USE OF LIPOSOMES TO ASSOCIATE FOREIGN GENETIC MATERIAL WITH SPERMATOZOA

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It is now possible with the aid of genetic engineering methods to modify inherited nature in a deliberate manner. This has led to a significant increase in productivity of live stock, the creation of immunity to diseases, and the breeding of artificial species of animals, the consequence of which research into the production of transgenic individuals has become of the most urgent importance [5]. One way of obtaining transgenic animals is to use spermatozoa as a natural vector in order to supply foreign DNA to the animal's oocyte [7]. Substances that may be transferred are protected against lysis and modification by enzymes or whatever by being incorporated into vesicles or liposomes, which are adsorbed on spermatozoa under assigned conditions. Spermatozoa loaded with liposomes settle at the site of fertilization, where they may enter the oocytes. Liposomes are produced in the presence of nucleic acids coding for one or more genes that are intended for transfer. On the formation of liposomes, the substance to be transferred is incorporated into their internal space, and the spermatozoa carry the liposomes into the cytoplasm of target cells. In the cytoplasm the liposomes are destroyed by the action of various intracellular enzymes, releasing the encapsulated substances. During nucleic acid transfer and transformation in the genome of the zygote, a new gene (or genes) may be present in all cells of the developing organism [7]. Encouring results have been obtained in investigations in which liposomes were used for gene transport [1].

The aim of this investigation was to chose the optimal composition of liposomes for incorporation of DNA into them and for adsorption on spermatozoa, and also to evaluate the functional activity of spermatozoa modified by liposomes.

EXPERIMENTAL METHOD

Positively charged lipids, synthesized at the chemical faculty of the V. I. Lenin Moscow Polytechnical State University, under the direction of D. A. Predvoditelev and E. E. Nifant'ev [4], were used: tristearoxypropylenediol-1-O-phosphohomocholine (PHC) and distearoxyethylenediol-1-O-thion-phosphohomocholine (thion-PHC). As the control we used egg 1,2-diacyl-sn-glycero-3-phosphocholine (PC), a product of Khar'kov Bacterial Preparations Factory. The purity of the lipid was verified by thin-layer chromatography [2]. In some cases the liposomes were produced with the addition of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). Frozen bovine sperm, obtained from the bank of the Department of Biotechnology, All-Union Institute of Animal Husbandry, was used. The concentration of spermatozoa was 34 million per dose, and activity was 8 points. To determine the percentage of incorporation of the substance into liposomes, radioactively labeled DNA, namely a solution of plasmid pBR322 (0.25-1 mg/ml) and the fluorescent dye calcein ("Sigma" USA) in a concentration of 126 mM. Liposomes of two compositions were made: PHC:DOPE and thion-PHC:DOPE (in both cases the molar radio was 1:1). As the control, liposomes were prepared from pure PC. The liposomes were made by freezing and thawing, and the substances for transfer were incorporated in them and unincorporated material was washed off them by the method described in [3, 6]. The radioactivity associated with the liposomes was determined on a "Compugamma" gamma-counter (LKB, Sweden). The intensity of fluorescence was measured on a "Hitachi F3000" spectrofluorometer. The

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TABLE 1. Incorporation of Radioactively Labeled DNA into Liposomes

Composition of liposome	Incorporation of labeled DNA into liposomes, percent of amount added
PHC:DOPE	80 (40 ng DNA/mg lipid)
Thion-PHC:DOPE	75 (37 ng DNA/mg lipid)
PC	20 (10 ng DNA/mg lipid

TABLE 2. Incorporation of Calcein into Liposomes

Composition of liposome	Increase in fluorescence on addition of Triton X-100
PHC:DOPE	7,5 times
Thion-PHC:DOPE	7,5 »
	4,5 »

Legend. Here and in Table 3 conditions of measurement of fluorescence were: excitation 490 nm, emission 520 nm, volume of cuvette 1 ml.

TABLE 3. Attachment of Calcein-Containing Liposomes to Spermatozoa

Composition of liposome	Increase in fluorescence on addition of Triton X-100
PHC:DOPE	8 times
Thion-PHC:DOPE	5,5 times
PC	3,5 »

TABLE 4. Results of the Count

Composition of liposome	Activity of sperm, points
PHC:DOPE	7
Thion -PHC:DOPE	6
PC	3
Control	3

Legend. Activity (motility) of sperm assessed on a 10-point scale. Sperm in which virtually all (100%) spermatozoa possessed forward motion in a straight line scored 10 points.

dose of frozen sperm was introduced into a test tube with 1 ml of sodium citrate, pH 7.0, and thawed at room temperature. Samples of 30 μl of sperm were then taken (each containing 1 million spermatozoa) in four tubes: to two of them 300 ng of the positively charged test liposomes was added, the same number of PC liposomes was added to the third tube, and the last remained as the control. Incubation was carried out for 1 h at room temperature, after which the unbound material was washed off by centrifugation at slow speed (3000g) for 3 min. After incubation and washing, the motility of the spermatozoa in each tube was estimated visually (in points) by the method described in "Instructions on the Organization and Technology of Work of an Artificial Insemination Station for Live Stock." The sperm was studied under an "Amplival" light microscope (Germany) with magnification of 200-300 times. Sperm not treated with liposomes was used as the control.

EXPERIMENTAL RESULTS

To determine the percentage uptake of substances by liposomes experiments were carried out to study incorporation of radioactively labeled DNA (Table 1) and of the fluorescent dye calcein (Table 2). In the case of labeled DNA the degree of uptake was determined by measuring radioactivity of the samples, whereas in the case of calcein, the maximal value of fluorescence achieved as a result of destruction of the liposomes by Triton X-100 and dilution of the incorporated dye, was measured.

It follows from Tables 1 and 2 that positively charged liposomes containing PHC and thion-PHC take up calcein and DNA about 2-4 times better than control liposomes obtained from PC.

Tables 1 and 2 show that positively charged liposomes containing PHC and thion-PHC assimilate calcein and DNA approximately twice to four times better than control liposomes obtained from PC. To determine the number of liposomes adsorbed on spermatozoa, in the course of the incubation period and after separation of unbound liposomes, each test tube was treated with 10% Triton, which caused lysis of the liposomes and release of the dye into the external medium, where it was diluted, with a corresponding increase in the intensity of fluorescence. The greatest increase in fluorescence was observed in samples to which positively charged liposomes had been added (Table 3). This indicates superior binding of liposomes of the PHC:DOPE and thion-PHC:DOPE composition with spermatozoa.

Immediately after the end of incubation motility of the sperm was estimated visually, and the result reflected preservation of their functional activity after treatment with liposomes. For this purpose the number of spermatozoa with forward motion in a straight line was counted and assessed on a 10-point scale (with the same concentration of spermatozoa in each sample). The experiment showed that fusion of spermatozoa took place with lipid in a concentration of over 300 ng/million cells. With lower lipid concentrations fusion was not observed and the spermatozoa were actively motile.

As the control, sperm untreated with liposomes was used. The results of the count are given in Table 4. They show that there were significantly more actively motile spermatozoa in tubes with liposomes containing PHC and thion-PHC than in the tube with PC and in the control. It can be postulated that "adhesion" of positively charged liposomes protects the sperm against stress during the experiment (temperature, centrifugation).

Positively charged liposomes with the PHC:DOPE and thion-PHC:DOPE composition, containing the new synthetic diol phospholipids, thus incorporate DNA and calcein better than traditional liposomes obtained from PC.

Thanks to their positive charge these liposomes interact actively with spermatozoa, which, like all other cells, have a weak negative charge. Liposomes of the composition used are not toxic for spermatozoa. Such liposomes may prove suitable for transferring foreign DNA into the cytoplasm of target cells (oocytes), which is the aim of our current research.

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