EFFECT OF GLYCOSIDASE INHIBITORS ON THE CAPACITATION OF HAMSTER SPERMATOZOA BY CUMULUS CELLS *IN VITRO*

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Rapid transport of spermatozoa to the site of fertilization in the oviduct has been demonstrated in several species (Howe & Black, 1963; Yanagimachi & Chang, 1963; Harper, 1973). In the mouse, the uterotubal junction was shown to close a few minutes after mating (Zamboni, 1972). These findings suggest that, at least in some species, normal physiological capacitation of the spermatozoa takes place in the oviduct rather than in the uterus. Using hamster gametes *in vitro*, we earlier demonstrated that the cumulus oophorus and not the oviducal fluid is responsible for the capacitating action of the postovulatory contents of the oviduct (Gwatkin, Andersen & Hutchison, 1972).

When hamster spermatozoa are incubated with cumulus cells *in vitro*, the spermatozoa bind to a neuraminidase-sensitive site on the cell surface and detach spontaneously 3 to 4 hr later (Gwatkin *et al.*, 1972). In the presence of a dialysable factor from the cumulus matrix, spermatozoa are produced which are capable of binding to a trypsin-sensitive receptor on the zona pellucida (Hartmann & Gwatkin, 1971) and of entering eggs (Gwatkin *et al.*, 1972).

Since our earlier studies provided evidence for the existence of conjugated glucuronides on the surface of hamster spermatozoa (Gwatkin & Hutchison, 1971), the possible rôle of glycosidases which split these mucopolysaccharides was explored with enzyme inhibitors. Various concentrations of glucaro (1→4) lactone (GL), a specific inhibitor of β -glucuronidase structurally related to the sugar moiety in its substrate (Levy, 1952) were prepared in TC medium 199 (Morgan, Morton & Parker, 1950), modified to contain 30 μ g sodium pyruvate/ml, 3 mg crystalline bovine plasma albumin/ml and 2 mg sodium bicarbonate/ml. This medium was designated 199M2. Aliquots (10 μ l) of these solutions were mixed in droplets under mineral oil with an equal volume of cumulus cells (2×10^5 /ml) dispersed in solubilized matrix. This was prepared by placing the cumulus clots formed by twenty to thirty eggs in 100 μ l hyaluronidase solution (450 U.S.P. units/ml Dulbecco's phosphate-buffered saline, pH 7.2), counting the dispersed cells by haemocytometers and then diluting to the appropriate cell concentration. Epididymal sperm suspension (20 μ l containing 10^7 motile spermatozoa/ml) were added to the droplets and the cultures were incubated on a rocker (five to six oscillations/min) at 37° C under 5% CO₂ in air for 5 hr. Cumulus-free eggs were then added and sperm penetration into the eggs was scored 90 min later, as previously described (Gwatkin *et al.*, 1972).

Table 1 shows that GL in concentrations as low as 2 $\mu\text{g/ml}$ completely inhibited capacitation by the frozen-thawed cells. Inhibition of capacitation by the intact cells, however, required an inhibitor concentration which was more than fifty times greater. The results suggest that β -glucuronidase is involved in the capacitating action of the cumulus oophorus and that the enzyme acts where it is relatively inaccessible to the inhibitor, probably while the sperm-

Table 1. Effect of glucaro (1 $\rightarrow$ 4) lactone (purchased from Calbiochem. Corp.) on hamster sperm capacitation by cumulus before and after disruption of the cells by freezing and thawing

Inhibitor ($\mu\text{g/ml}$)	No. of ova* penetrated by spermatozoa			
	Frozen and thawed cumulus cells		Intact cumulus cells	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
0	10	11	15	15
2	0	0		
20	0	0	15	11
100			12	7
1000			0†	0†

* Number of ova out of a total of twenty ova.

† Glucose (2 mg/ml) did not affect capacitation, hence action of inhibitor at high concentration is not attributable to such non-specific effects as increased osmolarity.

Table 2. Effect of inhibitors of N-acetylglucosaminidase and hyaluronidase on hamster sperm capacitation *in vitro*

Inhibitor	Concentration ($\mu\text{g/ml}$)	No. of ova* penetrated by spermatozoa			
		Frozen and thawed cumulus cells		Intact cumulus cells	
		5×10^5 cells/ml	10^6 cells/ml	5×10^5 cells/ml	10^6 cells/ml
None	—	3	4	10	10
ADGL	3	2	4	9	9
	10	0	3	8	7
	30	0	0	3	4
	100	0	0	1	3
AATC	10	4	4	3	8
	30	5	5	1	6
	300	0	0	0	0

ADGL = 2-Acetamido-2-deoxygluconolactone (Koch-Light Labs.).

AATC = Ammonium aurin tricarboxylate (K & K Labs.).

* Number of ova out of a total of ten ova.

atozoa are bound to the cells. These results are in agreement with our previous report that GL at 5 $\mu\text{g/ml}$ blocks capacitation by crude β -glucuronidase solutions but does affect capacitation by the cumulus oophorus *in vitro* (Gwatkin & Hutchison, 1971).

Glucuronides, such as hyaluronic acid and chondroitin sulphate, are not attacked by purified β -glucuronidase since this enzyme cleaves only terminal

glucuronic acid groups (Levy & Conchie, 1966). Complete hydrolysis of hyaluronic acid is believed to require the sequential action of two more glycosidases, β -N-acetylglucosaminidase and hyaluronidase. Table 2 presents the results of experiments similar to those already described, but using 2-acetamido-2-deoxygluconolactone (ADGL), a specific competitive inhibitor of β -N-acetylglucosaminidase, and ammonium aurintricarboxylate (AATC), a relatively unspecific inhibitor of hyaluronidase which acts by competing for the polycationic activators of this enzyme (Mathews & Dorfman, 1955). At a cell concentration of 5×10^5 /ml, ADGL completely inhibited capacitation by the frozen and thawed cells at a concentration of $10 \mu\text{g}/\text{ml}$. With the intact cells, capacitation was inhibited but the inhibition was not complete, even at $100 \mu\text{g}/\text{ml}$. A similar difference between frozen and thawed cells was observed at a cell concentration of 10^6 /ml. These results suggest that β -N-acetylglucosaminidase is involved in cumulus cell-induced capacitation and that, like β -glucuronidase, it may act on the spermatozoa when they are bound to the cumulus cells. In contrast to these inhibitors, AATC was about equally effective in inhibiting capacitation by the frozen-thawed and intact cells, suggesting that, if hyaluronidase is involved, it acts in solution where it would be accessible to the inhibitor.

The high degree of enzyme specificity exhibited by the lactones, which derives from their close structural relationship to the respective sugars in their substrates, provides strong evidence that capacitation by the cumulus oophorus involves the glycosidases, β -glucuronidase and β -N-acetylglucosaminidase. The differential response of the frozen-thawed cells and intact cells suggests that these enzymes may act on the spermatozoa while they are bound to the cumulus cells. The nature of this association is currently being explored both *in vivo* and *in vitro* by transmission and scanning electron microscopy to determine whether the spermatozoa fuse with the cell surface in such a way as to allow lysosomal enzymes to act on the plasma membrane of the sperm head.

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