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Effect of two ram sperm capacitating media on acrosome reaction and zona-free hamster oocyte penetration test

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Efeito de dois meios de capacitação dos espermatozóides de carneiros na reação acrossômica e no teste de penetração em oócitos de hamster

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SUMMARY

An *in vitro* zona-free hamster oocyte penetration test was utilized in 24 trials in order to evaluate the capacitation media used for ram sperm. A pool of fresh semen was collected from three crossed breed rams. Two semen drops were washed by centrifugation and incubated in high ionic strength treatment (HIS) or in a defined medium with HEPES, on heat ewe serum and heparin. After the incubation to promote capacitation, simplified triple-stain technique was used to evaluate the spontaneous acrosome reaction of the capacitated sperm. Superovulation in 96 golden hamsters was induced by PMSG and hCG. The oocytes were treated with hyaluronidase and trypsin to remove, respectively, the cumulus cells and the zona pellucida. Oocytes and capacitated sperm were incubated during 3 hours for further penetration. Then, oocytes were fixed and stained, being evaluated under phase contrast microscope. No significant statistical difference ($p > 0.05$) was found between media, concerning the penetration rate of the capacitated sperm and between number of sperm viable with acrosome reaction after the capacitation treatment using two different media. It was concluded that both media utilized were effective in capacitating ram sperm.

UNITERMS: Capacitation; Spermatozoa; Rams; Oocytes; Hamsters.

INTRODUCTION

Zona-free golden hamster oocytes allow the entry of spermatozoa of many mammalian species provided the spermatozoa have completed capacitation and acrosome reaction[20]. The relative accessibility of hamster ova makes this approach an attractive alternative to a homologous fertilization system for assessing sperm performance[2].

Brackett *et al.*[1] utilized a short exposure (5 minutes) in a high ionic strength medium for bovine sperm capacitation, since this medium removes the decapacitation factors and the sperm surface proteins cover. Thompson; Cummins[18] also washed ram spermatozoa in a high ionic strength medium to accelerate the capacitation *in vitro*.

Several authors incubated ram spermatozoa in a medium containing on heat-inactivated ewe serum to promote sperm capacitation[4,5,6,7,11,16]. Others utilized a medium containing heparin to capacitate ram spermatozoa[13,15]. Capacitation is required for sperm to undergo an egg-induced acrosome reaction but does not involve a visible change in sperm morphology. Therefore most assays to detect capacitation involve evaluating the ability of sperm to acrosome react in response to a stimulus[12]. In fact, Williams *et al.*[19] found 50% acrosome reaction in ram semen after incubation in medium containing heparin and Thompson; Cummins[18] found 9.3% spermatozoa with acrosome reaction after treatment in high ionic strength medium. Also, Martín-Lunas *et al.*[10] found 10.51% spermatozoa acrosome reacted after caprine semen treatment with in heat inactivated ewe serum.

After bovine semen treatment in high ionic strength medium or in medium containing heparin, Schellander *et al.*[14] utilized zona-free hamster oocytes penetration and found 32% and 18.8% penetration rate, respectively, after treatment in high ionic strength medium and TALP medium containing heparin. Williams *et al.*[19] found a 53% penetration rate after ram semen treatment in medium containing heparin.

The present investigation was undertaken in order

to evaluate two ram sperm capacitation media by zona-free hamster penetration test, and to determine the acrosome reaction rate from capacitated spermatozoa, providing a useful capacitation technique for ovine *in vitro* fertilization.

MATERIAL AND METHOD

Sperm treatment

A pool of fresh semen was collected from three crossed breed rams. In a first procedure, two semen drops were washed twice in 4 ml of DM - defined medium[3] by centrifugation (5 min/ 300 g) to remove the seminal plasma and then incubated for 5 minutes at 38.5°C in a HIS - high ionic strength treatment[3]. After the supernatant was discarded, the material was incubated in a chemically defined medium during 2 hours in a CO_2 incubator at 38.5°C. In a second procedure, 2 semen drops were washed by centrifugation in 4 ml of a defined medium with HEPES. After the supernatant was discarded, the material was diluted in 1 ml defined medium containing on heat ewe serum (20%) and heparin (2.5 µg/ml), and incubated during two hours in a 5% CO_2 incubator at 38.5°C to promote the swim-up (separation of motile from unmotile sperm cells) and capacitation.

Sperm staining

Simplified triple-stain technique was used to evaluate the spontaneous acrosome reaction of the capacitated sperm.

The sperm suspensions from the swim-up were diluted with an equal volume of the defined medium containing 0.2% trypan blue, incubated at 37°C for 15 minutes, smeared on prewarmed glass slides, and air dried. The slides were rinsed in water and blotted. The smears on slides were fixed in 3% glutaraldehyde solution in 0.2M phosphate buffer at room temperature for 45 minutes, rinsed in water, and air dried. The smears were stained in 0.5% Bismark Brown solution in 30% ethanol at 40°C for 10 minutes, rinsed briefly in water, and air dried. Finally, the smears were stained with Rose Bengal to evaluate acrosomal status, following distinction of live cells from dead ones using trypan blue. After staining, the slides were examined at 1,000x under phase-contrast microscopy and spermatozoa were classified into the following four categories[17]:

1. live spermatozoa with acrosome reaction - light rose postacrosomal regions and white "acrosomal regions";
2. dead spermatozoa with normal acrosomes - blue postacrosomal regions;
3. dead spermatozoa with abnormal acrosomes (i.e.; degenerative acrosome reactions) - blue postacrosomal regions with white "acrosomal regions";
4. live spermatozoa with normal acrosomes - light rose postacrosomal regions and pink acrosomes.

Sperm penetration assay

Superovulation in 96 golden hamsters was induced by intraperitoneal injections of 35 UI PMSG, followed by a 35 UI hCG injection, 56 hours later. The animals were killed by cervical dislocation and the Fallopian tubes were removed, ripped and the oocytes were treated with TL-HEPES-PVA medium containing 1 mg/ml of hyaluronidase to remove the cumulus cells. The same medium containing 0.1% of trypsin was used to remove the zona pellucida.

Sperm cells recovered from swim-up technique was adjusted to the concentration of 1 x 10^6 cells/ml. Oocytes were placed in a fertilization dish, inseminated with 25 µl capacitated sperm, and incubated in CO_2 at 38.5°C during 3 hours under mineral oil, for further penetration. Then, oocytes were fixed by 0.1% glutharaldeyde for 6 minutes and by 100 µl acetic acid : ethanol 3:1, covered with mineral oil during 15 hours. The oocytes were stained with 1% acetic orcein in 40% acetic acid, being evaluated under phase contrast microscope. Penetrated oocytes were considered those showing the sperm head decondensation and sperm tail.

RESULTS

From 96 golden hamsters, 3,069 oocytes were collected, resulting in an average of 31.97 oocytes per female.

When HIS medium was used, a penetration rate of 35.22% was achieved, indicating that 355 oocytes of the 1,008 inseminated were found penetrated. On the other hand, 375 oocytes (37.31%) of the 1,005 inseminated were penetrated when DM-HEPES-on heat ewe serum-heparin was utilized. No significant statistical difference ($p > 0.05$) was found between media, concerning the penetration rate of the capacitated sperm (Tab. 1 and Fig. 1).

Results showed that after the capacitation in a high ionic strength treatment, 16.5% of the 1,000 observed sperm were viable with acrosome reaction and 80.2% were viable however intact. 13.8% of the 1,000 sperm treated with DM-HEPES-in heat ewe serum-heparin were viable with acrosome reaction and 83.7% were viable and intact. No significant statistical difference ($p > 0.05$) was found between those results (Tab. 2 and Fig. 2).

Table 1

Penetrated oocytes by sperm treated in medium HIS and DM-HEPES-on heat ewe serum-heparin (São Paulo, 1996).

Medium	nº utilized oocytes	nº penetrated oocytes	% penetrated oocytes
High ionic strengh treatment	1008	355	35.22
DM-HEPES-on heat ewe serum- heparin	1005	375	37.31

Table 2
Proportions (%) of live, dead, intact and acrosome reacted spermatozoa after capacitation, assessed by a simplified triple-stain technique (São Paulo, 1996).

Medium	Spermatozoa			
	live and acrosome reacted	live and intact	dead and acrosome degenerated	dead and intact
High ionic strengh treatment	16.5%	80.2%	0.2%	3.1%
DM-HEPES-on heat ewe serum-heparin	13.8%	83.7%	0	2.5%

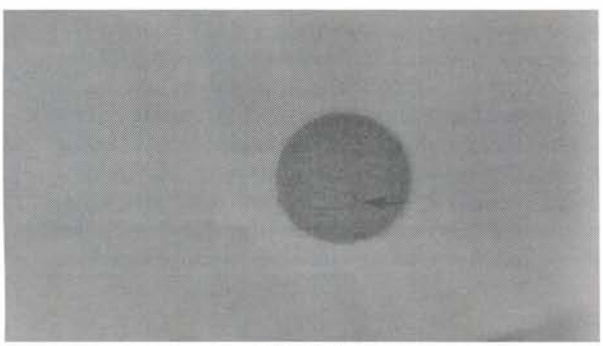

Figure 1
Ram spermatozoa penetration in zona-free hamster oocyte.

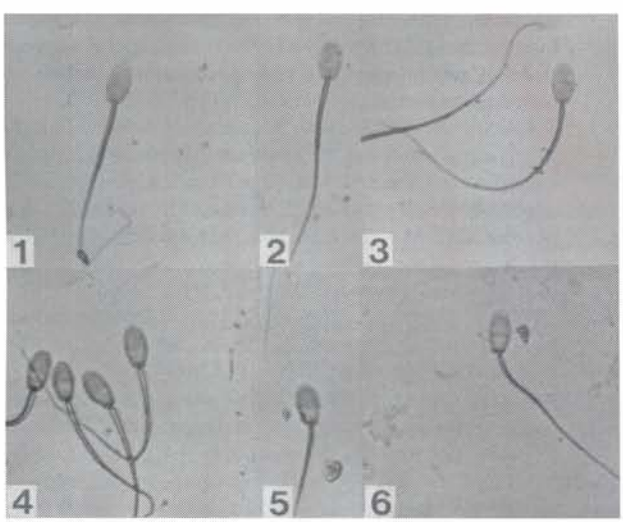

Figure 2
Spermatozoa stained by a simplified triple stain technique. 1. a live spermatozoon acrosome reacted; 2. a dead spermatozoon with an intact acrosome; 3. a dead spermatozoon with degenerative acrosome reaction; 4. live spermatozoa with intact acrosomes; 5 and 6. live spermatozoa with acrosome partially intact.

DISCUSSION

A pioneer study revealed that, after preincubation in an appropriate culture medium, sperm from fertile men were able to penetrate zona-free hamster oocytes[20]. This fact suggested that the sperm had undergone capacitation and acrosome reaction.

In the present study, we verified the ram sperm capacitation and acrosome reaction after incubation in two different culture media, high ionic strength treatment and defined medium-HEPES-on heat ewe serum-heparin.

The high ionic strength treatment was utilized by Brackett *et al.*[1] to induce the bovine sperm capacitation.

After that, Thompson; Cummins[18] utilized such treatment to observe the ram sperm acrosome reaction. Finally, the same treatment was utilized by Schellander *et al.*[14] to verify the bovine sperm penetration in zona-free hamster oocytes. Although this capacitation treatment is relatively unrelated, the results obtained by us are better than those obtained by the authors cited. Sperm acrosome reaction was observed after the high ionic strength treatment, followed by a triple stain technique (16.5% live spermatozoa acrosome reacted), a relatively higher result than 9.3% obtained by Thompson; Cummins[18].

After the high ionic strength treatment, the capacitated sperm penetrated 35.22% zona-free hamster oocytes. This result is similar to 32% penetration rate obtained by Schellander *et al.*[14].

In relation to defined medium plus HEPES, on heat ewe serum and heparin, it must be taken into consideration that some authors utilized defined medium containing 20% in heat ewe serum with 6 hours of incubation, like Martin-Lunas *et al.*[10]. Some other authors utilized 10 mg heparin with one hour of incubation[14] or two hours of incubation[19] to promote sperm capacitation.

In the present work we utilized both heparin and on heat ewe serum together in the same medium, with two hours of incubation. Next, spermatozoa were stained by a triple stain technique, and 13.8% was live and acrosome reacted. Working with caprine semen, Martin-Lunas *et al.*[10] obtained a similar result (10.51% live spermatozoa acrosome reacted), while Williams *et al.*[19] found a better result (50% live spermatozoa acrosome reacted).

After semen incubation in defined medium plus HEPES, on heat ewe serum and heparin, ram sperm penetrated 37.31% of zona-free hamster oocytes. Schellander *et al.*[14] found only 18.8% penetration rate while Williams *et al.*[19] reached better result (53% penetration rate).

Additional experiments are necessary to test the defined medium plus HEPES-on heat ewe serum and heparin with a variable sperm incubation time for capacitated ram sperm since it is known that heparin is a glycosaminoglycan

that induces changes in sperm during capacitation[8]. On the other hand, on heat ewe serum induces cholesterol efflux from sperm membrane[9] due to estrogen and albumin action.

CONCLUSION

Both media assayed (high ionic strength treatment and DM-HEPES-on heat ewe serum-heparin) were effective in capacitating ram sperm.

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RESUMO

Foram realizados 24 experimentos de penetração de espermatozóides de carneiros em oócitos *zona-free* de hamster com a finalidade de avaliar dois diferentes meios de capacitação espermática. Foi feito um "pool" do sêmen fresco de três carneiros mestiços, submetido à lavagem por centrifugação para retirada do plasma seminal e incubação em meio altamente iônico (HIS) ou em meio quimicamente definido acrescido de HEPES, soro de ovelha no cio e heparina, a fim de promover a capacitação dos espermatozóides. Foram utilizadas 96 hamsters fêmeas, superovuladas com os hormônios PMSG e hCG, que forneceram 3.069 oócitos, (média de 31,97 oócitos por fêmea). Os oócitos foram tratados em meio TL-HEPES-PVA com hialuronidase, a fim de retirar as células do cumulus. A zona pelúcida foi retirada através de lavagem dos oócitos em meio TL-HEPES-PVA com tripsina. Em seguida, os oócitos foram colocados em placas de fertilização, às quais se adicionaram os espermatozóides capacitados, permanecendo em estufa de CO_2 por 3 horas a 38,5°C, para promover a penetração. A seguir, procedeu-se à fixação e coloração dos oócitos. Foram realizados experimentos de coloração dos espermatozóides capacitados pela técnica de tripla coloração simplificada. Não houve diferença estatisticamente significativa ($p > 0,05$) entre os meios utilizados, no que diz respeito à taxa de penetração dos espermatozóides capacitados, assim como entre o número de espermatozóides viáveis com reação acrossômica após a capacitação em dois diferentes meios. Assim, ambos os meios utilizados, conjugados com o período de incubação, foram eficientes na capacitação dos espermatozóides de ovinos.

UNITERMOS: Capacitação; Espermatozóides; Carneiros; Oócitos; Hamsters.

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