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In vitro penetration of swine oocytes by homologous spermatozoa: Distinct systems for gamete's co-incubation and oocyte's cryopreservation

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ABSTRACT

In vitro penetration (IVP) of swine oocytes by homologous spermatozoa was evaluated in two experiments using four boars as semen donors. In experiment 1, the IVP rate and the number of penetrating spermatozoa (PSP) were compared using three co-incubation systems for vitrified oocytes and fresh sperm: (1) 35 mL petri dishes in a CO_2 incubator, (2) 35 mL petri dishes in bags (submarine system) and (3) glass flasks partially submerged in water bath with the same gas mixture used for the bag system. Mean PSP was 8.2 ± 10.1 and the IVP rate was 90.5%. The PSP differed across all systems ($P = 0.0006$): 15.5 ± 0.5 for flasks, 6.3 ± 0.4 for CO_2, and 3.9 ± 0.4 for bags. The IVP rate for flasks (95.0%) was greater ($P = 0.01$) than for CO_2 and bags (90.8% and 85.0%, respectively), but it did not differ between flasks and CO_2 for three boars ($P > 0.05$). In experiment 2, co-incubation was done as described for glass flasks in experiment 1. The IVP rate and PSP were compared for cryopreserved oocytes: either vitrified in open pulled straws (OPS), or frozen in cryotubes. Mean PSP was 5.4 ± 6.5 and IVP rate was 89.6%. Both PSP and IVP rate were greater ($P < 0.0001$) for oocytes frozen in cryotubes (7.0 ± 0.3% and 95.8%, respectively) than those frozen in OPS (3.7 ± 0.3% and 83.4%, respectively), with no differences found for three boars ($P > 0.05$). In summary, successful IVP of swine oocytes by homologous spermatozoa can be achieved using gametes incubated in glass flasks and oocytes frozen in cryotubes.

1. Introduction

Due to their benefits for genetic improvement in swine, advanced artificial insemination (AI) programs require precise methods of estimating fertility for selection of boars with the greatest reproductive potential (Tardif et al., 1999). Conventional methods such as sperm motility, morphology and concentration are routinely used to estimate reproductive potential, but such measures do not always correlate closely with actual fertility (Barth, 1992). Thus, ejaculates classified as having high quality by such variables do not always achieve greater fertility rates (Linford et al., 1976; Xu et al., 1998). In commercial AI programs, lesser quality ejaculates are easily identified and eliminated while those used for AI have sperm concentrations that are greater than needed for conception, which reduces the probability of detecting differences in boar fertility (Gadea et al., 2004). Thus, boars of lesser fertility may be used in AI programs, which would impact herd reproductive performance by reducing the sow:boar ratio (Flowers and Esbenshade, 1993). The influence of individual boars may

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be further increased by using intra-uterine AI with minimal sperm concentrations and which results in an even lesser sow:boar ratio (Watson and Behan, 2002).

Methods that evaluate the capacity of spermatozoa to penetrate or fertilize oocytes *in vitro* have been used to estimate fertility (Gadea et al., 1998; Xu et al., 1998; Macedo et al., 2006; Sanchez-Ruiz et al., 2006). One such method, the *in vitro* penetration (IVP) test provides information about all stages of fertilization (Martínez et al., 1993) and detects, after only 24 h of sperm storage, a reduction in penetrating capacity not detected by conventional evaluation of sperm quality even after storage for 72 h (Macedo et al., 2006). The IVP test is more effective with freshly recovered oocytes than with vitrified oocytes and when co-incubation is done in a standard CO_2 incubator rather than in a closed bag submerged in a water bath (submarine) (Macedo et al., 2006). Thus, because the IVP test is both expensive and labor intensive further experiments are needed to improve its' efficiency before routine use.

The purpose of the present study was to examine two factors that influence the efficiency and simplicity of the IVP test: type of incubation system and oocyte source. In the first experiment we compared the IVP rate and number of penetrating spermatozoa (PSP) after co-incubation of spermatozoa from four boars with vitrified oocytes in either a standard CO_2 (5%) incubator, a closed bag (submarine) or a glass flask. In the second experiment, the PSP and IVP rate were determined following co-incubation with oocytes after either vitrification in open pulled straws or after cryopreservation in cryotubes.

2. Materials and methods

All chemicals used in the present study were purchased from Sigma–Aldrich Chemical Company (St. Louis, MI, USA), except TCM 199 (Gibco-Invitrogen, Grand Island, NY, USA), DPBS (Nutricell, Campinas-SP, Brazil) and SupercoolTM(21st Century Medicine, Rancho Cucamonga, CA, USA).

2.1. Experiment 1—comparison among three co-culture systems

2.1.1. Oocytes harvesting

Ovaries from prepubertal gilts were transported within 60 min from a local abattoir to the laboratory in saline + gentamicin (40 mg/ml) at 30 °C. Upon arrival, 3–6 mm follicles were aspirated using a 12 g × 40 mm needle attached to a 10 ml syringe. The aspirate was placed into 15 ml tubes, the contents allowed to settle and the sedimented portion was deposited into a 35 mm Petri dish. Oocytes with intact cumulus cell masses and zona pellucida were selected using stereomicroscopy and cumulus oophorus cells were mechanically removed with a 200 μL micropipette.

2.1.2. Oocytes vitrification and thawing

Oocytes were vitrified using a two-step protocol previously described for cryopreservation of swine morula (Bertholot et al., 2000; Macedo et al., 2006). The first vitrification solution (VS1) consisted of 1.4 M DMSO and 1.8 M ethylene glycol in DPBS + 0.4 mg/ml BSA. The second vitrification solution (VS2) included 2.8 M DMSO, 3.6 M ethylene glycol and 0.6 M sucrose. SupercoolTM (1%) was added to reduce toxicity and ice formation in both solutions (Wowk et al., 2000). After oocytes (10/group) were washed five times with DPBS + 0.4 mg/ml BSA, they were exposed to VS1 for three min, at room temperature. Then, over a 1 min period, oocytes were put into a 3 to 5 μL drop of VS2, loaded into OPS, also at room temperature (Bertholot et al., 2000) and subsequently exposed to LN_2 vapor for 3 s before immersion in liquid nitrogen (LN_2).

Oocytes in OPS were thawed by exposure to air for 5 s and the OPS tip was immersed in a solution of 0.5 M sucrose. Fingertip pressure on the opposite end of the straw was used to expel oocytes from the OPS tip. After 5 min, oocytes were placed in 0.25 M sucrose and 0.125 M sucrose for an additional 5 min in each solution. The sucrose solutions consisted of DPBS + 0.4 mg/ml BSA. Both vitrification and warming solutions were maintained at 39 °C.

2.1.3. Semen collection and dilution

Semen samples were collected by the gloved hand method from four F1 (Landrace × Large White) boars located on a commercial farm. All boars were considered to be fertile based on previous evaluation of semen quality by conventional methods. The AI dose consisted of 3×10^9 spermatozoa in 100 mL of Beltsville Thawing Solution (Pursel and Johnson, 1975) stored at 17 °C for at most 12 h.

2.1.4. Sperm washing procedures and oocyte insemination

Twelve micro-litre aliquots of diluted semen were centrifuged for one min at 60 × *g*. Then, 6 mL of supernatant were removed from each tube and added to 6 ml of washing solution (31.9 mg $CaCl_2 \cdot 2H_2O$, 99.7 mg penicillin, 76.6 mg streptomycin and 1 g BSA in 1 L of water) in a new tube. Subsequently, each sample was centrifuged 3× for three min at 1200 × *g*. After each centrifugation, supernatant was removed and the pellet re-suspended in 12 mL of washing solution. After the final spin, the pellet was re-suspended at a ratio of 1:1 in incubation medium (0.91 mM pyruvate, 5.5 mM glucose, 50 μg/mL penicillin, 75 μg/mL streptomycin sulphate and 1.1 μg/mL Ca lactate; pH 7.8). The suspension was incubated in a humidified atmosphere of 5% CO_2 in air at 38.5 °C for 40 min. The sperm washing procedures were adapted from Funahashi and Day (1993). After thawing, vitrified oocytes were thawed and co-incubated for 18 h with spermatozoa at a concentration of 1×10^6 viable sperm/mL. That sperm concentration was used for each group of 35 oocytes in all the three tested systems.

2.1.5. Co-culture systems

In the CO_2 incubator system, oocytes and spermatozoa were co-incubated in a standard culture incubator with an 'open' continuous flow gas atmosphere of humidified 5% CO_2 in air at 38.5 °C. Groups of 35 oocytes (35/group) were placed in 300 μL drops of IVP medium (0.91 mM sodium pyruvate, 5.5 mM glucose, 50 μg/mL penicillin, 75 μg/mL streptomycin sulphate, 1.1 μg/mL calcium

lactate, and 2 mM caffeine; Macedo et al., 2006) under oil in a 35 mm Petri dish.

The closed bag system is based on a procedure developed by Vajta et al. (1997b) for *in vitro* production of cattle embryos and adapted for the swine IVP test by Macedo et al. (2006). The system uses plastic bags with a volume of nearly 100 cm^3, produced by modifying the bags used to extend swine semen. Oocytes (35/group) were put in 300 μL of IVP medium and covered with 3.5 mL of mineral oil in 35 mm Petri dishes as described above. Then, the dish was placed into the bag which was then filled with a commercially available gas mixture consisting of 5% CO_2, 5% O_2 and 90% N_2, sealed with a plastic heat sealer and submerged to a depth of 10 cm in a water bath at 38.5 °C with continuous movement.

In the flask system, groups of 35 oocytes were immersed in 1 mL of IVP medium, covered with 1 mL of mineral oil, and placed inside a glass flask (32 mm diameter). After each flask received the same gas mixture as described for the bag system, it was sealed with a rubber stopper and placed in a water bath. The flask was submerged only up to the top level of the mineral oil.

2.2. Experiment 2—comparison among oocyte vitirification systems

2.2.1. Oocyte freezing, cryopreservation, thawing and insemination

The solution used for freezing oocytes in cryotubes (FS1) consisted of 0.7 M DMSO, 0.9 M ethylene glycol and 1% SupercoolTM. Thirty two previously denuded oocytes were put into a 7 μL drop of FS1 for 120 s and transferred into a cryotube. The lower half of the cryotube was immersed in liquid nitrogen. Then, 1.0 mL of fertilization medium and 1.2 mL of mineral oil were added and the cryotubes were sealed and completely immersed in LN_2. Cryotubes were thawed by submersion into a 60 °C water bath for 150 s. The procedure for OPS vitrification and thawing of oocytes were the same as was described for experiment 1. Both the freezing and the thawing solutions were maintained at 39 °C.

2.2.2. Evaluation of IVP rate and PSP

In both experiments, after 18 h of incubation, spermatozoa and oocytes were washed in 400 μL drops of DPBS with 0.4% BSA, and loosely attached spermatozoa were removed by vigorous pipetting (Ivanova and Mollova, 1993). Oocytes were subsequently placed into 50 μL drops of the same solution, in which they were stored at 5 °C for up to 7 days. An oocyte was considered to be penetrated when its zona pellucida contained at least one spermatozoon, some of which had elongated and swollen heads (Ivanova and Mollova, 1993; Macedo et al., 2006). The IVP rate was calculated as follows: (number of penetrated oocytes/total number of oocytes) × 100.

2.3. Statistical analyses

For all analyses, each oocyte was considered as an experimental unit. The number of PSP was compared across boars and incubation systems in experiment 1, and across boars and cryopreservation systems in experiment 2 by analysis of variance after square-root transformation, since it did not follow a normal distribution. Potential interactions were tested and comparisons of means were conducted using the LSD test. For the sake of interpretation, the results were expressed in the original scale.

In both experiments, the effects of boars and systems on the IVP rate were evaluated by chi-square tests. Interactions between those effects were tested through logistic regression, considering combinations of boars and systems as the reference levels for comparisons. All analyses were conducted using Statistix® software (2003).

3. Results

3.1. Experiment 1

The mean number of PSP/oocyte was 8.2 ± 10.1. The number of PSP was greater for the flask system than for the other systems and the CO_2 system was greater than the bag system ($P = 0.0006$, Table 1). Boar B had the greatest and boar C the least number of PSP ($P = 0.0006$), but boars A and D did not differ ($P > 0.05$). Due to a system by boar interaction, the number of PSP was greater for boar B than for the other boars in all systems, but boar B had a greater number of PSP in the flask system than in the other systems ($P < 0.0001$). For boar B, the number of PSP was least in the bag system, but it did not differ between the bag and CO_2 systems ($P > 0.05$). The other boars also had greater num-

Table 1
Number of penetrating spermatozoa per oocyte by incubation system and by boar in experiment 1 ($n = 937$ oocytes).*.

Boar	Incubation system						Total
	CO_2 incubator		Bag		Glass flask		
	Spermatozoa	*n*	Spermatozoa	*n*	Spermatozoa	*n*	
A	6.8 ± 0.8^{cd}	96	4.2 ± 1.0^{e}	59	14.9 ± 0.9^{b}	81	8.3 ± 0.5^{B}
B	$8.7 \pm 0.1.0^{c}$	61	7.0 ± 0.9^{cd}	84	26.6 ± 1.0^{a}	55	13.1 ± 0.6^{A}
C	3.6 ± 0.8^{e}	109	0.9 ± 0.9^{f}	70	10.2 ± 0.9^{bc}	74	5.0 ± 0.5^{C}
D	6.9 ± 0.9^{cd}	73	5.0 ± 0.8^{de}	109	9.8 ± 0.8^{c}	66	7.3 ± 0.5^{B}
Total	6.3 ± 0.4^{Y}	339	3.9 ± 0.4^{Z}	276	15.5 ± 0.5^{X}	322	8.2 ± 10.1

Means ± SEM having distinct superscripts (a, b, c, d, e, f) differ across boars and systems by $P < 0.0001$.
Means ± SEM having distinct superscriptsc (A, B, C) differ across boars by $P = 0.0006$.
Means ± SEM having distinct superscripts (X, Y, Z) differ across systems by $P = 0.0006$.
* Analysis of variance considered response variable after square root transformation.

Table 2
In vitro oocyte penetration rate (%) by incubation system and by boar in experiment 1 ($n = 937$ oocytes).

Boar	CO_2 incubator	Bag	Glass flask	Total
A	70.8	72.9	97.5	80.4[c]
B	100.0	96.4	98.2	98.2[a]
C	93.6	80.0	94.6	89.4[b]
D	98.6	90.8	90.9	93.4[b]
Total	90.8[A]	85.0[A]	95.0[B]	90.4

Frequencies having distinct superscripts (A, B) differ across systems by $P = 0.01$.
Frequencies having distinct superscripts (a, b, c) differ across boars by $P < 0.01$.

bers of PSP in the flask system, with the exception of boar D, which had similar numbers in both the CO_2 and flask systems ($P > 0.05$). Although boar C had the least overall number of PSP, specifically in the flask system, its number of PSP was similar to that of boars A and D ($P > 0.05$) and greater than those observed for itself and for other boars in the other tested systems, ($P < 0.05$).

For 937 oocytes, the IVP rate was 90.5% and the monospermy rate was 11.5%. The IVP rate was greatest for the flask system ($P = 0.01$), but it did not differ ($P > 0.05$) for the CO_2 and bag systems (Table 2). IVP rates did not differ for boars C and D ($P > 0.05$). Boar A had the least and boar B the greatest IVP rates ($P < 0.01$). The effect of the system by boar interaction on the IVP rate, with the CO_2 system considered to be the reference level (Table 3), indicated that, in comparison with boar A, only boar B in the CO_2 system and both boars A and C in the bag system presented similar IVP rates ($P > 0.05$). However, no boar per system combination presented an IVP rate that was different from boar B in the CO_2 system ($P > 0.05$). In comparison with either boar C or boar D, distinct rates were observed only for boar A in both the CO_2 and bag systems and for boar C in the bag system ($P < 0.05$). When boar A in the bag system was used as a reference (Table 4), similar rates were observed for boars A and B in the CO_2 system and for boar C in the bag system ($P > 0.05$). When boar B was the reference, only boar A in both the CO_2 and bag systems and boar C in the bag system had distinct rates ($P < 0.0.5$). When boar C was the reference, similar rates occurred for boars A and B in the CO_2 system and for boar D in the flask system ($P > 0.05$). When boar D was the reference, the only distinct rates were for boar A in both the bag and CO_2 systems and boar C in the bag system ($P < 0.05$).

3.2. Experiment 2

The mean PSP/oocyte was 5.4 ± 6.5. The number of PSP was greater for boar A than for the other boars and less

Table 3
Effect of an incubation system by boar interaction on *in vitro* oocyte penetration rate, considering the CO_2 incubator as the reference incubation system in experiment 1 ($n = 937$ oocytes).[*].

Treatment		Boar A	Boar B	Boar C	Boar D
CO_2 incubator	Boar A	–	0.5269	<0.0001	0.0010
	Boar B	0.5269	–	0.6335	0.6816
	Boar C	<0.0001	0.6335	–	0.3712
	Boar D	0.0010	0.6816	0.3712	–
Bag	Boar A	0.7837	0.5313	0.0001	0.0017
	Boar B	0.0001	0.6348	0.9711	0.4002
	Boar C	0.1819	0.5485	0.0014	0.0059
	Boar D	0.0005	0.5887	0.1087	0.0613
Glass flask	Boar A	0.0002	0.6528	0.6419	0.6270
	Boar B	0.0027	0.6679	0.5234	0.8401
	Boar C	0.0004	0.6147	0.5753	0.2109
	Boar D	0.0034	0.5892	0.1470	0.0712

[*] Numbers in cells are the *P* values from logistic regression models.

Table 4
Effect of an incubation system by boar interaction on *in vitro* oocyte penetration rate, considering Bag as the reference incubation system in experiment 1 ($n = 937$ oocytes).[*].

Treatment		Boar A	Boar B	Boar C	Boar D
CO_2 incubator	Boar A	0.7837	0.0001	0.1819	0.0005
	Boar B	0.5313	0.6348	0.5485	0.5887
	Boar C	0.0001	0.9711	0.0014	0.1087
	Boar D	0.0017	0.4002	0.0059	0.0613
Bag	Boar A	–	0.0004	0.3418	0.0032
	Boar B	0.0004	–	0.0038	0.1372
	Boar C	0.3418	0.0038	–	0.0424
	Boar D	0.0032	0.1372	0.0424	–
Glass flask	Boar A	0.0005	0.6813	0.0032	0.0795
	Boar B	0.0043	0.5529	0.0134	0.1103
	Boar C	0.0015	0.5787	0.0131	0.3518
	Boar D	0.0113	0.1721	0.0793	0.9852

[*] Numbers in cells are the *P* values from logistic regression models.

Table 5
Number of penetrating spermatozoa per oocyte by freezing system and by boar in experiment 2.*.

Boar	OPS		Cryotube		Total
	n	Spermatozoa	*n*	Spermatozoa	
A	73	6.2 ± 0.6^{b}	59	14.7 ± 0.7^{a}	10.5 ± 0.5^{A}
B	43	4.0 ± 0.8^{bc}	39	7.3 ± 0.9^{b}	5.6 ± 0.6^{B}
C	68	3.9 ± 0.7^{c}	86	5.2 ± 0.6^{bc}	4.5 ± 0.4^{BC}
D	74	3.1 ± 0.6^{c}	88	3.8 ± 0.6^{c}	3.5 ± 0.4^{C}
Total	258	3.7 ± 0.3^{X}	272	7.0 ± 0.3^{Y}	5.4 ± 6.5

Means ± SEM having distinct superscripts (a, b, c) differ across boars and systems by $P < 0.0001$.
Means ± SEM having distinct superscripts (X, Y) differ across systems by $P < 0.0001$.
Means ± SEM having distinct superscripts (A, B, C)differ across boars by $P < 0.0001$.
* Analysis of variance considered variable after square root transformation.

for boar D than for boar B ($P < 0.0001$) (Table 5). The number of PSP was greater for oocytes frozen in cryotubes than for those vitrified by OPS ($P < 0.0001$). Due to a system by boar interaction, a greater number of PSP was observed for oocytes in cryotubes only for boar A ($P > 0.0001$), with no differences observed for the other boars ($P > 0.05$). Overall, IVP rate ($n = 530$ oocytes) was 89.6% and the monospermy rate was 13.8%. The IVP rate was greater ($P < 0.0001$) for oocytes in cryotubes than for those in OPS (Table 6). The IVP rate was greater for boar A than for boars B and C ($P = 0.02$), but no other differences were observed among boars ($P > 0.05$). Considering a system by boar interaction (Table 7), when boar A was the reference, distinct rates occurred only for boars B and C in OPS ($P < 0.05$).

When boar B was the reference, the only similar rates were for boar C in OPS and for boars B and D in cryotubes ($P > 0.05$). In comparison with boar C in OPS, the only similar rates were for boar B in both OPS and cryotubes ($P > 0.05$). When boar D was the reference, different rates occurred for boars B and C in OPS ($P < 0.05$).

4. Discussion

The greater IVP rates with the flask system than with either the CO_2 or the bag systems represents a novel finding, because no previous study reported the use of glass flasks for gamete co-culture in the IVP test as tested in the present study, including the fertilization medium and the mineral oil together. Considering the system by boar interaction, boar A had the least overall IVP rate, but had a greater rate in the flask system than in either the CO_2 or the bag systems. Also, the overall number of PSP was greater in the flask than in the other systems for three of four boars, including boar C, which had the least number of PSP across boars and a greater IVP rate in the flask system than in the bag system. Thus, sperm from all four boars generally performed better in the flask system, even without performing as well in the other systems. Overall, boar B had the greatest number of PSP and the greatest IVP rate. Of the three systems, boar B had the greatest PSP number in flasks, but his IVP rate was greater in all systems.

Table 6
In vitro oocyte penetration rate (%) by oocyte freezing system and by boar in experiment 2 ($n = 530$ oocytes).

Boar	OPS	Cryotube	Total
A	94.5	98.3	96.4^{a}
B	76.7	100.0	88.3^{b}
C	70.6	95.3	82.9^{b}
D	91.9	89.8	90.8^{ab}
Total	83.4^{A}	95.8^{B}	89.6

Frequencies having distinct superscripts (A, B) differ across systems by $P = 0.0001$.
Frequencies having distinct superscripts (a, b) differ across boars by $P < 0.02$.

Due to the 100% IVP rate observed in the CO_2 system, differences could not be detected in the logistic regression analysis in comparison with boar B in this system because the lack non-penetrated oocytes produced extremely large confidence intervals. As CO_2 incubators are expensive and the bag system did not produce completely satisfactory results in a previous study (Macedo et al., 2006), the flask system represents a feasible alternative because it is easier to perform and the lesser rate of gas exchange with the external environment reduces changes in pH of the fertilization medium (Macedo et al., 2006). In experiment 1, the overall number of PSP was highest for the flask system, possibly because the spermatozoa:oocyte ratio was greater than in the other systems, even though the number of spermatozoa per mL of fertilization medium was the same for all systems. Even though the overall number of PSP in the bag system was less than for the CO_2 system, results from the present study with the bag system were greater than those reported by Macedo et al. (2006). In the previous study, the poorer results with the bag system using vitrified oocytes were inferior to those obtained for the CO_2 system possibly because CO_2 was expired into the bags (Vajta et al., 1997b). In the present study, the efficiency of the bag system was improved by the use of a defined gas mixture (Olivier et al., 1998) which provided an environment similar to that of a CO_2 incubator, as indicated by the fact that the results observed within boars for the CO_2 and bag systems were similar. Similar results were seen in comparisons of the flask and CO_2 systems. Such findings indicate that both the bag and the flask systems can be used in the IVP test, but the flask system has the advantage of easier execution.

There are no previous descriptions of the use of cryotubes to freeze oocytes, as conducted in the present study, in which frozen oocytes, freezing solutions and mineral oil are all together, although Chian et al. (2004) used cryotubes to store oocytes of cattle vitrified in straws, obtaining greater survival rates. Cryotubes are not widely used because they are thicker-walled than 0.25 mL straws, which could slow the freezing process and reduce cell viability (Vajta et al., 1998a). In the present study, the results with the cryotubes were superior to those obtained with OPS, likely due to the simultaneous presence of the fertilization medium and mineral oil during co-culture of the gametes after the oocytes were thawed. Also, the use of cryotubes simplifies the execution of IVP test because there is no need to either prepare fertilization medium or to use a stereoscopic microscope to process oocytes on the day of the test. The higher number of PSP observed for cryotubes

Table 7
Effect of a freezing system by boar interaction on *in vitro* oocyte penetration rate considering OPS as the reference freezing system in experiment 2 ($n = 530$ oocytes).[*].

System		Boar A	Boar B	Boar C	Boar D
OPS	Boar A	–	0.0085	0.0007	0.5293
	Boar B	0.0085	–	0.4777	0.0271
	Boar C	0.0007	0.4777	–	0.0020
	Boar D	0.5293	0.0271	0.0020	–
Cryotube	Boar A	0.2841	0.0075	0.0023	0.1359
	Boar B	0.5630	0.4708	0.4540	0.5387
	Boar C	0.8120	0.0036	0.0002	0.3735
	Boar D	0.2783	0.0523	0.0033	0.6437

[*] Numbers in cells are the *P* values from logistic regression models.

than for OPS may be due to the presence of TCM 199 during freezing and thawing, which may aid in oocyte preservation due to the presence of amino acids (Bertholot et al., 2002).

The IVP test can be conducted with swine oocytes preserved in a solution of fetal bovine serum and 10% DMSO (Lynham and Harrison, 1998). The concentration of cryoprotectants used to freeze oocytes in experiment 2 was one-half of the cryoprotectant concentration of VS1 used in experiment 1. The use of cryoprotectant solutions with lesser concentrations of cryoprotectants allows cell dehydration and prevents cell damage due to osmotic shock which may be caused by vitrification solutions which contain higher concentrations of cryoprotectants (Mahmoudzadeh et al., 1995). Although greater concentrations of cryoprotectants may be used (Vajta et al., 1997a; Vajta et al., 1998b; Vajta et al., 1999; Isachenko et al., 1998; Macedo et al., 2006), the protocol used in the present study consisted of only one freezing solution, so oocytes were thawed directly in the fertilization medium, instead of passing through the multi-step re-hydration process usually used. Thus, no manipulation of oocytes during and after thawing was necessary. As the fertilization medium and both freezing and thawing solutions were kept together, the oocytes were exposed to the freezing solution in the cryotubes and were partially immersed in liquid nitrogen during at least 45 s to prevent dilution of the freezing solution. Furthermore, as the fertilization medium was put in the cryotubes with a 1000 μL micropipette, it slowly flowed down the internal surface, which was cooled by direct contact with the liquid nitrogen. So, the rapid freezing of the fertilization medium prevented temperature exchange with the freezing solution.

In conclusion, the IVP test for swine oocytes can be conducted using a flask or bag system that does not require the use of a CO_2 incubator for co-culture of gametes. Also, cryotubes can replace OPS for cryopreservation of oocytes to be used in the IVP test.

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