Livestock Science 154 (2013) 235–239

Contents lists available at SciVerse ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Distinct incubation for homologous *in vitro* spermatozoa binding on swine oocytes subjected to different storage conditions

C.D. Corcini [a,b,*], A.S. Varela Junior[d], E.C.S. Santos [a,b], S.M.M. Gheller [b], V.M. Danieli [b], R.M.R. Brizolara [c], B.S. Elert [c], A.D. Vieira [b], D.C. Bongalhardo [c], T. Lucia Jr.[b]

[a] *Centro de Biotecnologia, Universidade Federal de Pelotas, Campus Universitário Capão do Leão, 96010-900 Pelotas-RS, Brazil*
[b] *ReproPel, Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Universitário Capão do Leão, 96010-900 Pelotas-RS, Brazil*
[c] *Instituto de Biologia, Universidade Federal de Pelotas, Campus Universitário Capão do Leão, 96010-900 Pelotas-RS, Brazil*
[d] *Instituto de Ciências Biológicas, Universidade Federal do Rio Grande, 96201-900 Rio Grande-RS, Brazil*

ARTICLE INFO

Article history:
Received 26 October 2012
Received in revised form
10 January 2013
Accepted 1 March 2013

Keywords:
In vitro penetration
Oocytes
Swine sperm
mTBM
BTS
Frozen ovaries

ABSTRACT

The sperm *in vitro* binding assay in homologous oocytes can be used to estimate the boar fertility potential, but its usefulness may be limited by laboratorial structure and oocytes availability. This study aimed at determining the effect of distinct methods of oocytes conditioning and incubation media for the *in vitro* penetration (IVP) test. Oocytes used in the IVP test were: fresh and conditioned in PBS (T1); cooled and conditioned in PBS at 5 °C for 48 h (T2); or stored in ovaries frozen at −20 °C (T3). For each treatment, two incubation media were tested at 39 °C for 6 h: modified TRIS buffer medium (mTBM); or Beltsville Thawing Solution (BTS) extender. The responses of interest were: IVP and polyspermy rates; and the number of penetrating spermatozoa per oocyte. All responses observed with incubation in BTS were inferior to those observed with incubation in mTBM ($P < 0.0001$). When incubation was done in mTBM, none of the responses differed across treatments ($P > 0.05$). However, when incubation was in BTS, all the three responses were superior for T1 than for T2 and T3 ($P < 0.05$). Thus, the IVP test may be conducted with ovaries either cooled or recovered from frozen ovaries with results similar to those observed with fresh oocytes, if incubation is done in mTBM.

1. Introduction

Assays that evaluate some stages of the fertilization process *in vitro*, such as sperm binding to the zona pellucida (Fazeli et al., 1995; Ivanova and Mollova, 1993) and *in vitro* fertilization (Martinez et al., 1993; Peláez et al., 2006; Sellés et al., 2003; Xu et al., 1998) have been used to estimate potential boar fertility. Other such assays evaluates the *in vitro* penetration (IVP) of swine oocytes by either homologous or heterologous spermatozoa (Corcini et al., 2012a; Gadea et al., 1998; Matás et al., 1996). The IVP test detected reduction in the penetrating capacity of boar sperm in swine oocytes only after 24 h of storage, although sperm quality was considered acceptable after 72 h of storage, according to conventional evaluation methods (Macedo et al., 2006). However, the IVP test is sensitive to individual variation among the boars used as sperm donors (Macedo et al., 2010; Popwell and Flowers, 2004).

* Corresponding author at: ReproPel, Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Universitário Capão do Leão, 96010-900 Pelotas-RS, Brazil. Tel./fax: +55 53 3275 9004.
E-mail address: corcinicd@gmail.com (C.D. Corcini).

1871-1413
http://dx.doi.org/10.1016/j.livsci.2013.03.002

The IVP test is commonly more effective using freshly recovered oocytes and standard CO_2 sperm co-incubation. However, this generates logistic and financial constraints that limit the application of such assay in routine conditions, especially due to difficulties related to short-term oocytes preservation. Eventually, CO_2 incubators may be replaced by alternative incubation systems (Corcini et al., 2012a; Macedo et al., 2006, 2010) and vitrified oocytes can be used instead of fresh oocytes (Macedo et al., 2006, 2010). Nevertheless, the zona pellucida of vitrified oocytes may be altered, becoming non-penetrable by spermatozoa (Lynham and Harrison, 1998). Thus, testing alternative methods of preserving oocytes for IVP assay would be justifiable. Cooling oocytes at 5 °C for up to 7 days may enable fertilization and pronucleus formation, as observed for mouse oocytes in contact with homologous spermatozoa (Tsuchiya et al., 2001). Additionally, bovine oocytes recovered from frozen ovaries can be penetrated *in vitro* by bull spermatozoa, but their IVP rates were lower than those observed for fresh oocytes *in vitro* matured (Tatemoto et al., 1994). However, cooled swine oocytes and oocytes obtained from frozen ovaries were not yet tested for use in IVP tests.

The modified TRIS buffer medium (mTBM) has been successfully used as the incubation medium for IVP of boar spermatozoa in homologous oocytes (Abeydeera and Day, 1997). Nonetheless, mTBM requires pH adjustment to 7.0 before the IVP test and also includes some chemicals commonly unavailable in artificial insemination (AI) studs. Thus, using the Beltsville Thawing Solution (BTS) (Pursel and Johnson, 1975) as the incubation medium may contribute to make the IVP test more feasible and cost-effective because BTS is the most commonly used extender for swine sperm cooled at 15–18 °C and is available in most swine AI studs.

This study evaluated the IVP of boar spermatozoa in homologous oocytes that were either fresh, cooled or recovered from frozen ovaries, testing mTBM and BTS as incubation media.

2. Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solutions were prepared using Milli-Q Synthesis System ultrapure water (Millipore, Bedford, MA, USA).

Sperm donors were sexually mature boars which were under routine semen collection in a commercial AI stud. Over 14 weeks, ejaculates from four boars (e.g., A, B, C and D) were collected each week through the gloved hand method, in plastic bags with a disposable filter within a plastic bottle. Immediately after collection, the ejaculates were extended in BTS (Pursel and Johnson, 1975) and the sperm concentration was adjusted to 3.5×10^9 spermatozoa in 100 ml, using a Neubauer chamber. Sperm samples were cooled at 17 °C and transported to the ReproPel laboratory for further processing, which occurred only for ejaculates presenting sperm motility of at least 70% and normal sperm morphology of at least 85%. All those evaluations were conducted by the same trained technician, using phase contrast microscopy. Thereafter, within an interval of at most 12 h after collection, ejaculates were combined in six distinct pooled samples. Each sample included sperm from two of the four boars, so sperm from each boar was present in three samples (e.g., A–B, A–C, A–D, B–C, B–D and C–D). After 2 weeks, the boars used as sperm donors were replaced by other four boars, but the composition of the pooled sperm samples followed the same structure. Therefore, after 14 weeks, 56 ejaculates from 28 boars were processed, totaling 84 pooled ejaculates.

Oocytes harvested from prepubertal gilts slaughtered in a local abattoir were transported to the ReproPel laboratory, within 60 min, in a saline/gentamicin solution (40 mg/ml) at 30 °C. The oocytes were allocated to three treatments: fresh oocytes, collected in the same day of the IVP test (T1); oocytes collected 48 h prior to the IVP test and stored at 5 °C in PBS (Nutricell, Campinas-SP, Brazil) (T2); and oocytes collected prior to the IVP test from ovaries frozen at −20 °C for 48 h (T3).

Subsequently, 3–6 mm follicles were aspirated using a 18G 1/½″ needle attached to a 10 ml syringe. The aspirate was placed into 15 ml tubes, the contents allowed to settle during 30 min and the sediment was placed into a 35 mm Petri dish. The oocytes having cumulus cell masses and zona pellucida were selected using stereomicroscopy and the *cumulus oophorus* cells were mechanically removed with a 200 μL micropipette. Twenty oocytes were allocated to each sperm pool, within each treatment, representing a total of 10,800 oocytes (1800 per treatment).

The IVP test was conducted following the protocol described by Abeydeera and Day (1997), with modifications. Two fertilization media were tested: mTBM and BTS. Both media received the inclusion of: 0.4% BSA; 2.5 mM caffeine; and 1.1 μg/mL of calcium lactate, to promote sperm capacitation. Spermatozoa were centrifuged at $900 \times g$ for 3 min and re-suspended in the fecundation media. The sperm concentration used in fertilization drop was 1×10^6/mL, as determined with a Neubauer chamber. The gametes were incubated in water bath at 39 °C for 6 h. Then, the oocytes were recovered, washed and stained with 10 μg/mL Hoechst 33342 to be evaluated in an epifluorescent microscope (Olympus BX 51) at 400 ×. Oocytes were considered penetrated when their zona pellucida contained one or more spermatozoa, which might have elongated and swollen heads (Ivanova and Mollova, 1993). The IVP and polyspermy rates and the number of spermatozoa per oocyte were determined as described elsewhere (Macedo et al., 2006, 2010).

The effects of combinations of treatments and incubation media on the number of spermatozoa per oocyte were tested using the Kruskal–Wallis analysis of variance for non parametric data, whereas the effects on the IVP and polyspermy rates were tested using logistic regression. All statistical analyses were conducted with Statistix® (2008, Florida, USA).

3. Results

The total IVP rate observed in this study was 70.7%. The total polyspermy rate was 43.4%, with 1.8 ± 2.0 spermatozoa per oocyte.

With incubation in BTS, IVP and polyspermy rates (Table 1) and the number of spermatozoa per oocyte (Fig. 1) were inferior to those observed with incubation in mTBM for all treatments ($P < 0.0001$). The IVP and polyspermy rates observed with mTBM were greater than 80% and 70%, respectively, whereas both rates were at least 10% points lower with BTS.

When incubation was done in mTBM, the IVP and polyspermy rates (Table 1) and for the number of spermatozoa per oocyte (Fig. 1) were similar ($P > 0.05$). However, with incubation in BTS, the IVP rate for T1 was greater than for both T2 and T3 ($P < 0.001$) by nearly 15% points (Table 1). Likewise, in the BTS medium, the polyspermy rate for T1 was nearly 10% points greater ($P < 0.0001$) than for both T2 and T3 (Table 1). The number of spermatozoa per oocyte was almost twice greater in T1 than with both T2 and T3 ($P < 0.0001$), when incubation occurred in BTS (Fig. 1). Considering all responses, no differences were observed between T2 and T3 ($P > 0.05$).

4. Discussion

This is the first study using swine oocytes that were either cooled or recovered from frozen ovaries for IVP test. Using this strategy, similar responses to those obtained from freshly recovered oocytes were observed when incubation was performed in mTBM. Bovine oocytes harvested from ovaries frozen for up to 3 months have been used in the IVP test, with IVP rate inferior to that observed with fresh oocytes matured *in vitro* (Tatemoto et al., 1994), which was observed in the present study only with incubation in BTS. The use of cooled oocytes may contribute to simplify the IVP test, since it would not need to be executed immediately after oocytes harvesting. Vitrified oocytes may be used for the IVP test (Macedo et al., 2006, 2010), but the use of oocytes recovered from frozen ovaries prevents limitations related to the long-term storage of vitrified oocytes in hyper saturated solutions that may alter their penetrability by spermatozoa, which reduces both the IVP rate and the number of spermatozoa per oocyte (Lynham and Harrison, 1998). Despite the recommendations to use cryoprotectant solutions in the IVP test to preserve oocytes structures (Holst et al., 2000; Macedo et al., 2006), no such solutions were used in the present study, which is in agreement with reports that more than one third of the human ovaries frozen at −20 °C without cryoprotectants presented follicles with integer oocytes that could be used for *in vitro* fertilization (Maltaris et al., 2006). Therefore, oocytes recovered from frozen ovaries may be stored as raw material to be used for the IVP test in larger scale and over longer periods than those currently used. The use of oocytes recovered from frozen ovaries has a great potential for further use in assays aimed to estimate potential male fertility *in vitro*, which should be determined by future research.

Table 1
Rates of *in vitro* penetration (IVP) and polyspermy for boar spermatozoa in homologous oocytes according to distinct methods of oocytes processing and incubation media.

Oocytes	Incubation medium	IVP (%)	Polyspermy (%)
Fresh	mTBM	83.0 (1494/1800)[a]	71.6 (1069/1494)[a]
	BTS	70.4 (1267/1800)[b]	52.8 (669/1267)[b]
Cooled (5 °C for 48 h)	mTBM	80.3 (1445/1800)[a]	72.2 (1044/1445)[a]
	BTS	53.3 (959/1800)[c]	43.1 (414/959)[c]
From frozen ovaries (−20 °C)	mTBM	84.0 (1512/1800)[a]	74.5 (1126/1512)[a]
	BTS	54.5 (981/1800)[c]	43.0 (422/981)[c]

[a] Rates presenting distinct superscripts differ by at least $P < 0.0001$.
[b] Rates presenting distinct superscripts differ by at least $P < 0.0001$.
[c] Rates presenting distinct superscripts differ by at least $P < 0.0001$.

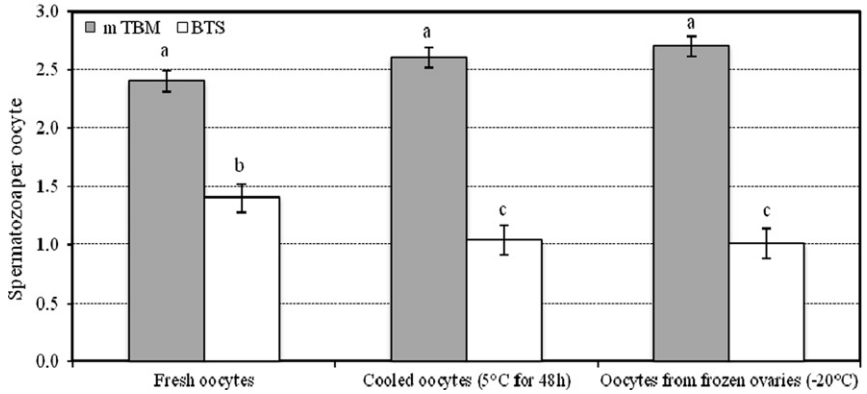

Fig. 1. Number of boar spermatozoa penetrating in homologous oocytes according to distinct methods of oocytes processing and incubation media. a,b,c Numbers presenting distinct supercripts differ by at least $P < 0.0001$.

The IVP rates observed with incubation in mTBM during 6 h were similar to those reported by Zhao et al. (2002), regardless of the method of oocytes conditioning, using a conventional CO_2 incubator. The results of the present study indicate that CO_2 incubators may be unnecessary for the IVP test, as also reported elsewhere (Corcini et al., 2012a; Macedo et al., 2006, 2010). The greater IVP and polyspermy rates and number of spermatozoa per oocyte observed with mTBM than with BTS may be attributed to the high calcium concentration in the mTBM, which originally contained 0.11% (or 7.5 mM) calcium chloride (Abeydeera and Day, 1997) and was further supplemented with 1.1 μg/mL calcium lactate, whereas BTS does not include calcium chloride (Pursel and Johnson, 1975). That high calcium concentration likely favored calcium influx, which is necessary for the acrosome reaction during incubation. Even though mTBM does not include sodium bicarbonate, which is present in BTS, such chemical does not influence the activation of the adenylate cyclase (Abeydeera and Day, 1997), which is responsible for the capacitation of swine spermatozoa. Nonetheless, sodium bicarbonate may have negatively influenced the evaluated responses by increasing sperm agglutination (Harayama et al., 1999), which may reduce the number of spermatozoa available for fertilization. Despite such findings, the IVP rate observed with incubation in BTS can be considered acceptable (Gadea et al., 1998). That may have resulted from the enrichment of BTS with BSA and caffeine (Gil et al., 2004), since BSA is responsible for thinning the plasma membrane of spermatozoa by removing cholesterol, whereas caffeine inhibits the phosphodiesterase, allowing an increase of cyclic AMP, which promotes sperm hyper-activation. The reduced IVP rate observed with BTS in both T2 and T3 may have occurred because of structural changes in the N-acetylglucosamine terminals due to the reaction between CO_2 and the medium, which alters the 55Kα and 55Kβ binding glycoproteins, preventing the correct spermatozoa–oocyte interaction (Berger et al., 1989).

In *in vitro* fertilization assays, when spermatozoa concentration is reduced, oocytes penetration by multiple spermatozoa decreases but overall penetration rates decrease as well (Abeydeera and Day, 1997). In the present study, with the same spermatozoa concentration for the two tested incubation media (1×10^6/mL), polyspermy was reduced with incubation in BTS, which justifies further research testing BTS as an alternative incubation medium. However, reduced polyspermy in swine may have negative effects for subsequent reproductive performance, since polyspermy in IVP assays (expressed by the number of accessory spermatozoa bound to the zona pellucida) has been positively correlated with total litter size of sows, when fecundation occurred *in vivo* (Braudmeier et al., 2004; Gadea et al., 1998). Nevertheless, in the study of Gadea et al. (1998), gametes were co-incubated for 16–18 h. Thus, if incubation is performed in BTS during the same period, it is possible that polyspermy and the number of spermatozoa per oocyte can be greater than those observed in the present study.

As also observed with other tests aimed to estimate fertility *in vitro*, the IVP test is prone to individual variation attributed to the boars used as sperm donors (Macedo et al., 2006, 2010; Popwell and Flowers, 2004). Therefore, some of the reported responses might vary if boars are evaluated individually. However, the effects of the methods of oocytes conditioning and incubation media observed in the present study were not influenced by the effect of the boars because potential individual effects were diluted due to the use of distinct pooled sperm samples each week. As a total of 28 boars were used as sperm donors, the responses observed in the present study considered a broader range of boars in comparison with studies aimed to estimate individual boar fertility, that commonly include only 3–5 boars (Corcini et al., 2012b; Peña et al., 2006; Saravia et al., 2009).

5. Conclusions

Swine oocytes that were either cooled or harvested from frozen ovaries can be penetrated *in vitro* by homologous spermatozoa as efficiently as freshly recovered oocytes, when incubated in mTBM. Although incubation can also be done in BTS, a medium widely available at swine AI studs, acceptable *in vitro* penetration rates can only be achieved using freshly recovered oocytes and responses are generally lower than those observed with incubation in mTBM.

Conflict of interest statement

There are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Acknowledgments

The authors thank the CAPES foundation for the scholarship given to the first author.

References

Abeydeera, L.R., Day, B.N., 1997. *In vitro* penetration on pig oocytes in a modified TRIS-buffered medium: effect of BSA, caffeine and calcium. Theriogenology 48, 537–544.

Berger, T., Davis, A., Wardrip, N.J., Hedrick, J.L., 1989. Sperm binding to the pig zona pellucida and inhibition of binding by solubilized components of the zona pellucida. J. Reprod. Fertil. 86, 559–565.

Braudmeier, A.G., Demers, J.M., Shanks, R.D., Miller, D.J., 2004. The relationship of porcine sperm zona-binding ability to fertility. J. Anim. Sci. 82, 452–458.

Corcini, C.D., Stephan, M.H.L., Colares, E.P., Santos, E.C.S., Varela Jr., A.S., Bongalhardo, D.C., Lucia Jr., T., 2012a. *In vitro* assays for vesper mice (*Calomys laucha*) sperm using heterologous substrates from non-rodent species. J. Exp. Zool. Part A Ecol. Gen. Physiol. 317, 96–102.

Corcini, C.D., Varela Jr., A.S., Pigozzo, R., Rambo, G., Goularte, K.L., Calderam, K., León, P.M.M., Bongalhardo, D.C., Lucia Jr., T., 2012b. Pre-freezing and post-thawing quality of boar sperm for distinct portions of the ejaculate and as a function of protein bands present in seminal plasma. Livst. Sci. 145, 28–33.

Fazeli, A.R., Holt, C., Steenweg, W., Bevers, M.M., Holt, W.V., Colenbrander, B., 1995. Development of a sperm hemizona binding assay for boar semen. Theriogenology 44, 17–27.

Gadea, J., Matás, C., Lucas, X., 1998. Prediction of porcine semen fertility by homologous *in vitro* penetration (hIVP) assay. Anim. Reprod. Sci. 56, 95–108.

Gil, M.A., Ruiz, M., Vazquez, J.M., Roca, J., Day, B.N., Martínez, E.A., 2004. Effect of short periods of sperm-oocyte co-incubation during *in vitro* fertilization on embryo development in pigs. Theriogenology 67, 620–626.

Harayama, H., Magargee, S.F., Kunze, E., Shidara, O., Iwamoto, E., Arikawa, S., Miyake, M., Kato, S., Hammerstedt, R.H., 1999. Changes in epididymal protein anti-agglutinin on ejaculated boar spermatozoa during capacitation *in vitro*. Reprod. Fertil. Dev. 11, 193–199.

Holst, B.S., Larsson, B., Linde-Forsberg, C., Rodríguez-Martínez, H., 2000. Sperm binding capacity and ultrastructure of the zona pellucida of stored canine oocytes. J. Reprod. Fertil. 119, 77–83.

Ivanova, M., Mollova, M., 1993. Zona-penetration *in vitro* test for evaluation boar sperm fertility. Theriogenology 40, 397–410.

Lynham, J.A., Harrison, R.A.P., 1998. Use of stored pig eggs to assess boar sperm fertilizing functions *in vitro*. Biol. Reprod. 58, 539–550.

Macedo Jr., M.C., Deschamps, J.C., Lucia Jr., T., Bordignon, J., Serret, C.G., Rambo, G., Pivato, I., Schmitt, E., 2006. *In vitro* penetration of fresh and vitrified swine oocytes by homologous spermatozoa using different incubation systems. Anim. Reprod. Sci. 92, 334–348.

Macedo Jr., M.C., Lucia Jr., T., Rambo, G., Ferreira Filho, E.B., Rosa, A.P., Fabiane, C., Cabral, M., Dechamps, J.C., 2010. *In vitro* penetration of swine oocytes by homologous spermatozoa: distinct systems for gamete's coincubation and oocyte's cryopreservation. Anim. Reprod. Sci. 117, 295–301.

Maltaris, T., Dragonas, C., Hoffmann, I., Mueller, A., Beckmann, M.W., Dittrich, R., 2006. Simple prediction of survival of follicles in cryopreserved human ovarian tissue. J. Reprod. Dev. 52, 557–582.

Martinez, E.A., Vázquez, J.M., Matas, C., Roca, J., Coy, P., Gadea, J., 1993. Evaluation of boar spermatozoa penetrating capacity using pig oocytes at the germinal vesicle stage. Theriogenology 40, 547–557.

Matás, C., Martinez, E.A., Vazquez, J.M., Rota, J., Gadea, J., 1996. *In vitro* penetration assay of boar sperm fertility: effect of various factors on the penetrability of immature pig oocytes. Theriogenology 46, 503–513.

Peláez, J., Breininger, E., Alegre, B., Peña, F.J., Domínguez, J.C., 2006. *In vitro* evaluation of the quality and fertilizing capacity of boar semen frozen in 0.25 ml straws. Reprod. Domest. Anim. 41, 153–161.

Peña, F.J., Saravia, F., Núñez-Martínez, I., Johannisson, A., Wallgren, M., Rodriguez-Martinez, H., 2006. Do different portions of the boar ejaculate vary in their ability to sustain cryopreservation? Anim. Reprod. Sci. 93, 101–113.

Popwell, J.M., Flowers, W.L., 2004. Variability in relationships between semen quality and estimates of *in vivo* and *in vitro* fertility in boars. Anim. Reprod. Sci. 81, 97–113.

Pursel, V.G., Johnson, L.A., 1975. Freezing of boar spermatozoa: freezing capacity with concentrated semen and a new thawing procedure J. Anim. Sci. 40, 99–102.

Saravia, F., Wallgren, M., Johannisson, A., Calvete, J.J., Sanz, L., Peña, F.J., Roca, J., Rodríguez-Martínez, H., 2009. Exposure to the seminal plasma of different portions of the boar ejaculate modulates the survival of spermatozoa cryopreserved in MiniFlatPacks. Theriogenology 71, 662–675.

Sellés, E., Gadea, J., Romar, R., Matás, C., Ruiz, S., 2003. Analysis of *in vitro* fertilizing capacity to evaluate the freezing procedures of boar semen and to predict the subsequent fertility. Reprod. Domest. Anim. 38, 66–72.

Statistix®, 2008. Statistix® 9 Analytical software. Tallahassee, FL, USA.

Tatemoto, H., Horiuhi, T., Maeda, T., Terada, T., Tsutsumi, Y., 1994. Penetration by bull spermatozoa into the zona pellucida of dead bovine oocytes recovered from frozen-thawed ovaries. Theriogenology 42, 465–474.

Tsuchiya, H., Ogonuki, N., Kuwana, T., Sankai, T., Kanayama, K., 2001. Short-term preservation of mouse oocytes at 5 °C. Exp. Anim. 50, 441–443.

Xu, X., Pommier, S., Arbov, T., Hutchings, B., Sotti, W., Foxcroft, G.R., 1998. *In vitro* maturation and fertilization techniques for assessment of semen quality and boar fertility. J. Anim. Sci. 76, 3079–3089.

Zhao, X.M., Songa, X.X., Kawai, Y., Niwa, K., 2002. Penetration *in vitro* of zona-free pig oocytes by homologous and heterologous spermatozoa. Theriogenology 58, 995–1006.