of all motile sperm obtained from 104 thawing processes (26 dairy farms x 2 canisters x 2 assessments) were clustered in a single data group that represented 16,710 spermatozoa. Each spermatozoid was defined according to the eight motility patterns described above. A multivariate K-means clustering analysis was carried out in order to classify the 16,710 spermatozoa into a reduced number of subpopulations taking into account their pattern of movement. According to this classification, each sperm belonged to a single cluster. The spermatozoa which were very close to each other were assigned to the same group while those which were further away from each other were classified into different groups. The K-means clustering procedure uses Euclidean distances of 8 quantitative variables so that the core of the clusters was the means of the observations assigned to each cluster. In order to define the exact number of clusters, a prior analysis of hierarchic dendograms was carried out (Holt 1996). This analysis was based on individual ejaculates using the Ward method. For each of the canisters belonging to the 26 sperm tanks, contingency tables were used in order to determine the percentage of spermatozoa assigned to the different clusters at different times (0h and 2h).

A General Linear model (GLM) was used to analyse the frequency of distribution of the spermatozoa inside the subpopulations after subjecting the straws to the post-defrosting incubation and to a manipulation consisting of raising and lowering the canisters inside the sperm tank. The GLM procedure was also used to evaluate the influence exerted on the distribution of spermatozoa inside the subpopulations by dependent variables as the following: the level of liquid nitrogen inside the tanks, insulation of the tank, preservation conditions of the tanks and size of the cattle farms. All the statistical analysis were carried out using the SPSS software (SPSS Inc., Chicago, IL, USA), and the differences were considered significant when $P<0.05$.

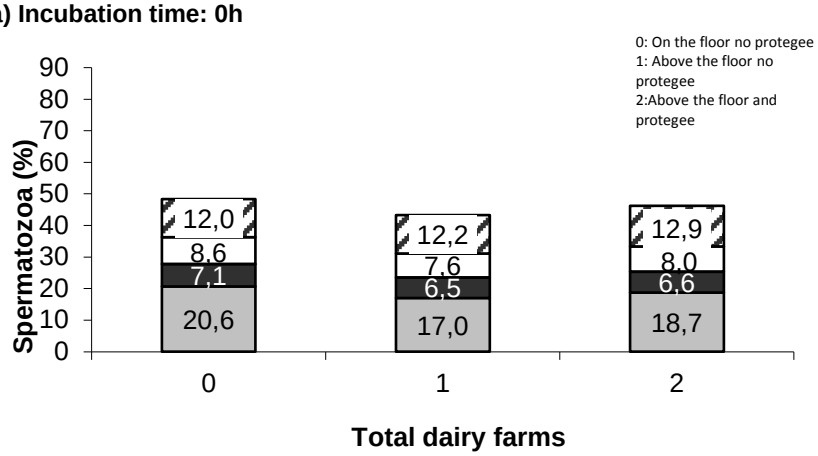
Results

Sperm total motility

The sperm managed in the 26 dairy farms had a lower mean sperm motility, objectively determined with the CASA system, compared to the doses

stored in the AI centre. Immediately after the thawing process (Figure 1.a), the percentage of sperm total motility was 45.2 ± 6.9%, 46.9 ± 8.9% and 59.0%, for thawed samples in frequently used canisters, in rarely used canisters and the control doses respectively. As demonstrated, no significant differences were observed among the treatment groups. The variables, such as the conditions of the storage tank, and the size of the cattle farms, did not have any interaction over the objective sperm motility immediately after thawing (Table 2). However, the differences in the insulation of the tanks of nitrogen had a significant effect on the total sperm motility (Figure 2.a). Thus, the doses stored in the tanks placed on the floor and without insulation showed a mean sperm motility of (48.5 ± 7.2). Similar results were found for the doses stored in tanks insulated inside a box and kept upright (46.3 ± 7.2). A significant decline in sperm motility was obtained for the doses stored in tanks placed on the floor, although insulated inside a box (43.5 ± 8.5). After 2 h of incubation (Figure 2.b), the percentage of total sperm motility diminished in all the tanks located in different places. However, the doses that registered the highest decline were those stored in tanks placed on the floor and not insulated (35.6 ± 9.4) whereas the values of motility for tanks placed on the floor and insulated were 40.3 ± 7.3 and the values of sperm motility for tanks kept upright and insulated were 41.0 ± 8.7. The frequency with which all the tanks of the collaborating farms were refilled was correct. Therefore, it did not influence the total sperm motility, nor did the size of the dairy farms (Table 2).

*indicate significant differences between subpopulations (P<0.05).



*indicate significant differences between subpopulations (P<0.05).

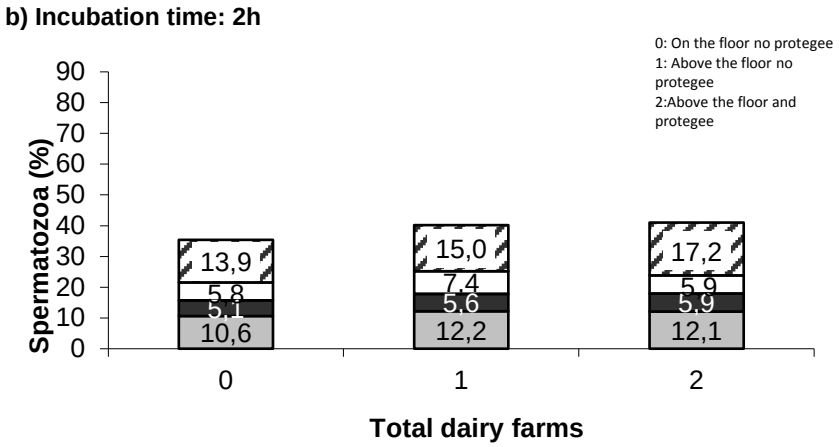


Figure 2. Relative frequency distribution of motile spermatozoa within subpopulations of straws storage in different insulates tanks, thawed at 37ºC during 40s, after 0 (a) and 2 h (b) of incubation at 37ºC.

Motile sperm subpopulations.

Four sperm subpopulations were defined after carrying out a multivariate cluster analysis of a total of 16,710 individual sperm. The characteristics of motility of those subpopulations are shown in table 1 and its patterns of motion can be described as follows:

Subpopulation 1 is represented by spermatozoa with low velocity (mean VCL and VAP) but with high progressiveness (high LIN, STR, WOB and low ALH). This subpopulation represents 35% of the total motile spermatozoa.

Subpopulation 2 is made up of spermatozoa with high activity but with non progressive motion as indicated by high values of VCL and ALH together with low values of LIN and STR and moderate BCF. These spermatozoa could be considered to have a movement similar to hyperkinesis. About 14% of the total motile spermatozoa were assigned to this subpopulation.

Subpopulation 3 is represented by 17% of the total spermatozoa that move slowly and not progressively. These spermatozoa show low values of VCL, ALH and BCF together with low LIN and STR.

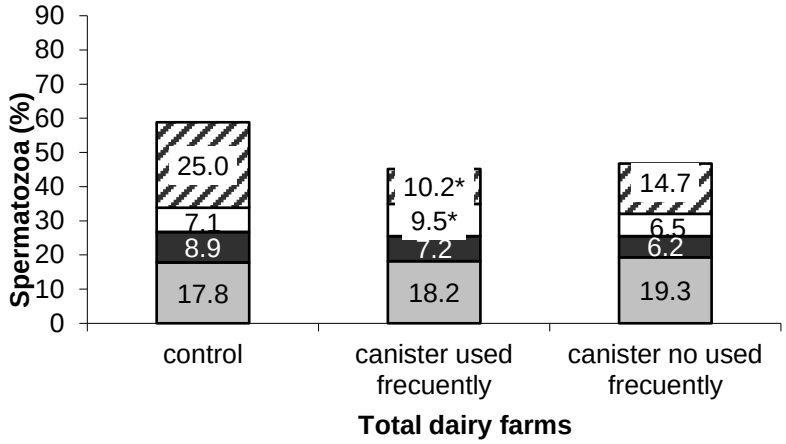
Subpopulation 4, which contains about 31% of the total population, is represented by spermatozoa with faster and progressive movement as its high values of VCL, VAP, LIN, STR and also BCF indicate.

Frequency of distribution of the spermatozoa inside the subpopulations according to management of doses in different canisters.

Immediately after thawing, the use of doses placed in a commonly used canister had a significant effect ($P< 0.05$) on the proportion of spermatozoa assigned to subpopulations 4 and 3. (Figure 1.a). Just after thawing, the samples of

semen placed in a frequently used canister by the farmer showed a significant reduction ($p<0.05$) in the subpopulation of spermatozoa with rapid and progressive movement (SP 4). It also showed an important increase in the proportion of spermatozoa with slow and non progressive movement (SP 3). Meanwhile, the sperm doses stored in our laboratory showed a high proportion of spermatozoa assigned to subpopulation 4. The management of semen did not alter the percentage of spermatozoa assigned to subpopulations 1 and 2.

a) Incubation time: 0h



*indicate significant differences between subpopulations ($P<0.05$).

b) Incubation time: 2h

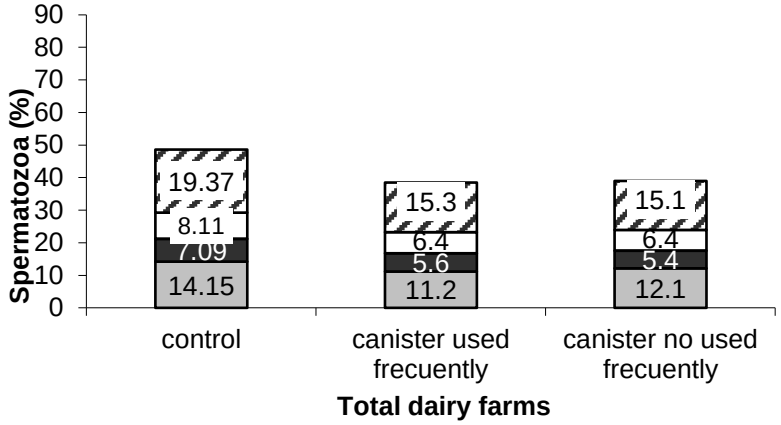


Figure 1. Relative frequency distribution of motile spermatozoa within subpopulations (1: grey columns, 2: black columns, 3: white columns, 4: striped columns) of straws storage in canister used frequently and other canister no used frequently, thawed at 37ºC during 40s, after 0 (a) and 2 h (b) of incubation at 37ºC.

After 2 h of incubation (Figure 1.b), the percentage of sperm assigned to subpopulations 3 and 4 diminished in all the samples of semen placed in the farmer and our laboratory. However, the doses that registered the highest decline were those stored in semen placed in a frequently used canister and rarely used canisters. The best results remained for controlling dose , more sperm number assigned to the SP4, and less proportion of spermatozoa with SP3. No significant differences were observed among the different groups

Other variables, such as the differences in conditions of tank insulation and the frequency of refill with liquid nitrogen, did not have any effect over the frequency of distribution of the spermatozoa inside the subpopulations (Table 2).

Discussion

The results of the present study indicated that, in general terms, there were no significant differences in the post-thaw sperm motility between the control doses and the doses stored in the dairy farms. These results disagree with other previously published studies that show significant differences between the semen stored in the farms and the one stored in the AI centre *(Pace and Sullivan, 1978; Linewear et al., 1979; Senger, 1980)*. In those prior studies, different periods of storage were used as well as different methods of sperm package. Furthermore, the sperm motility was assessed subjectively *(Barth, 1993; Dalton, 2002)*.

In this experiment, four sperm subpopulations were established in samples of frozen-thawed bull semen. They were determined according to the eight kinetic parameters studied. The present study confirms previous studies *(Muiño et al., 2008)* in which a very similar structure was described. That structure consisted of four sperm subpopulations in fresh and frozen-thawed Holstein bull sperm. Despite the fact that both experiments were developed in bulls of different breeds, the cryopreservation protocols used were similar. The presence of three or four well defined sperm subpopulations was shown in other species *(Holt et al., 1996; Abaigar et al., 1999; Quintero et al., 2003; Nuñez Martinez et al., 2006)*.

The doses that were managed and stored for 7 mo induced considerable changes in the distribution of the spermatozoa in the subpopulations. These changes were evident immediately after thawing the samples.

A significant increase in the proportion of the spermatozoa assigned to the subpopulation of spermatozoa with low and non progressive motion (SP 3) was observed in the samples of frozen-thawed semen. In these samples we could also observe a significant decline in the proportion of rapid and progressive spermatozoa (SP 4). This fact has recurred in all the sperm doses that were stored in the tanks of the dairy farms studied.

The damage caused to the frozen sperm when it is exposed to room temperature, around 20º C *(Larson and Graham, 1958; Rapaz, 1966; Holt, 2000)*, has been pointed out in several studies. This is common during manipulations such as extracting the straw and then reintroducing it in liquid nitrogen with the aim of transferring the straws from one tank to another, or simply to see their identification. Such manipulations can result in a partial thawing of the content of the straw. As a consequence, the spermatozoa can undergo a phenomenon called re-crystallisation by which the sperm becomes unviable.

The present study suggests that the decline in the percentage of spermatozoa observed in subpopulation 4 at the moment of thawing (the proportion of the most rapid and progressive spermatozoa might be the most appropriate pattern of movement for fertilization to take place) may be caused by the phenomenon of re-crystallisation. The increase in the size of some crystals might damage the sperm membrane and make some of the spermatozoa in SP 4 move to SP 3 (formed by non-progressive spermatozoa with little movement). The transfer from SP 4 to SP 3 could represent a late stage in cell deterioration. As the differences between the doses in the farms of study and the control doses were mainly found in the proportion of the spermatozoa assigned to subpopulation 4, this subpopulation might also determine the differences in fertility on site. For this reason, the doses stored in the AI centre might have a better fertility on site than the ones stored in the collaborating farms. The correlation between subpopulation 4 and the number of spermatozoa bound to the zona pellucida, the penetration rate and the rate of pronucleos formation were determined by *Ferraz et al., (2013)*. They found a significant ($P<0.05$) and positive correlation between the zone pellucid, the penetration rate and the pronucleus formation with sperm subpopulation 4 ($r=0.79$, $r=0.66$ and $r=0.63$, respectively).

Berndtson et al., (1976) showed that when the 0.25 ml straws in the globet were exposed to 20 ± 0,6ºC, that is room conditions, the temperature of re-crystallisation was reached in 40-60 s of exposure. However, re-crystallisation was reached in less time, in 10-15 s, when the straws were exposed to the air at 20 ± 0,6 ºC and a forceps was used. *Barth (1993)* suggests that the sperm in the neck of the tank must not be handled for more than 5 s in order to avoid re-crystallisation.

Other studies suggested that the sperm stored and used on site can be subjected to gradual deterioration *(Pace and Sullivan, 1978; Janett et al., 2008)*. However, these changes were not observed by other authors *(Lineweaver et al., 1979; Senger et al., 1980)* when they compared sperm stored in farms with sperm stored in AI centres as they obtained similar values of viability and sperm motility. Furthermore, they did not find any differences in viability between the sperm located in the upper part of the canister and the one in the lower part.

Other possible sources of sperm damage have been described by different researchers and taken into account in the present study. For example, the level of liquid nitrogen in the farm´s cryogenic storage tanks, the package of sperm in 0.25

ml straws and the insulation of the tanks. When the level of liquid nitrogen is high enough to cover the globets, the sperm is much less sensitive to changes in temperature. Such changes are produced especially when the sperm doses are removed in order to carry out the AI or to transfer them from one tank to another *(Berndtson et al., 1976)*. In our experiment, the level of liquid nitrogen was always kept above the minimum level. The tanks were frequently refilled and the level of liquid nitrogen was periodically checked with a dipstick. In order to study whether the frequency of refill with liquid nitrogen was correct, we analysed the motility and the subpopulations in the doses stored in canisters. Then, we compared them with the doses stored in the AI centre and no differences were found between them. This result confirmed our hypothesis that the frequency of refill was correct.

With regards to the type of straws for packaging semen, the 0.25 ml straws are the most commonly used in Galicia (in the Northwest of Spain) freeze and store bovine sperm due to their high storage efficiency in liquid nitrogen.

It is well known that 0.25 ml straws respond faster to the changes in temperature than 0.50 ml straws *(Stevenson et al., 2009)*. However, the fertility rate of semen frozen in 0.25 ml straws is the same or higher than that of semen frozen in 0.50 ml straws *(Pickett and Berndtson, 1974; Johnson et al., 1995; Stevenson et al., 2009)*. In this experiment, the 0.25 ml and 0.50 ml doses, which were stored at the same time, did not show any differences between them in sperm motility nor in the distribution of the subpopulations after being stored in the farms for 7 mo. Similar results were obtained by *Berndtson and Foote (1972) and Coulter and Foote (1973)* who did not observe any differences in the sperm motility of the sperm doses stored for 0, 6 and 18 mo.

Finally, when the insulation of the tanks (usually of 18 L) was revised, we could see that most of them were located in the offices of the farms. They were kept upright and protected from direct sunlight in a place where they were easily accessible. The place was fresh, dry and clean, as well as being ventilated and free of dust. Nine tanks were placed directly on the floor and subjected to acid corrosion that might deteriorate them. The total motility after thawing diminished considerably in the tanks kept directly on the floor as compared to those kept upright.

In conclusion, once the sperm doses in the tanks studied in this experiment were assessed, the results obtained in terms of total sperm motility after thawing were better in the case of the doses stored in the AI centre as well as in those kept in the rarely used canister. However, the study of the different sperm subpopulations present in the thawed sperm samples, and especially the dynamic subpopulations, revealed slight functional differences between both ways of handling the doses (frequent management of the canister / no management of the canister). A high percentage of spermatozoa with rapid and progressive movement was shown, after the thawing process, in the doses kept in the AI centre. However, it is not known whether this slight difference (SP 4) observed in these doses with

respect to the ones stored in the farms can affect fertility *in vivo*. Nevertheless, a more detailed study of the sperm subpopulations, which coexist in a sperm sample, might open up new possibilities to improve semen mishandling. Moreover, it could help avoid its harmful effects on the sperm doses kept in the farm tanks and in particular the sperm stored in the commonly used canister. It seems likely that this problem could be accentuated in the farms in which the inseminators are the owners of the herds because they very often have minimum training and no supervision. Consequently, as time goes by they may develop bad habits and a lax attitude towards the sperm management.

Acknowledgments

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Skladištenje, čuvanje zamrznute sperme na farmama muznih krava

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Rezime

Ukupno 26 kontejnera sa tečnim azotom je izabrano sa različitih farmi muznih krava. Tri doze semena su stavljene u kontejner/kanister koji se često koristi, dok je još tri slamke položeno u drugi kanister koji nije sadržavao nijednu dozu sperme, da se utvrdi da li se dopunjavanje sa tečnim azotom radi na odgovarajući način. Zatim, 10 dozi sperme koje pripadaju istoj zamrznutoj partiji su čuvane u našoj laboratoriji pod idealnim uslovima, i koristile su se kao kontrolne doze. Doze su posle određenog vremensko perioda prikupljene sa farmi i analizirane kako bi se dobili podaci o ukupnom motilitetom sperme i pojedinačnim kinetičkim parametrima svake sperme. Četiri subpopulacije sperme (SP) sa različitim obrascima pokretljivosti su identifikovani pomoću multivariacione analize klastera.

Rezultati pokazuju da je srednja ukupna pokretljivost sperme neznatno opala za doze uskladištene u kontejneru/kanisteru koji se često koristi (45,2 ± 6,9%) u odnosu na doze pohranjene u kontejner/kanister koji se retko koristi (46,9 ± 59,0). Međutim, smanjenje ukupnog motiliteta bilo je veće u poređenju sa kontrolnim dozama (59,0%). Što se tiče sperme SP, (SP4 brza i progresivna sperma) koja je sadržala 31% od ukupnog broja spermatozoida (kontrolne doze), razlikovala se najviše kada su kontrolne doze poređene sa dozama koje se čuvaju u

kontejnerima na farmama. Procenat potonjih je smanjen na 10% nakon što su skladištene u kontejnerima na farmama u periou od 7 meseci.

Takva oštećenje kod SP 4 je progresivno i kumulativno i verovatno će doći do drastičnog smanjenja, što ugrožava plodnost navedenih doza sperme.

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