

Boar spermatozoa cryopreservation (Maxi-straws method)

Tirapicos Nunes J., Charneca R., Milhano A., Almeida J.A.

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 85-91

Article available on line / Article disponible en ligne à l'adresse :

<http://om.ciheam.org/article.php?IDPDF=600119>

To cite this article / Pour citer cet article

Tirapicos Nunes J., Charneca R., Milhano A., Almeida J.A. **Boar spermatozoa cryopreservation (Maxi-straws method)**. In : Almeida J.A. (ed.), Tirapicos Nunes J. (ed.). *Tradition and innovation in Mediterranean pig production*. Zaragoza : CIHEAM, 2000. p. 85-91 (Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 41)



<http://www.ciheam.org/>
<http://om.ciheam.org/>

Boar spermatozoa cryopreservation² (Maxi-straws method)

J. Tirapicos Nunes, R. Charneca, A. Milhano and J.A. Almeida
Universidade de Evora, Ap. 94, 7002-554 Evora, Portugal

SUMMARY - Artificial insemination (AI) using frozen semen, still possesses some limitations. Fertility and prolificacy rates are quite low when frozen semen is used, compared with fresh semen. However, some progress has been achieved by using improved new technologies. One of such techniques is the freezing of semen by using the method proposed by Westendorf *et al.* (1975). Our trial is also based on Westendorf *et al.* (1975) method, tested the efficacy of freezing semen, assessing it *in vitro*, after thawing and dilution into 4 different solutions. These were the 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); the BTS – Beltsville thawing solution; the MR-A - Commercial extender for fresh semen and the ACROMAX - Commercial extender for fresh semen. Semen *in vitro* evaluations took place at the 10th and 20th day after preservation into liquid nitrogen. Semen *in vivo* evaluation was carried out on 15 Alentejano breed sows artificially inseminated with thawed semen. The quality of semen decreased progressively after 30 minutes of thawing. Significant effects on semen quality were associated with thawing solutions, genotypes and months of semen collection. The % of positive pregnancies was 20% as revealed by echography at 28 days after AI.

Key words: Boar, freezing, thawing, semen quality, AI.

RESUME - "Congélation des spermatozoïdes de verrats (méthode des maxi-palettes)". L'utilisation de l'IA porcine avec semence congelée n'est pas encore comparable en raison soit de son coût soit de ses résultats. L'utilisation de semence fraîche et l'introduction de nouvelles méthodes permettent cependant d'améliorer de façon significative la fertilité. La congélation en palettes et macrotubes préconisée par Westendorf *et al.* en 1975, a été légèrement modifiée afin d'améliorer les résultats de la congélation. Ce travail se base sur une adaptation de cette méthode. Pour tester l'efficacité du processus de congélation, la semence a été évaluée *in vitro*, après décongélation et dilution dans quatre solutions différentes : solution glucose-citrate de sodium (adapté de Pursel et Johnson, 1975) ; dilueur BTS ; dilueur MR-A et dilueur ACROMAX. Les observations ont été réalisées 10 et 20 jours après la conservation dans l'azote liquide. L'efficacité *in vivo* a été testée en inséminant 15 truies de race Alentejana avec des doses de semence décongelée. *In vitro*, on observe une diminution progressive de la mobilité 30 minutes après la décongélation. Par ailleurs, la solution de décongélation affecte la mobilité à partir des 30 minutes. Les génotypes étudiés et l'époque de récolte ont des effets significatifs. Enfin, le pourcentage de gestation par IA a été de 20% (diagnostic de gestation par échographie au 28^{ème} jour).

Mots-clés : Verrats, spermatozoïdes, congélation, décongélation, qualité de la semence, IA.

Introduction

Extensive pig production systems are vulnerable. Several factors can endanger the viability of these systems production rows, which have been based upon natural resources being directly consumed by pigs, autochthon swine breeds and the exploitation of traditional ways of processing and commercializing end food-products.

Local breeds are presently spread apart in small size populations, whose survival is questioned by: excessive consanguinity, genetic drift, wrong crosses, and high exposure to diseases.

Nowadays, preservation of many of these breeds is socially and economically important, taking in account that they should be maintained within traditional systems (*in situ* management). On the other hand, cryopreservation (preservation *extra situ*), must be applied, if the risk of inadequate genetic strategies and sanitary problems is to be conveniently reduced.

This experiment aimed at assessing in our conditions, the interest of applying an adaptation to the cryopreservation method of Westendorf (1975), by using proper equipment, and choosing from several freezing rates.

Material and methods

The sperm rich fractions of four boars [three Alentejano swine breed (AL) and one Large White (LW), from 8 months to 24 months of age] were collected by the gloved-hand method.

Procedures before freezing

Semen was diluted 1:2 with Beltsville thawing solution (BTS) at 32°C, maintained during an equilibrium period (1 h) at room temperature ($\pm 22^\circ\text{C}$), allowed to cool down to 15°C within an one hour period and then kept at this temperature during another 3 hours period.

Semen was split into 100 ml centrifuge flasks and centrifuged (900 g, 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 cooled down to 5°C, and 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 product had an approximate concentration of 1×10^9 spermatozoa/ml.

Freezing procedures

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 the chamber of the programmable biological freezing device.

The following freezing programme was run: 5 to -6°C , following a $-3^\circ\text{C}/\text{min}$ freezing rate; with a final decrease in temperature, from -6 to -140°C , being achieved at a different freezing rate of $-20^\circ\text{C}/\text{min}$. Frozen straws were stored in liquid N_2 until thawing.

Thawing procedures

The thawing solutions used were:

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.

ACROMAX - Commercial extender for fresh semen.

Maxi-straws were thawed during a 45 s period in a water bath at 50°C. The thawed semen was transferred into the various thawing solutions, all kept in a water bath at 37°C.

Preparation of the artificial insemination (AI) doses

Two or three identified maxi-straws were thawed as previously described. The thawed semen was loaded into an AI bottle half filled with BTS extender at 37°C, when done bottle was completely filled with more BTS at the same temperature. A microscopical observation and evaluation of semen was made and the AI was performed as rapidly as possible.

Preparation and AI of the sows

The heat detection was made, with an Alentejano swine breed adult boar, twice a day (morning and afternoon). After the observation of the immobility reflex the sows were placed on a restraining crate and the AI was performed in the presence of a boar.

The following procedure was adopted:

Three AI were performed using the same boar's frozen semen: the 1st insemination with 7×10^9 spermatozoa; the 2nd one with 10.5×10^9 spermatozoa (± 8 h after the 1st AI) and finally a 3rd insemination with 7×10^9 spermatozoa (± 24 h after the 1st insemination).

Pregnancy rates were tested with ecographie performed on the 28th day after last AI.

Results

The results of our experiment showed an influence of month, genotype, thawing solution and cryopreservation period on the quality of the studied semen before freezing and after thawing up to 1 hour of incubation at 37°C.

Firstly, there was a decrease on semen motility related with semen handling before freezing. After thawing we frequently observed an increasing spermatozoa motility until 30 minutes of incubation at 37°C. Thereafter we found a progressive decrease on spermatozoa motility up to 60 minutes of incubation. These changes on spermatozoa motility depended on several of the studied factors.

If we consider the influence of month and genotype on sperm motility before freezing and after thawing, there were no significant differences until the semen cooled down to 15°C. However, in July, at 5°C, spermatozoa motility was significantly higher in the LW animal ($P < 0.001$). Immediately after thawing (MOT 0') statistically significant differences between genotypes were not observed in July. On the contrary, during the months of August and September, semen of Alentejano animals had higher spermatozoa motilities immediately after thawing (MOT 0') than the LW animal ($P < 0.05$ and $P < 0.01$ respectively). Comparing semen incubated at 37°C, sperm motilities at 15, 30, 45 and 60 minutes were significantly higher on the Alentejano animals than on the LW one in every studied month (Table 1).

Table 1. Influence of month and genotype on sperm quality, both before freezing and after thawing (means \pm sem)

Month	Breed	MOT 5°C	MOT 0'	MOT 15'	MOT 30'	MOT 45'	MOT 60'
July	AL	71.6 \pm 0.6	33.1 \pm 1.0	40.0 \pm 0.9	41.8 \pm 0.9	37.7 \pm 1.2	29.7 \pm 1.2
	LW	77.3 \pm 0.9	33.2 \pm 1.7	36.4 \pm 1.5	33.5 \pm 1.6	28.7 \pm 2.0	19.3 \pm 2.1
	SL	$P < 0.001$	NS	$P < 0.05$	$P < 0.001$	$P < 0.01$	$P < 0.001$
August	AL	76.6 \pm 0.4	31.2 \pm 0.8	39.3 \pm 0.7	40.8 \pm 0.7	35.9 \pm 0.9	29.9 \pm 0.9
	LW	76.5 \pm 0.9	26.0 \pm 1.5	27.9 \pm 1.4	23.5 \pm 1.4	16.0 \pm 1.8	8.1 \pm 1.9
	SL	NS	$P < 0.05$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$
Sept	AL	70.6 \pm 0.6	36.3 \pm 1.1	40.2 \pm 1.0	37.6 \pm 1.0	33.2 \pm 1.2	27.4 \pm 1.3
	LW	69.8 \pm 1.1	30.5 \pm 1.9	31.8 \pm 1.7	25.9 \pm 1.8	17.0 \pm 2.2	9.9 \pm 2.4
	SL	NS	$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

MOT 5°C: Spermatozoa motility (%) at 5°C

MOT 0': Spermatozoa motility (%) after thawing (0 minutes)

MOT 15': Spermatozoa motility (%) at 15 minutes after thawing

MOT 30': Spermatozoa motility (%) at 30 minutes after thawing

MOT 45': Spermatozoa motility (%) at 45 minutes after thawing

MOT 60': Spermatozoa motility (%) at 60 minutes after thawing

SL: Significance level

The influence of month, when separately studied for each genotype, showed us some interesting results. Spermatozoa motilities observed on AL animals before freezing were significantly higher during August ($P<0.001$). Nevertheless, those higher motilities had no effect on the post-thawing sperm quality of these animals (Table 2).

Table 2. Influence of month on sperm quality, before and after freezing/thawing semen procedures carried out on the Alentejano swine breed boars (means \pm sem)

Month	MOT 15°C	MOT 5°C	MOT 0'	MOT 15'	MOT 30'	MOT 45'	MOT 60'
July	77.0 \pm 0.5 ^a	71.6 \pm 0.6 ^a	33.1 \pm 1.0 ^{ab}	40.0 \pm 0.9	41.8 \pm 0.9 ^a	37.7 \pm 1.2	29.7 \pm 1.2
August	80.0 \pm 0.4 ^b	76.6 \pm 0.4 ^b	31.2 \pm 0.8 ^a	39.3 \pm 0.7	40.8 \pm 0.7 ^a	35.9 \pm 0.9	29.9 \pm 0.9
Sept	75.2 \pm 0.5 ^a	70.6 \pm 0.6 ^a	36.3 \pm 1.1 ^b	40.2 \pm 1.0	37.6 \pm 1.0 ^b	33.2 \pm 1.2	27.4 \pm 1.3
SL	$P<0.001$	$P<0.001$	$P<0.01$	NS	$P<0.05$	NS	NS

MOT 15°C: Spermatozoa motility (%) at 15°C
 MOT 5°C: Spermatozoa motility (%) at 5°C
 MOT 0': Spermatozoa motility (%) immediately after thawing (0 minutes)
 MOT 15': Spermatozoa motility (%) at 15 minutes after thawing
 MOT 30': Spermatozoa motility (%) at 30 minutes after thawing
 MOT 45': Spermatozoa motility (%) at 45 minutes after thawing
 MOT 60': Spermatozoa motility (%) at 60 minutes after thawing
 SL: Significance level

The spermatozoa motility before freezing (at 15°C and 5°C) observed on the LW animal was also higher during August but, surprisingly, the spermatozoa motility after thawing of frozen semen in August was always lower (sometimes significantly) than spermatozoa motility of frozen/thawed semen on the other months (Table 3).

Table 3. Influence of month on sperm quality, before and after freezing/thawing semen procedures carried out on the Large-White boar (means \pm sem)

Month	MOT 15°C	MOT 5°C	MOT 0'	MOT 15'	MOT 30'	MOT 45'	MOT 60'
July	76.3 \pm 0.8 ^a	76.3 \pm 0.9 ^a	33.2 \pm 1.7	36.4 \pm 1.5 ^a	33.5 \pm 1.6 ^a	28.7 \pm 2.0 ^a	19.3 \pm 2.1 ^a
August	80.1 \pm 0.7 ^a	76.5 \pm 0.9 ^a	26.0 \pm 1.5	27.9 \pm 1.4 ^b	23.5 \pm 1.4 ^b	16.0 \pm 1.8 ^b	8.1 \pm 1.9 ^b
Sept	74.8 \pm 0.9 ^b	69.8 \pm 1.1 ^b	30.5 \pm 1.9	31.8 \pm 1.7 ^c	25.9 \pm 1.8 ^b	17.0 \pm 2.2 ^b	9.9 \pm 2.4 ^b
SL	$P<0.001$	$P<0.001$	NS	$P<0.001$	$P<0.001$	$P<0.001$	$P<0.001$

MOT 15°C: Spermatozoa motility (%) at 15°C
 MOT 5°C: Spermatozoa motility (%) at 5°C
 MOT 0' – Spermatozoa motility (%) immediately after thawing (0 minutes)
 MOT 15': Spermatozoa motility (%) at 15 minutes after thawing
 MOT 30': Spermatozoa motility (%) at 30 minutes after thawing
 MOT 45': Spermatozoa motility (%) at 45 minutes after thawing
 MOT 60': Spermatozoa motility (%) at 60 minutes after thawing
 SL: Significance level

Regarding the other factors also studied in this experiment [i.e., the cryopreservation period (10 or 20 days of conservation) and the type of thawing solution], on thawed sperm quality, no statistically significant effect of the cryopreservation period was observed, even when month and genotype were considered as sources of variation. Nevertheless, the thawing solution played a significant influence on thawed sperm quality (Fig. 1).

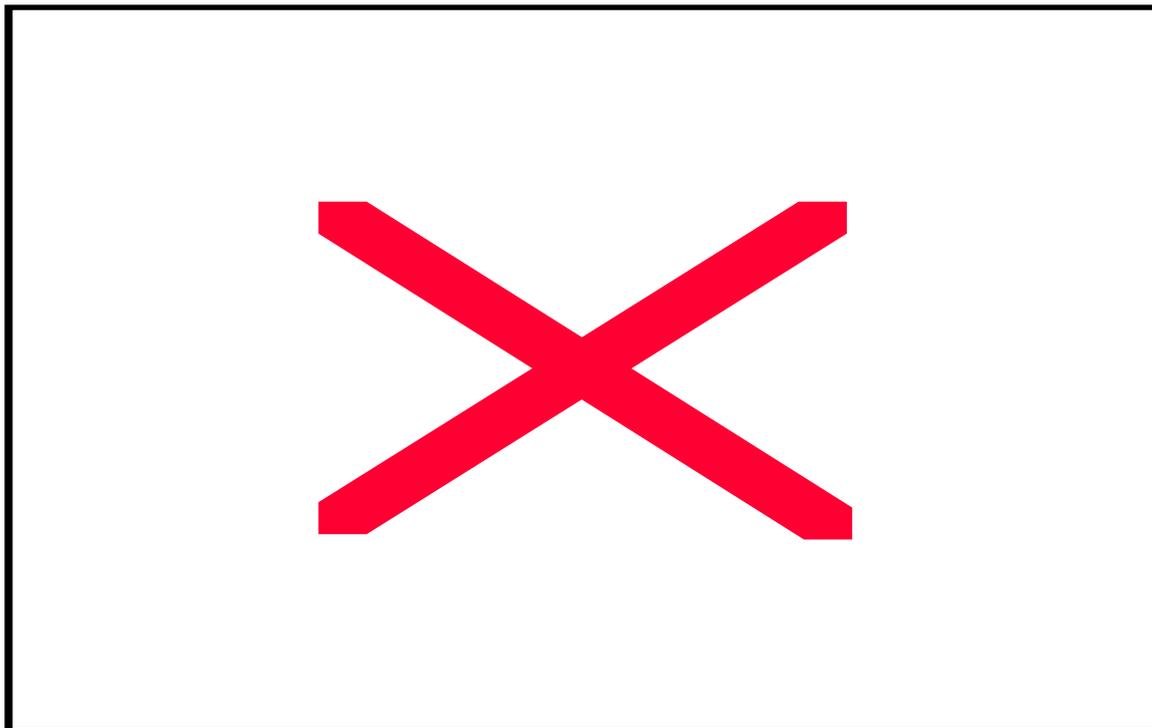


Fig. 1. Influence of thawing solution on AL and LW boars spermatozoa motility (%) until 1 h of incubation at 37°C.

Regarding the AL boars the Beltsville Thawing Solution (BTS) performed significantly better (Table 4), allowing for a higher sperm motility than the other studied solutions (from 30 minutes of incubation at 37°C, until the last observation we carried out 30 minutes later).

Table 4. Influence of thawing solutions on AL thawed sperm quality until 1 hour of incubation at 37°C (means ± sem)

Solutions	MOT 0'	MOT 15'	MOT 30'	MOT 45'	MOT 60'
SD	32.9 ± 1.1	38.5 ± 0.9	37.8 ± 1.0 ^a	33.0 ± 1.2 ^a	25.9 ± 1.3 ^a
BTS	33.0 ± 1.1	40.7 ± 0.9	42.4 ± 1.0 ^b	38.6 ± 1.2 ^b	32.5 ± 1.3 ^b
MR-A	34.7 ± 1.1	39.6 ± 0.9	40.2 ± 1.0 ^a	35.8 ± 1.2 ^a	28.7 ± 1.3 ^a
ACR	34.1 ± 1.1	40.5 ± 0.9	39.9 ± 1.0 ^a	35.2 ± 1.2 ^a	28.9 ± 1.3 ^a
SL	NS	NS	<i>P</i> <0.05	<i>P</i> <0.05	<i>P</i> <0.01

MOT 0': Spermatozoa motility (%) immediately after thawing (0 minutes)

MOT 15': Spermatozoa motility (%) at 15 minutes after thawing

MOT 30': Spermatozoa motility (%) at 30 minutes after thawing

MOT 45': Spermatozoa motility (%) at 45 minutes after thawing

MOT 60': Spermatozoa motility (%) at 60 minutes after thawing

SL: Significance level

The evaluation of the LW thawed sperm showed that there was also a BTS superiority, although it was only statistically significant on spermatozoa motility at 30 minutes after thawing (Table 5).

Table 5. Influence of thawing solutions on LW thawed sperm quality until 1 hour of incubation at 37°C (means ± sem)

Solutions	MOT 0'	MOT 15'	MOT 30'	MOT 45'	MOT 60'
SD	27.4 ± 1.9	31.0 ± 1.7	26.9 ± 1.8 ^a	17.6 ± 2.2	10.0 ± 2.4
BTS	30.5 ± 1.9	34.3 ± 1.7	31.7 ± 1.7 ^b	25.2 ± 2.1	15.6 ± 2.3
MR-A	31.4 ± 1.9	32.1 ± 1.7	26.1 ± 1.7 ^a	20.5 ± 2.1	13.1 ± 2.3
ACR	30.2 ± 1.9	30.9 ± 1.7	25.8 ± 1.7 ^a	18.9 ± 2.1	11.2 ± 2.3
SL	NS	NS	P<0.05	NS	NS

MOT 0': Spermatozoa motility (%) immediately after thawing (0 minutes)

MOT 15': Spermatozoa motility (%) at 15 minutes after thawing

MOT 30': Spermatozoa motility (%) at 30 minutes after thawing

MOT 45': Spermatozoa motility (%) at 45 minutes after thawing

MOT 60': Spermatozoa motility (%) at 60 minutes after thawing

SL: Significance level

The pregnancy rate of the *in vivo* experiment was 20%.

Discussion

The results of our experiments showed that AL boars always had a better post-thawing semen quality, compared with the LW boar, even when sperm quality before freezing/thawing procedures was similar. As we didn't find any bibliography concerning the cryopreservation of Alentejano swine breed semen, it appears that this particular breed has a good semen cryopreservation potential.

The mean percentage of spermatozoa motility on AL frozen semen, observed after a 30 minutes incubation period, was identical or superior to those observed by Bwanga *et al.* (1990) and Almid and Johnson (1988), 37.8% and 27%, respectively. Large-White thawed semen results are, in general, inferior to those observed by the same authors. Bwanga *et al.* (1990), also observed a motility decrease after 30 minutes of incubation at 37°C. A similar pattern was also observed in our experiments, as far as, AL thawed semen was concerned. However, the LW thawed semen showed an earlier decrease of spermatozoa motility, i.e., from 15 minutes of incubation onwards.

A seasonal effect on thawed semen quality was observed on the LW animal. Actually, post-thawing spermatozoa motilities during August and September were significantly inferior to those observed during July.

Martinat-Botté *et al.* (1986) and Hafez (1993), reported the sensibility of the boar to extreme temperature conditions. The total sperm cells per ejaculate, the % of alive spermatozoa, the motility and fertility rate have all been reported to decrease during high temperature periods. Our results may therefore mirror this particular relationship between the former variables and the environmental temperature boars were submitted to. Since daily temperatures during June, July and August tend to be very high in this region (ranging from 30 to 43°C), they may affect semen quality and its capability of being successfully subjected to freezing processes. On this field, the slightly better performance of AL boars may result from a better adaptation of this swine breed to Alentejo climatic conditions.

The cryopreservation period had no effect on thawed semen quality, leading us to consider that, after freezing, the immersion time into liquid nitrogen doesn't affect the quality of semen. Thus allowing it to be preserved during an almost unlimited period of time without considerable depreciation to its quality.

In our experiment, better results were achieved by using BTS as thawing solution, particularly with AL frozen semen. Pursel and Johnson (1975), also reported the advantage of using BTS for semen thawing.

Although relatively low pregnancy and farrowing rates were observed on our experiment (20%), they were identical to the ones reported by Paquignon (1984).

Several advances on frozen/thawing procedures shall be achieved in order to improve reproductive parameters resulting from AI with preserved frozen semen to the point when they become identical to the ones obtained with fresh semen. Posterior experiments, e.g., changes to some techniques and packing to 0.5 ml straws have already lead us to significant increases on both rates (unpublished data).

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.

References

- Almid, T. and Johnson, L. (1988). Effects of glycerol concentration, equilibration time and temperature of glycerol addition on post-thaw viability of boar spermatozoa frozen in straws². *J. Anim. Sci.*, 66: 2899-2905.
- Bwanga, C.O., Braganca, M.M., Einarsson, S. and Rodríguez-Martínez, H. (1990). Cryopreservation of boar semen in mini- and maxi-straws. *J. Vet. Med.*, A37: 651-658.
- Hafez, E.S.E. (1993). *Reproduction in Farm Animals*. 6th Edition. 1 Vol. Hafez, E.S.E. (ed.). Lea & Febiger, Philadelphia.
- Martinat-Botté, F., Bosc, M. Paquignon, M. and Courot, M. (1986). Reproduction. In: *Le Porc et Son Elevage (Bases Scientifiques et Techniques)*. Editions Maloine, S.A., Paris, pp. 23-53.
- Paquignon, M. (1984). Semen technology in the pig. *Curr. Top. Vet. Med. Anim. Sci.*, 30: 202-218.
- Pursel, V.G. and Johnson, L.A. (1975). Freezing of boar spermatozoa. Fertilizing capacity with concentrated semen and a new thawing procedure. *J. Anim. Sci.*, 40: 99-102.
- Westendorf, P., Richter, L. and Treu, H. (1975). Zur tiefgefrierung von ebersperma: labor und besamungsergebnisse mit dem Hulsenberger pailletten verfahren. *Dtsch. Tieraztl. Wschr.*, 82: 261-267.