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in

Almeida J.A. (ed.), Tirapicos Nunes J. (ed.).
Tradition and innovation in Mediterranean pig production

Zaragoza : CIHEAM
Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 41

2000
pages 99-103

Boar spermatozoa cryopreservation[1] (pelets *vs* maxi-straws freezing)

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SUMMARY - Experiments were carried out to study the effect of two techniques of deep-freezing semen (*P* = Pellets and *M* = Maxi-straws). Ejaculates were obtained from 5 Alentejano swine breed boars and 2 contrast boars (1 Duroc x LW and 1 LW). Regarding P-method, semen drops were previously frozen (on a carbonic ice plate at, -76°C), before being deep-frozen into liquid nitrogen (Paquignon *et al.*, 1974). As far as the M-method was concerned, it was adapted from Westendorf *et al.* (1975), using a programmable freezing chamber. Concerning *in vitro* sperm evaluation, pellets were placed directly into four different thawing solutions at 42°C (SD-glucose-sodium citrate solution, BTS, MR-A, SD + BTS) and maxi-straws were thawed for 40s in, a water bath at 40°C. After thawing was completed, semen of the later method was also diluted into the same four solutions previously mentioned. During evaluation procedures, diluted semen was maintained at 37°C. Heterospermic inseminations (fresh semen + frozen semen) were practised in 12 sows of the Alentejano swine breed. The main results obtained from our experiments shown that the percentage of spermatozoa motility was higher in method M, after thawing, at 15, 30, 45, and 60 minutes ($P<0.001$). Heterospermic artificial inseminations (AI) showed a strong difference, favourable to fresh semen.

Key words: Cryopreservation, semen, AI, alentejano swine breed.

RESUME - *"Cryopreservation de spermatozoïdes de verrat (congélation en pastilles* vs *macrotubes". Des essais ont été réalisés pour comparer deux techniques de préparation de la semence pour la congélation (*P *= pastilles et* M *= macrotubes). Les éjaculats provenaient de 5 verrats de race Alentejana et 2 témoins à savoir 1 verrat croisé (Duroc x LW) et 1 verrat LW. Le procédé de congélation* P*, préconisé par Paquignon* et al*. (1974), consiste en pré-congélation en pastilles dans de la glace carbonique suivie par une immersion dans l'azote liquide. Le procédé de congélation* M *est une adaptation de la méthode préconisée par Westendorf* et al.*, (1975). Pour le test* in vitro *de la mobilité des spermatozoïdes après la décongélation, dans le cas de la méthode* P *nous avons plongé les pastilles directement dans les solutions de décongélation suivantes : solution glucose-citrate de sodium (adapté de Pursel & Johnson, 1975) ; dilueur BTS ; dilueur MR-A, et une quatrième solution constituée par la solution glucose-citrate, dans laquelle du BTS a été ajouté 15 minutes après la décongélation. Pour la méthode* M*, la décongélation a été réalisée par immersion des macrotubes dans l'eau à 37°C pendant 45 secondes puis dans les solutions de décongélation citées précédemment. Pendant l'observation et l'évaluation, les solutions ont été maintenues à 37°C. L'efficacité* in vivo *a été testée par l'insémination hétérospermique de truies de race Alentejana (semence fraîche + semence décongelée). La mobilité des spermatozoïdes après la décongélation est significativement supérieure dans la méthode* M *(*$P<0,001$*). Les résultats des inséminations hétérospermiques ont été très favorables à la semence fraîche.*

Mots-clés : *Cryopréservation, semence, IA, race Alentejana.*

Introduction

The remarkable progress observed on intensive pig production systems and the vicissitudes of the extensive systems endangered to disappearance many local swine breeds all over the world. Nowadays, ecological awareness make us to realize how important is it to preserve the biodiversity of both animals and their natural environment, which sustains their production based on perfectly balanced (sustainable) systems.

Freezing of boar semen has been practised long ago, in France, to preserve swine germplasm of endangered swine breeds, namely Gascon and Blanc de L'Ouest (Paquignon *et al.,* 1976).

Results obtained out of freezing methods, evaluated either *in vitro* or by AI, have been successively improved after various adaptations.

This trail aimed at comparing two classical techniques, namely pellets by Paquignon *et al.* (1974) and maxi-straws by Westendorf *et al.* (1975), to select the best of the tested methods, regarding its application on Alentejano swine breed boars semen, tacking into account the prevalent outdoor breeding system used in Alentejo.

Material and methods

The rich sperm fractions of five Alentejano swine breed, one Duroc x Large White and one Large White boar were collected by the gloved-hand method.

The procedures before freezing

Pellets

Semen was split into 100 ml centrifuge flasks and was centrifuged (800 g, for 15 min) at 28-30°C.

The concentrated semen was diluted with 8.5 ml of the extender *A* (5.67 g of glucose; 22.5 ml of egg yolk and 77.5 ml of distilled water), allowed to cool down during 1 h until reaching 15°C and remaining at this temperature during another 4 h period.

This solution was than re-diluted with extender *B* (extender A with 4.4% of glycerol) and allowed to cool down, during 1 h until it reached 5°C.

Maxi-straws

Semen was diluted 1:2 with Beltsville thawing solution (BTS) at 32°C, kept during an equilibrium period (1 h) at room temperature (±22°C), allowed to cool down to 15°C (within an 1 h period) and then maintained at this temperature during another 3 h period.

Semen was split into 100 ml centrifuge flasks and centrifuged (900 g, for 15 min) at 15°C. The concentrated semen was diluted with 3.5 ml of the extender *C* (8.8 g of lactose, 20 ml of egg yolk and 80 ml of distilled water), slowly cool down to 5°C and further remaining at this temperature for a 2 h period. Finally, a third dilution was made at 5°C with 1.5 ml of the extender *D* (93.5 ml of extender C; 1.5 ml of Orvus Es Paste and 5 ml of glycerol) and homogenized. The final solution had an approximate concentration of 10^9 spermatozoa/ml.

Freezing procedures

Pellets

Pellets volume was 0.1 ml; drops of semen were shed in concavities previously moulded over a carbonic ice plate at -76°C, remaining there during 2-3 min. The frozen pellets were immediately plunged in liquid N_2, before being stored in liquid N_2 until thawing.

Maxi-straws

After a third dilution, processed semen was put into 3.5 ml maxi-straws, closed manually with metallic sealing balls. Filled straws were then placed into a programmable biological freezing chamber.

The following freezing program was run (5 to -6°C, according to a -3°C/min freezing rate; followed by a further decrease in temperature from -6 to -140°C, at a -20°C/min freezing rate. Frozen straws were stored in liquid N_2 until thawing.

Thawing procedures

The thawing solutions used

SD – glucose – sodium citrate solution (37 g of glucose; 1.25 g of sodium bicarbonate; 6 g of sodium citrate; 1.25 g of EDTA; 0.75 g of potassium chloride and 1000 ml of distilled water).

BTS – Beltsville thawing solution (commercial).

MR-A – Commercial extender for fresh semen.

Thawing solution with SD and BTS added 15 min after thawing.

Pellets

Pellets were thawed by direct immersion in four thawing solutions at 42°C. After homogenization and stabilization, the solutions were kept at 37°C in a water bath. Samples were taken, observed and evaluated immediately after thawing, and at 15, 30, 45, and 60 min post-thawing. The evaluation procedure consisted of assessing the % of motile spermatozoa and the % of progressive motility of each thawing solution.

Maxi-straws

Maxi-straws were thawed by immersion in a 40°C water bath during 40 s. The thawed semen was put into the various thawing solutions; all kept at 37°C (in a water bath). The same observation and evaluation, as already mentioned for pellets, were also followed in this situation.

Statistical analysis

Data were subjected to analysis of variance by using the Least square mix model (maximum likelihood; Harvey, 1990). The general mathematical model used was:

$$Y_{ijkl} = \mu + Gen_i + Est_j + Met_k + \in_{ijkl}$$

Y_{ijkl} = value for dependent variable
μ = Mean common effect
Gen_i = fixed effect of genotype (i = 1, 2, 3)
Est_j = fixed effect of season (j = 1, 2, 3, 4)
Met_k = fixed effect of method (k = 1, 2)
$\in_{ijkl}$ = standard error of mean

Results

Statistically significant differences on initial (before freezing) percentage of spermatozoa motility were not observed (Table 1). However, immediately after thawing and during the next 60 minutes of incubation at 37°C, the *M* method was associated to significantly higher percentage of spermatozoa motility ($P<0.001$), compared with the *P* method (Fig. 1).

Discussion

This experiment showed that the procedures carried out before the freezing of semen affected its motility in a similar way, on both methods, given that significant differences on spermatozoa motility were not observed before freezing. Nevertheless, the freezing/thawing procedures differentially affected the post-thawing quality of semen. Thus, all the percentages of spermatozoa motility were significantly higher in the thawed semen processed by method *M*. Bwanga *et al.* (1990) observed decreases on spermatozoa motility (semen frozen in maxi-straws)

from 30 minutes to 2 h after thawing (incubated at 37°C). Our experiment also revealed the same tendency, our results being in agreement with the ones reported by Almid, *et al.* (1988) and Bwanga *et al.* (1990) (37.8% and 27% of spermatozoa motility at 30 min after thawing respectively). The work of Paquignon, *et al.* (1986), where they used *P* and *M* semen freezing methods, pellets and in maxi-straws, showed a higher spermatozoa motility associated with semen preserved under pellets method (20.1%) than in maxi-straws method (14.6%), both at 15 minutes after thawing. This situation was not confirmed by our results, where the use of method *M* lead to a higher average spermatozoa motility (20.3%, 15 min after thawing) compared to method *P* (18.3% at the same occasion).

In spite of the disappointing results obtained on *in vivo* experiments, our *in vitro* evaluation of frozen/thawed semen suggests that the *M* method may potentially allow better results to be achieved, compared with method *P*. Further research is required to confirm the potential field for application of both methodologies.

Table 1. Mean (± sem) percentage of spermatozoa motility, as observed before freezing and after thawing and incubation

	BFM†	PTM††	M 15'†††	M 30'	M 45'	M 60'
Pellets	69 ± 0.9	0 ± 1.2	6 ± 1.8	11 ± 2.0	4 ± 1.8	1 ± 1.7
Maxi-straws	71 ± 1.1	18 ± 1.3	27 ± 2.7	37 ± 2.3	35 ± 2.1	29 ± 1.9
SL	NS	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001

†BFM: Before freezing % of spermatozoa motility
††PTM Post-thawing % of spermatozoa motility
†††M 15'; 30'; 45' and 60': Percentage of spermatozoa motility observed at 15, 30, 45 and 60 minutes of incubation (37°C)

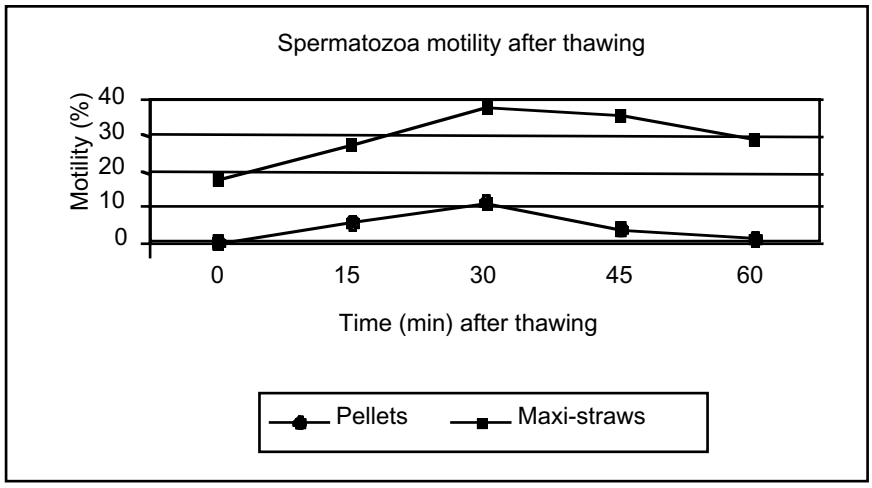

Fig. 1. Mean percentage of spermatozoa motility after thawing.

Acknowledgements

Authors would like to thank to programme PAMAF for the financial support of Project No. 3032 which allowed them to run up this experiment and express their gratitude to the Professor A. Murilhas for his help on English version revision.

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