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Hamster Zona Free Oocyte Sperm Penetration Test: A Bioassay to Test Ovine Sperm Capacitation

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ABSTRACT. *Zona-free hamster oocyte sperm penetration assay (SPA) is an efficient in vitro technique to assess sperm capacitation and fertilizability. Its use in assessing penetration rate of ram spermatozoa was studied. The eggs were cultured in Biggers, Whitting and Whittingham medium and sperm were prepared by swim-up technique using modified Brackett's defined medium. Seventy three percent (8 out of 11) of the ram sperm samples capacitated in heparin (10 µg/ml) showed signs of penetration. The sperm penetration rates of the oocytes incubated for 1.5, 3, 4 and 5 h were 6, 0, 16.7 and 10.5% respectively, indicating that the 4 h incubation was the optimum period for capacitated ram spermatozoa to penetrate hamster oocytes. From the results, it seems feasible to use this bioassay to assess sperm capacitation in the ram.*

INTRODUCTION

Zona-free hamster oocyte penetration assay (SPA) is the most commonly used method to evaluate sperm fertilizability in many mammalian species (Yanagimachi, 1984). Zona free hamster eggs are unique because they allow heterologous spermatozoa penetration. The cytoplasm of hamster eggs is translucent, and pronuclei and other signs of fertilization can easily be seen even without fixation and staining. The polar bodies neither degenerate nor become fragmented soon after fertilization unlike in some other species. Other advantages of SPA are the simplicity, use of simple culture media and the ability to use relatively low sperm concentrations (1-2x10^4 sperm/ml or about 100-200 sperm per egg). The latter prevents high incidence of polyspermic fertilization (Bavister, 1989). SPA can also be used in testing fertilization potential of spermatozoa stored for a prolonged period (Padilla *et al.* 1991). In this study SPA was used as a bioassay to check penetration rate of ram spermatozoa capacitated with heparin.

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MATERIALS AND METHODS

All glassware were first thoroughly washed with diluted 7x solution (Flow laboratories,USA), then washed five times in double distilled water and oven dried at 170°C for 30 to 45 min.

The egg culture medium used in this study was Biggers, Whitten and Whittingham (BWW) or modified Kreb's Ringer's solution (Hafez, 1987). The medium was sterilized by passing through 0.2 μm sterile Acrodisc filter (Gelman Sciences, (UK) and stored at 4°C in aliquots of 20 ml, until used. Before use, the aliquots were equilibrated overnight at 39°C in the CO_2 incubator, with 5% CO_2 in air.

Procedure for superovulation of golden hamsters (*Mesocricetus auratus*) and preparation of zona free ova were similar to the technique described by Hafez (1987). The cumulus cell masses released from the ampullary region of the oviducts were transferred to a droplet of BWW medium containing 0.1% hyaluronidase (Sigma, UK) at 37°C, to remove the cumulus cells. Cumulus free ova, were washed twice in a droplet of BWW medium and transferred into another droplet of BWW medium containing 0.1% trypsin (Sigma), to remove the zona pellucida. Healthy and good looking zona free ova were washed twice in a droplet of BWW medium and stored in 200 μl droplets of fresh BWW medium containing 3 mg/ml serum albumin pre saturated with 5% CO_2 in air under light mineral oil at 39°C, until used.

Semen was collected from a proven Polled Dorset x Malaysian local (Malin) crossbred ram using an artificial vagina. Six hundred μl of fresh semen was diluted with 8 ml PBS(+) solution. Phosphate buffered saline(+) solution was prepaired by dissolving 1 tablet of PBS in 180 ml of distilled water and mixing with 20 ml distilled water containing 0.026 g of $CaCl_2$. $2H_2O$ and 0.02g $MgCl_2$. $6H_2O$. The diluted semen sample was washed twice by centrifugation at 500 g for 5 min. The sperm pellet was washed once again with 8 ml DM-H-SS (Modified Brackett's defined medium, Brackett and Oliphant, 1975) solution. The supernatant was removed and 0.25 ml of the pellet was slowly layered under a tube containing 1 ml DM-H-SS using a 1 ml disposable syringe fitted to a 25 G needle. Four such tubes were prepared and the sperm were allowed to swim-up for 1 h at 39°C. The top 0.5 ml of the supernatant from the tubes containing highly motile spermatozoa were pooled and centrifuged at 500 g for 5 min. The sperm concentration of the pellet was counted and the final dilution was made with DM-Ca + 20% sheep serum (SS) to get $10x10^6$ motile sperm/ml. The sperm suspension for fertilization was prepared by adding 10 μg of heparin into 1 ml of sperm suspension. After pre-incubation for 15 min at 39°C under 5%

CO_2 in air, 200 μl fertilization droplets were prepared under light mineral oil and 8-10 ova/fertilization droplet were added. Ova and sperm were incubated at 39°C and 5% CO_2 in air under maximum humidity for 1.5 - 5 h. Incubated ova were washed twice with fresh BWW medium and mounted on glass slides. Ova were fixed with aceto-ethanol over night in an air tight container and stained with 0.3% aceto-lacmoid stain (Hafez, 1987).

RESULTS AND DISCUSSION

Presence of swollen (Hafez, 1987) or decondensed (Howard *et al.* 1988) sperm head/s with attached or closely associated sperm tail/s, (Figure 1) was the main criterion considered as a sign of penetration. Oocytes with male pronucleus/pronuclei and corresponding sperm tail/s (Hafez 1987), oocytes with 2nd polar body and at least with two pronuclei (Figure 2) with or without corresponding sperm tails (Niwa *et al.* 1988) were also considered as penetrated. Out of 11 sperm samples tested with 187 zona free oocytes, 8 (73%) showed signs of sperm penetration. The percentages of ova penetrated after different periods of incubation are shown in Table 1.

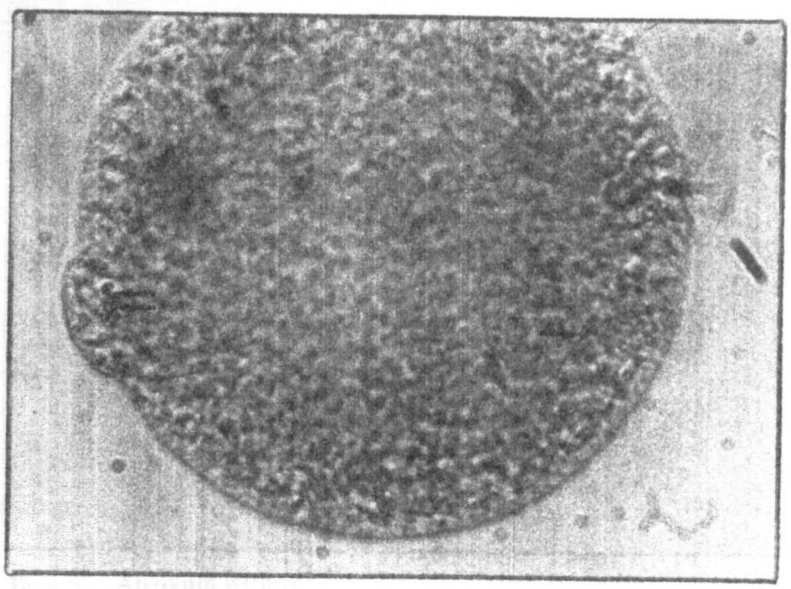

Figure 1. **An ovum with very closely associated spermatozoon. (arrow indicates the tail of the sperm). The oocyte is at the metaphase of 2nd meiotic division and chromatin condensation (two arrows) is clearly visible (x400).**

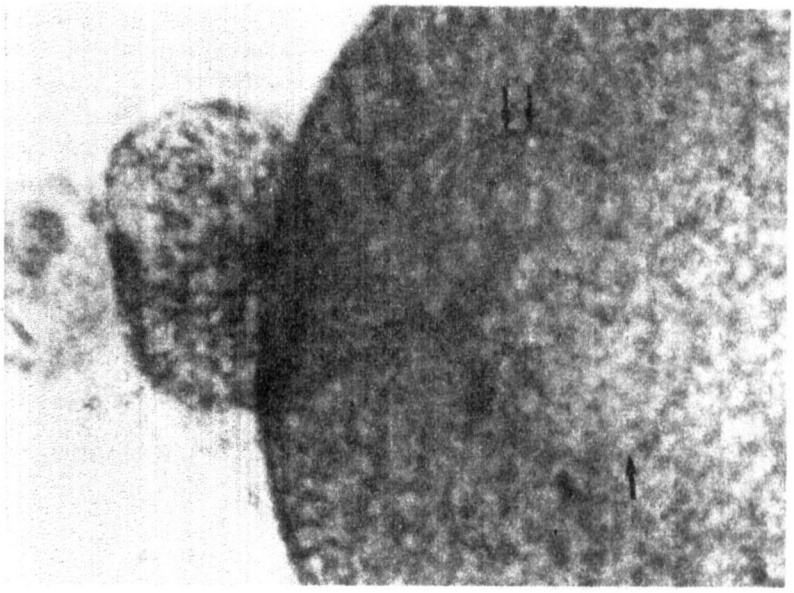

Figure 2. **An ovum with male (one arrow) and female (two arrows) pronuclei just before syngamy. Two polar bodies are clearly visible (x400).**

Table 1. **Number of ova used and percentage penetrated after different periods of incubation.**

No. of ova tested	Incubation period (h)	Average penetration rate (%)
15 (1)	1.5	6
13 (3)	3.0	0
102 (6)	4.0	16.7
57 (4)	5.0	10.5

(Numbers within parenthesis represent the number of experiments)

The maximum penetration rate of 16.7% was observed at 4h of incubation period. In the six experiments, the sperm penetration rate varied between 9.4-33.3%. Further incubation did not improve the penetration rate. A

shorter incubation period resulted in reduction of the percentage of penetrated eggs. The overall penetration rate of hamster oocytes in this experiment was lower than the penetration rates observed in other studies. Harayama *et al.* (1993) has observed penetration rates of 55 and 97% for boar spermatozoa collected from the rete testis incubated for 2 and 3h, respectively. Blue *et al.* (1989) reported oocyte penetration rates of 36 and 46% for stallion spermatozoa capacitated with calcium ionophore and lysophosphatidyl serine, respectively. According to the findings of Ramesha *et al.* (1993) hamster egg penetration rate was 40-95.5% with frozen buffalo sperm; and that reported by Tizol *et al.* (1990) in different culture media with bull sperm was between 37-75%.

Capacitated spermatozoa were very sticky and showed strong affinity to ova. In many instances they were heavily attached to the oocytes as shown in Figure 3. In the oocytes incubated for 3h this phenomenon was very prominent.

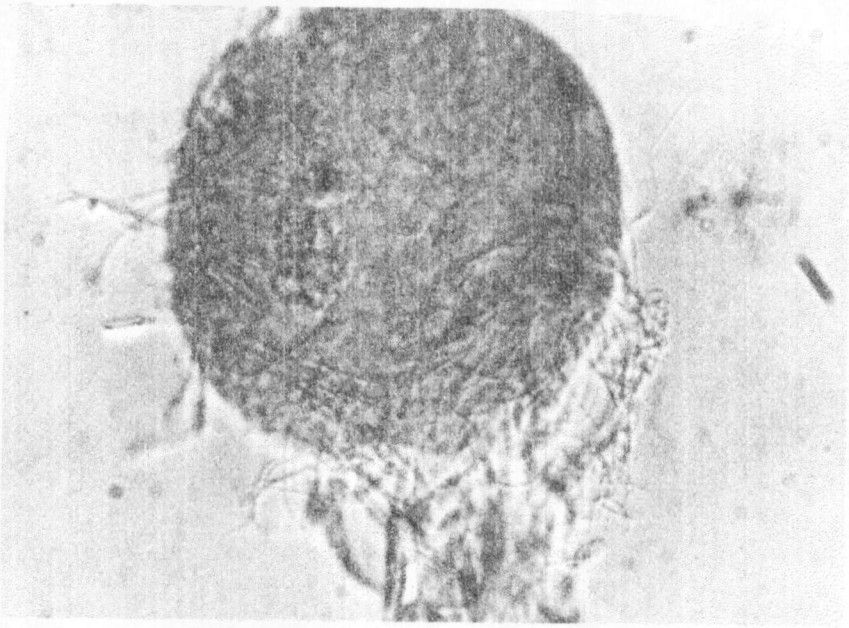

Figure 3. An ovum under differential interference contrast (x20). Note the clusters of spermatozoa attached to it.

It is possible that ram sperm capacitation is taking place at 3h of incubation. Examination of oocytes for sperm penetration was difficult due to heavy attachment of spermatozoa. This can be a reason for zero penetration rate in the

oocytes incubated for 3h and the overall lower penetration rates observed. It was very difficult to remove the spermatozoa by suction device. Eggs were fragile and sometimes got lost during the removal of attached spermatozoa by suction. This could have been avoided to a greater extent if lower sperm concentrations (100-200 sperm/egg) as suggested by Bavister (1989) were used in the fertilization droplets. Too low sperm concentrations are detrimental, because sperm survival is concentration dependent (Bavister, 1974); and only a minority of the total sperm population has the capacity to penetrate eggs (Bavister, 1986). According to Bavister (1989), when relative gamete ratio (sperm/egg x ml) was $10^{3.5}$ or greater, >90% of eggs were fertilized in vitro.

CONCLUSIONS

These results suggest that SPA can be used as a bioassay to check ram sperm capacitation. It appeared that 4h of incubation period was optimum for capacitated acrosome reacted ram spermatozoa to interact with zona free hamster oocytes. However, more information is required to confirm this finding. As no morphological changes are observed in capacitated spermatozoa (Roldan and Gomendio 1992) SPA can be successfully used in the studies of *in vitro* fertilization to test the efficiency of different capacitation agents and different media.

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REFERENCES

Bavister, B.D. (1974). The effect of variation in the culture conditions on the motility of hamster spermatozoa. J. Reprod. Fert. 38: 431-440.

Bavister, B.D. (1986). Animal *in vitro* fertilization and embryo development. pp 81-148, *In*: Gwatkin, R.B.L. (Ed.) Manipulation of mammalian development, Plenum Press, New York.

Bavister, B.D. (1989). A consistently successful procedure for *in vitro* fertilization of golden hamster eggs. Gam. Res. 23: 139-158.

Blue, B.J., McKinnon, A.O., Squires, E.L. Siedel, G.E. Jr. and Musca ri, K.T. (1989). Capacitation of stallion spermatozoa and fertilization of equine oocytes *in vitro*. Equine Embryo Transfer II. Equine Vet. J. Supp. 8:111-116.

Brackett,B.G. and Oliphant, G. (1975). Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod. 12: 260-274.

Hafez, E.S.E. (1987). Reproduction in farm animals, 5th edition, Lea and Febiger, Philadelphia.

Harayama, H., Kusunoki, H. and Kato, S. (1993). Capacity of rete testicular and cauda epididymal boar spermatozoa to undergo the acrosome reaction and subsequent fusion with egg plasma membrane. Mol. Rep. Dev. 35(1): 62-68.

Howard, J.G., Barone, M.A., Chingerman, K.J.,Bush, M. and Wildt, D,E. (1988). Influence of teratospermia, culture media and sperm swim-up technique on penetration of zona free hamster ova by domestic cat spermatozoa. Proc. 11th Int. Congr. Anim. Reprod. and A.I. Dublin.

Niwa, K., Ohgoda, O., Yuhara, M. (1988). Effects of caffeine in media for pretreatment of frozen thawed sperm on *in vitro* penetration of cattle oocytes. Proc. 11th Int. Congr. Anim. Reprod. and A.I. Dublin.

Padilla, A.M., Tobback, C. and Foote, R.H. (1991). Penetration of frozen thawed zona free hamster oocytes by fresh and slow-cooled stallion spermatozoa. J. Reprod. Fert. Supp. 44: 207-212.

Ramesha, K.P., Goswami, S.L. and Das, S.K. (1993). Zona free hamster egg penetration test for assessing fertility of Murrah buffalo (*Bubalus bubalis*) bulls. Buffalo J. 9(3): 259-263.

Roldan, E.R.S. and Gomendio, M. (1992). Morphological, functional and biochemical changes underlying the preparation and selection of fertilizing spermatozoa *in vivo*. Anim. Reprod. Sci. 28: 69-78.

Tizol, G., Faundez, R. and Olszewski A. (1990). The use of different culture media for the *in vitro* capacitation of bull spermatozoa. Medycyna- Weterynaryjna. 46(6): 200-202.

Yanagimachi, R. (1984). Zona free hamster eggs: Their use in assessing fertilizing capacity and examining chromosomes of human spermatozoa. Gam. Res. 10(87): 232.