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Inhibitory Effect of Synthetic Phospholipid Vesicles Containing Cholesterol on the Fertilizing Ability of Rabbit Spermatozoa (39374)

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Investigations undertaken in this laboratory (1-3) indicate that two distinct classes of membrane vesicles are responsible for reversible inhibition of fertilizing ability in rabbit spermatozoa by seminal plasma from intact and vasectomized bucks (4, 5). Fertilization *in vitro* of eggs from superovulated immature rats by cauda epididymal sperm cells was also inhibited when vesicles isolated from rabbit seminal plasma were added to the medium (6). These findings are consistent with a direct, nonspecific action by seminal plasma vesicles on the sperm cell.

In the present investigation, the effect of vesicles composed of dipalmitoyl phosphatidylcholine (DPPC), dimyristoyl phosphatidylcholine (DMPC), and cholesterol have been examined on the fertilizing ability of uterine-capacitated rabbit spermatozoa.

Materials and methods. Lipid suspensions. Five lipids were employed in these experiments: cholesterol (Steraloids), β-γ-dipalmitoyl-DL-α-glycerol-phosphorylcholine (Sigma), β-γ-dimyristoyl-L-α-glycerol-phosphorylcholine (Sigma), phosphatidyl-L-serine (Nutritional Biochemical), and L-α-glycerol-phosphorylcholine (Sigma). Purity in these preparations was established, prior to use, by thin-layer chromatography on silica gel plates that were developed first with chloroform, methanol, and water (65:25:4) and then petroleum ether, ethyl ether, and acetic acid (80:20:1) (7). Mixtures of phospholipid and cholesterol were prepared by dissolution in benzene, or chloroform, and subsequent lyophilization to remove the organic solvent. The lipids were suspended (50 mg/ml) in 1 ml of Hanks solution (Difco), modified to contain 3.8 m*M* Ca^{2+} (8), and ultrasonic irradiation was performed with a titanium probe (0.4-cm diameter) fitted to a Biosonik III sonicator (Bronwill) that was operated at 20 kHz. During sonication, which lasted about 15 min, a presterilized 5-ml plastic tube (Falcon) containing the lipid suspension was immersed in an ice bath. Vesicle formation was established by examination of a sample placed on a carbon-coated formvar-copper grid (200 mesh) under an electron microscope (Zeiss, EM 9S2), after negative staining with 10 mg/ml of uranyl acetate.

Assay of sperm fertilizing capacity. Treatment effects on the fertilizing capacity of rabbit spermatozoa were determined from the fertilization rates achieved among eggs obtained from does following insemination with treated and untreated sperm cells into contralateral oviducts, as previously described (3). Spermatozoa flushed from the uterus of a donor in 4 to 6 ml of Ca^{2+}-enriched Hanks solution, about 12 hr after mating, were subdivided into two equal portions, before sedimentation at 600 *g* for 15 min. Both cell pellets were then resuspended in the same volume (0.3 to 1.0 ml) of medium. Experimental (Hanks + 0.5 to 10 mg of lipid/ml) and control (Hanks) sperm suspensions, containing 0.2 to 20 × 10^6 sperm cells/ml, were preincubated for 45 min at 37° before insemination. An iv injection of 70 to 90 USP units human chorionic gonadotrophin (Squibb) induced ovulation in these animals. To assay for decapacitation activity, 10- or 25-μl control and test sperm suspensions were instilled into opposite oviducts of a doe, which had been anesthetized with 30 mg of sodium pentobarbital/kg body weight, 2 to 4 hr after the anticipated time of ovulation. The female rabbits used in these experiments were of the New Zealand strain, of proven fertility, and 3.5 to 5.5 kg body weight. The recovery of fertilizing capacity in treated spermatozoa was determined from fertilization rates observed following intrauterine injection of 0.1-ml sperm suspension with a 1-ml disposable syringe having an 0.5-in. 26-gauge needle. Sperm cell concentrations were determined with a hemocytometer. The sperm suspensions used in this investigation

showed motility at the completion of each experiment, which usually involved insemination of five does. The inseminated recipients were autopsied after 1 day and eggs flushed from their oviducts were mounted on a glass slide, stained with 10 mg/ml of lacmoid, and inspected microscopically for chromatin, polar body formation, and associated spermatozoa. There were spermatozoa in contact with the eggs recovered from all does included in the results. Differences between control and experimental fertilization rates have been assessed for statistical significance with a χ^2 test, corrected for data discontinuity.

Results. In Fig. 1 are shown vesicles prepared from DPPC and DPPC with 10 (w/w)% cholesterol. The vesicles range in apparent diameter from approximately 500 to 3000 Å in each preparation.

Data presented in Table I reveal that uterine-capacitated rabbit spermatozoa fertilized significantly fewer eggs when suspended in medium with DMPC and DPPC vesicles with 10 to 40 (w/w)% cholesterol, at concentrations of 1 to 10 mg of lipid/ml, following insemination 2 to 4 hr postovulation. Among a total of 79 eggs from oviducts of does inseminated with sperm cells exposed to these vesicles there were only 8 fertilized eggs, whereas 67 of 87 eggs from the contralateral oviduct had been fertilized by untreated spermatozoa. Fewer eggs were fertilized by sperm treated with cholesterol containing vesicles prepared with DPPC than DMPC. The DMPC–cholesterol vesicle suspensions were more inhibitory at concentrations of 9 to 10 mg of lipid/ml than at 1 mg of lipid/ml, as might be expected. In three experimental groups concerning DMPC and DPPC vesicles with cholesterol shown in Table I, the mean number of sperm cells per egg for each group ranged from 2.7 to 15.4, while control groups averaged between 7.7 and 83.8 sperm per egg. The number of spermatozoa reaching the egg does not appear to be the main cause in preventing fertilization, however, for these ranges in the mean number of sperm per egg can be seen to overlap. It was noticed that spermatozoa treated with these vesicles became less motile. It was also noted that 9.4 mg of lipid/ml of DMPC vesicles with 40 (w/w)% cholesterol, prepared from mixtures that did not involve dissolution by an organic solvent, caused a decrease in fertilization rate from 82% (18/22) with untreated spermatozoa to 5% (1/19) by treated sperm cells. In one experiment, 10 (w/w)% phosphatidylserine was added to DMPC-cholesterol vesicles, with no apparent influence on decapacitation activity. Parenthetically, there was an average fertilization rate of 84% after oviductal insemination of untreated spermatozoa and, it may be noted, an inverse relationship, albeit a weak one (regression coefficient = 0.697), exists between fertilization rate and the number of sperm inseminated, over the range (2.5 to 50.0 $\times$ 10^4 sperm per oviduct) used in these experiments.

Intrauterine insemination of uterine-capacitated spermatozoa treated with DMPC and DPPC vesicles containing 40 (w/w)% cholesterol at a concentration of 1 mg of lipid/ml, 5 to 6 hr prior to ovulation, resulted in 53% (18/34) fertilization (Table I). By comparison, 55% (23/42) eggs were fertilized with untreated spermatozoa on the control side. This suggests that the inhibitory effect of these synthetic vesicles on sperm fertilizing capacity is reversed after treated sperm cells have been returned to the uterus for recapacitation. The possibility that these spermatozoa may not have been initially in a decapacitated state can be ruled out since insemination of these sperm into the uteri of two does, about 4 hr postovulation, resulted in 8% (1/12) fertilization with DPPC–cholesterol-treated sperm cells and 92% (11/12) fertilization with untreated spermatozoa, which is a statistically significant difference ($\chi_1^2 = 13.25$, $0.05 \geqslant P$).

Vesicles composed of DMPC and DPPC, without cholesterol, displayed no apparent decapacitation activity, at concentrations of 1 and 10 mg of lipid/ml, respectively. Table I shows that among both groups 80% (28/35) eggs were fertilized by sperm cells treated with phosphatidylcholine vesicles and, by comparison, 86% (37/43) eggs were fertilized by untreated spermatozoa. It seems clear from Table I that cholesterol is responsible for the inhibitory action of the synthetic vesicles, as cholesterol suspensions lacking phospholipid significantly lowered fertilization. Sperm cells suspended in medium containing 0.4 to 4.0 mg cholesterol/

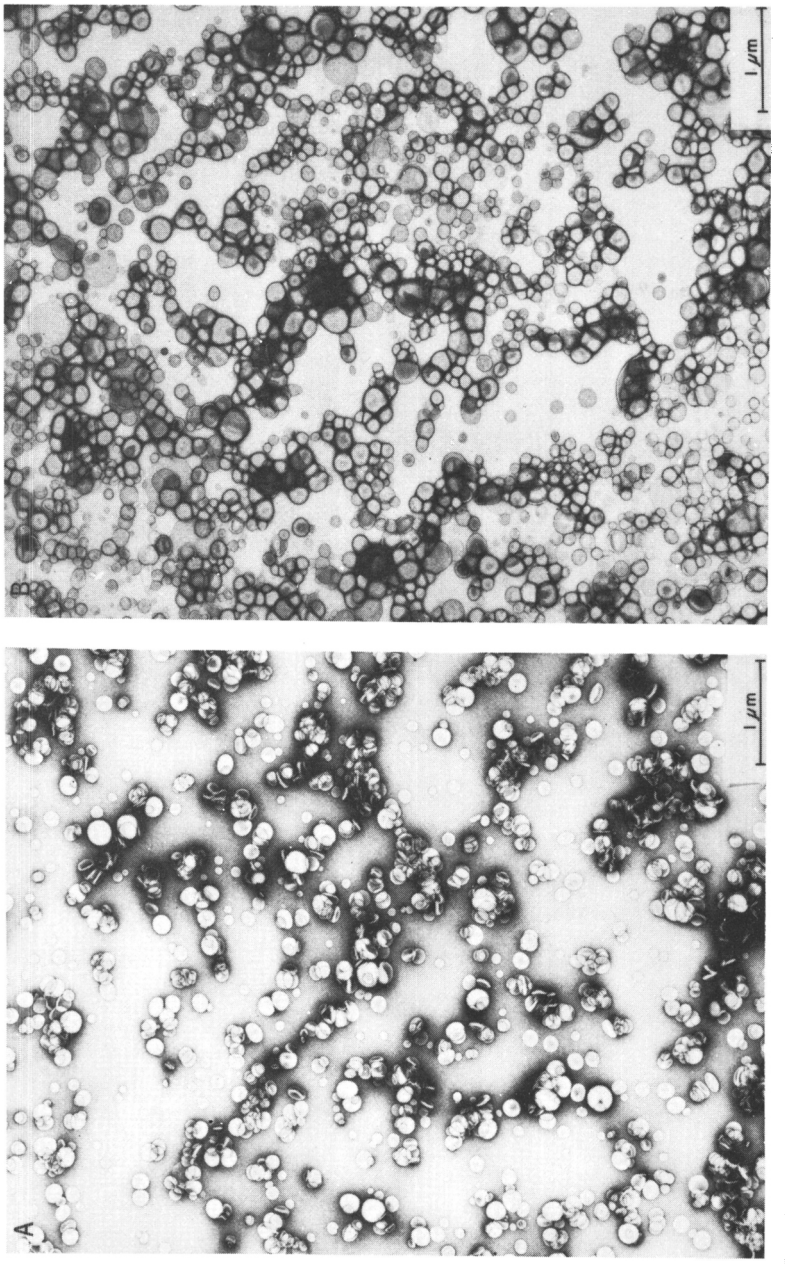

FIG. 1. Electron micrographs of negatively stained vesicles prepared from (A) DPPC and (B) DPPC with 10 (w/w)% cholesterol.

TABLE I. INFLUENCE OF VARIOUS LIPID SUSPENSIONS ON THE FERTILIZING CAPACITY OF RABBIT SPERMATOZOA

Lipid suspension				Eggs			
				Control oviduct		Experimental oviduct	
Composition (w/w)%	Amount (mg/ml)	Number of inseminations	Number of sperm cells ($\times 10^4$)	Total	No. fertilized (%)	Total	No. fertilized (%)
				Oviductal insemination 2 to 4 hr postovulation			
DPPC (100)	10	4	2.5	19	17 (89)	17	12 (71) NS[a]
DMPC (100)	1	4	50.0	24	20 (83)	18	16 (89) NS
				43	**37** (86)	**35**	**28** (18) NS
DPPC (60–80) + cholesterol (10–40) + phosphatidylserine (0–10)	1–2	7, 8[b]	43.0	26	15 (58)	30	3 (10)*
DMPC (60) + cholesterol (40)[c]	1	4	22.5	15	10 (67)	17	4 (24)*
	9–10	9	11.2	46	42 (91)	32	1 (3)*
				87	**67** (77)	**79**	**8** (10)*
Glycerophosphocholine (control)	0.5–1	9	52.0–1.5	28	15 (54)	36	21 (58) NS
Glycerophosphocholine (83) + cholesterol (17) (experimental)							
Cholesterol (100)	0.4	3	3.8	17	17 (100)	10	2 (20)*
	4	3	5.0	13	13 (100)	15	0 (0)*
				30	**30** (100)	**25**	**2** (8)*
				Uterine insemination 5 to 6 hr preovulation			
DPPC (60) + cholesterol (40)	1	6	67.0	21	9 (43)	20	9 (45) NS
DMPC (60) + cholesterol (40)	1	6	20.0–0.5	21	14 (67)	14	9 (64) NS
				42	**23** (55)	**34**	**18** (53) NS

[a] * Statistically significant difference ($P < 0.05$). NS, nonsignificant difference.
[b] Seven control and eight experimental inseminations.
[c] Four inseminations were made in each group with untreated sperm cells in the contralateral oviduct.

ml fertilized 8% (2/25) eggs, while all eggs (30/30) recovered after insemination of untreated spermatozoa were fertilized.

In the presence of 0.5 to 1 mg of lipid/ml of glycerophosphorylcholine and 17 (w/w)% cholesterol (0.085 to 0.17 mg cholesterol/ml) the fertilization rate was 58% (21/36), and this compares with 54% (15/28) fertilization with untreated spermatozoa (Table I). The ineffectiveness of glycerophosphorylcholine and cholesterol in this experiment may indicate the fatty acid side chain of the phospholipid aids in the inhibitory action, presumably by helping to disperse cholesterol, since phospholipid vesicles bearing 0.2 mg of cholesterol blocked fertilization.

Discussion. Suspensions of DMPC and DPPC vesicles containing cholesterol were found to reversibly inhibit fertilization by uterine-capacitated rabbit spermatozoa. The ability of these synthetic vesicles to mimic the action of membrane vesicles from seminal plasma (3) suggests that lipids in the latter are implicated in sperm decapacitation. Cholesterol was shown to be necessary for decapacitation activity and the sterol is known to be present in both classes of vesicles in seminal plasma from rabbits (9).

Concentrations of 1 to 10 mg of lipid/ml were present in the vesicle suspensions used in this work. By comparison, 0.02 to 1.0 mg of protein/ml (approximately 0.014 to 0.43 mg lipid/ml) vesicles from seminal plasma induced decapacitation of rabbit spermatozoa (1, 3). When sperm numbers are taken into account, DPPC–cholesterol vesicles were found to be inhibitory at a concentration equivalent to 5.8 μg of lipid/10^5 sperm cells, and this compares with an effective level of 10 μg of protein/10^5 sperm cells (about 7 μg lipid/10^5 sperm cells) obtained

for low density seminal plasma vesicles (1). Viewed from this perspective, synthetic and natural vesicles have similar potency.

Phosphatidylcholine (DPPC) vesicles have been reported to fuse with the plasma membrane of living cells at 37° (9). Addition of cholesterol to the sperm plasma membrane of living cells at 37° (10). Addicontaining cholesterol could prevent the acrosome reaction, for the sterol is known to inhibit membrane fusion (11, 12). The activity of some membrane-associated enzymes could also be influenced by the presence of cholesterol. Decreased fluidity resulting from a partial restriction of motional freedom among fatty acid side chains in liquid crystalline phospholipid bilayers has been suggested as the basis of the action by cholesterol (12, 13).

These experiments obviously do not exclude the possibility that phospholipid suspensions containing cholesterol, that have not been dispersed as vesicles, could also inhibit sperm-fertilizing capacity. In this connection, it may be noted that presaturation of albumin with cholesterol prevented fertilization of rat eggs *in vitro* (14). The present findings give added credence to the suggestion that addition of lipid (cholesterol) to the sperm plasma membrane could be implicated in sperm decapacitation (15). As DPPC, unlike DMPC, has a critical temperature for "gel to liquid" transition in layers of the phospholipid (13) that is above physiological temperature, it may be anticipated to exert a stabilizing influence on membranes. The inability of DPPC vesicles to decapacitate rabbit spermatozoa possibly results from rapid hydrolysis by sperm phospholipase (16) following incorporation.

If the reversible inhibition of sperm-fertilizing capacity observed in these experiments is equivalent to decapacitation by seminal plasma, then involvement of polypeptide and glycoprotein in sperm decapacitation (17, 18) perhaps indicates alternative (nonlipid) ways of inducing decapacitation exist. Further experimentation should help clarify the significance of this possibility.

Summary. Suspensions of dimyristoyl and dipalmitoyl phosphatidylcholine vesicles bearing 10 to 40 (w/w)% cholesterol inhibited the fertilizing ability of uterine-capacitated rabbit spermatozoa at concentrations of 1 to 10 mg of lipid/ml. Recovery of fertilizing ability by treated sperm cells was observed following insemination into the uterus 5 to 6 hr before ovulation. Vesicles lacking the sterol were not inhibitory under the conditions employed. Suspensions of cholesterol (0.4 to 4 mg of sterol/ml) without phospholipid, in contrast, inhibited fertilization. Implication of cholesterol in sperm decapacitation by seminal plasma membrane vesicles is discussed in terms of these results.

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