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Effect of Hypercholesterolaemia on Testicular Function and Sperm Physiology

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The effect of hypercholesterolaemia on testicular endocrine and exocrine function was evaluated. Furthermore, the influence of hypercholesterolaemia on sperm quality, quantity, and fertilizing potential was appreciated. Eight mature rabbits (group A) were fed chow containing 3% cholesterol for 12 weeks. An additional six rabbits (group B) were fed normal chow for the same period. At the end of the experimental period hormonal profiles and sperm parameters were evaluated. In addition, the sperm reproductive potential was assessed by in vitro fertilization (IVF) procedures. Peripheral serum responses to testicular stimulation with human chorionic gonadotropin, epididymal sperm content and motility, and IVF outcome were significantly lower in group A than in group B. In contrast, serum cholesterol levels were significantly higher in group A. It appears that hypercholesterolaemia has a detrimental effect on Leydig cell function, spermatogenesis, the epididymal sperm maturation process, and the overall sperm fertilizing capacity.

Key words: cholesterol; infertility; sperm; temperature; testis

Cholesterol is secreted in the seminal plasma by the prostate and protects spermatozoa against environmental shock (Sofikitis and Miyagawa, 1991). High sexual satisfaction prior to/during ejaculation is accompanied by an enhanced prostatic secretion of cholesterol (Sofikitis and Miyagawa, 1993a).

Several reports refer to the role of cholesterol in sperm capacitation and the acrosome reaction process (Benoff et al., 1993). Changes in sperm cholesterol profiles appear to be involved in the asynchronism of capacitation (Benoff et al., 1993). During the sperm's passage through the female reproductive tract, a loss of cholesterol from the spermatozoal membrane is involved in the mechanism of sperm capacitation in mammals. Cholesterol has previously been demonstrated to limit protein insertion into phospholipid bilayers, to restrict lateral mobility of functional receptor sites embedded in the cell surface lipid core, and to modulate the activity of membrane proteins by changing their conformation (Yeagle, 1985). Furthermore, serum albumin, known to serve as a capacitating factor, induces its effect by promoting the efflux of cholesterol from the human sperm plasma membrane (Langlais et al., 1988). There is strong evidence that cholesterol modifies the fusion of the plasma membrane with the outer acrosomal membrane in the same way other decapacitating factors do (Davis and Hungund, 1976). Cholesterol provides rigidness to the membrane, whereas polyunsaturated agents promote membrane fluidity.

A cholesterol enriched diet produces a decrease in the kinetics of the acrosome reaction in live rabbit spermatozoa (Diaz-Fontdevilla and Buston-Obregon, 1993). This effect is consistent with the studies of Sebastian and colleagues (1987) showing that human male infertility might be associated with altered lipid metabolism in seminal plasma. The decapacitating properties of cholesterol have also been clearly demonstrated by Diaz-Fontdevilla and

Abbreviations: DPBS, Dulbecco's phosphate buffered saline; hCG, human chorionic gonadotropin; IVF, in vitro fertilization

Buston-Obregon (1993). In that study a cholesterol-enriched diet was found to alter the filipin-sterol complexes in the plasma membrane of the acrosomal region, suggesting that sperm membrane lipid domains induced by hypercholesterolaemia are the cause of the modification in sperm capacitation and acrosome reaction kinetics. However, there are no reports investigating the influence of cholesterol-enriched diets on spermatogenesis and Leydig cell function. Our objective was to estimate the effects of hypercholesterolaemia on testicular endocrine and exocrine function, and on overall sperm fertilizing capacity.

Materials and Methods

Eight mature (6-month-old) New Zealand white male rabbits were fed chow containing 3% cholesterol for 12 weeks (group A). An additional six rabbits were fed normal rabbit chow for the same period (group B). The animals were caged individually during the experimental period and received water and feeding ad libitum. At the end of the experimental period, blood was aspirated from a peripheral vein (ear vein) and cholesterol, total lipids and testosterone were evaluated. Then, all the animals received intramuscular administration of human chorionic gonadotropin (hCG; 1,500 IU). Three hours later, peripheral blood was aspirated and testosterone responses to hCG stimulation were measured. One week later, all the animals were anesthetized with intravenous sodium pentobarbital (30 mg/kg, Nembutal, Abbot Laboratories, Chicago, IL). Intravenous heparin sulfate (120 units/kg) was given for anticoagulation. Intra-abdominal and left testicular temperature were assessed. One-hundred milligrams from the left cauda epididymis (just prior to the origin of the vas deferens) were resected, and the epididymal sperm content, motility and fertilizing capacity were appreciated. The left testis was resected, and the left testicular weight was recorded. Cholesterol concentration in the left testicular tissue was assessed.

Serum/tissue cholesterol and lipid evaluation

Cholesterol was extracted from testicular tissue using the modified Bligh-Dyer method (Bligh and Dyer, 1959; Kate,1986). Then, serum or testicular cholesterol and serum total lipid were evaluated by the Special Reference Laboratory (Hiroshima, Japan) as previously described (Sofikitis and Miyagawa, 1993a).

Serum testosterone assay

Testosterone levels were assessed by radioimmunoassay according to the method of Coyotupa and coworkers (1972) using kits from Nihon DPC Corporation (Tokyo, Japan).

Measurement of testicular temperature

Left testicular temperature was assessed by percutaneous insertion of a 29-gauge needle probe attached to a digital thermometer (Unique Medical, PTC 201 model, Tokyo). Intra-abdominal temperature was monitored with a rectal probe, and body temperature was maintained between 36.7˚C and 37.3˚C with radiant heat throughout the procedure. The difference between the intra-abdominal and left intratesticular temperature was recorded.

Testicular weight

The testes were dissected free of surrounding tissue and weighed on a Mettler Basbal scale (Tokyo).

Epididymal caudal sperm content and motility

Epididymal caudal sperm content was measured as previously described (Sofikitis and Miyagawa, 1993b). The epididymal fragment was trimmed and minced in 5 mL of modified Dulbecco's phosphate buffered saline (DPBS; Sigma Chem., St. Louis, MO) (Sofikitis et al., 1996). A stereo microscope facilitated cutting the tissue into the smallest possible pieces. The

minced epididymal tissue was separated from the liberated spermatozoa by filtration through a 20-µm-diameter paper filter (Whatman Co., New York, NY). Six droplets (a volume of each sample was 0.06 mL) of the filtrate were used for the sperm count (number of spermatozoa/mL), and the average number was calculated. A Makler Counting Chamber (Sefi Medical Instruments, Haifa, Israel) was placed on the slide of an ordinary microscope, and a 20-power objective and 10-power eyepiece were used. Ten droplets were counted to calculate the percentage of motile spermatozoa in the filtrate immediately after its preparation. The proportion of motile spermatozoa in each drop was determined by counting 300 cells in randomly selected fields. Motility was also graded qualitatively and assigned a value from 0 to 4 as previously described (Sofikitis et al., 1991).

Epididymal caudal sperm fertilizing capacity

The ability of rabbit spermatozoa to penetrate and fertilize ova was tested in vitro. Epididymal caudal rabbit sperm samples were centrifuged at 200 × *g* for 10 min. The sperm pellet was transferred to modified Brackett medium (containing 3.2 g/L bovine albumin) (Brackett, 1970; Brackett and Oliphant, 1975). Then, sperm samples underwent a standard swim-up procedure. In brief, 1 mL-aliquots were incubated at a 45˚ angle at 37˚C under 5% carbon dioxide for 30 min. Supernatants (0.5 mL) were recovered. Spermatozoa in supernatants from group B exhibited motility ranging from 81% to 96%. Supernatants from group A rabbits showed sperm motility ranging from 53% to 70%. To avoid differences of sperm motility between both groups, spermatozoa from group A were filtrated via Sperm Prep (ZBL, Lexington, KY) as previously described (Sofikitis et al., 1993). The filtrates recovered from Sperm Prep columns exhibited percentages of motile spermatozoa ranging from 73% to 91%. Post-swim-up supernatants from group B rabbits and Sperm Prep filtrates from group A rabbits were centrifuged at 200 × *g* for 15 min, and sperm samples of a concentration equal to 2,000,000/mL were prepared (final samples) and incubated in modified Brackett medium at 37˚C under 5% carbon dioxide for 3 h. Cumulus masses containing oocytes were recovered from female mature rabbits as previously described (Sofikitis et al., 1996). Ten cumulus masses were inseminated with sperm samples from each male rabbit. Each cumulus mass placed in 0.9 mL of RD medium containing 10 mM of taurine [RD medium, Dulbecco's low-glucose Modified Eagle's medium (GIBCO Co., Grand Island, NY) mixed 1:1 (v/v) with RPMI 1640 (GIBCO)] (Carney et al., 1991) was inseminated with 0.1 mL of the final sperm sample and incubated at 37˚C under 5% carbon dioxide (Sofikitis et al., 1996). Oocytes were observed at 18 and 36 h after insemination. The percentage of oocytes with two pronuclei at 18 h and the percentage of cleaved oocytes at 36 h were recorded.

Statistical analysis

Wilcoxon's test was used for statistical analysis. A probability of less than 5% (< 0.05) was considered to be statistically significant. Values were expressed as mean ± SD. Measurements of hormonal and sperm parameters were performed in blind procedure.

Results

Cholesterol assay

Peripheral serum cholesterol levels were significantly higher in group A than in group B (Table 1). In contrast, there were no significant differences in cholesterol contents in testicular tissue between groups A and B.

Total lipid profiles

Total lipid levels in peripheral serum were significantly higher in group A than in group B (Table 1).

Table 1. Testicular and peripheral serum cholesterol/total lipid concentrations in rabbits fed cholesterol-enriched chow and rabbits which received normal chow

Group	Chow	Cholesterol		Total lipids
		Serum (mg/dL)	Testicular tissue (nmol/10^7 cells)	Serum (mg/dL)
A	Cholesterol-enriched	1803 ± 113*	15.2 ± 1.4	6986 ± 189*
B	Control	36 ± 8	14.4 ± 1.1	184 ± 16

Values are expressed as mean ± SD.
*$P < 0.05$ compared with the control group.

Testosterone assay

Basal peripheral serum testosterone profiles were not significantly different between groups A and B. In contrast, testosterone responses to hCG stimulation were significantly lower in group A (Table 2).

Testicular temperature

Left testicular versus intra-abdominal temperature difference was not significantly different between groups A and B (3.9 ± 0.4˚C and 4.1 ± 0.3˚C, respectively).

Testicular weight

Differences in mean values of left testicular weight between groups A and B were not significantly different (3153 ± 101 mg and 3208 ± 94 mg, respectively).

Table 2. Peripheral blood testosterone profiles in hypercholesterolaemic and control animals

Group	Chow	Testosterone (ng/mL)	
		Basal	Response to hCG
A	Cholesterol-enriched	2.4 ± 0.4	4.9 ± 0.7*
B	Control	2.2 ± 0.7	6.8 ± 0.9

Values are expressed as mean ± SD.
*$P < 0.05$ compared with the control group.

Epididymal sperm content, motility and fertilizing capacity

Epididymal sperm content, percentage of motile spermatozoa, and motility grade were significantly lower in group A than in group B (Table 3). Although differences in percentage of motile spermatozoa between group A after swim-up plus Sperm Prep filtration and group B after swim-up were not significantly different (see Materials and Methods), the percentage of oocytes with two pronuclei at 18 h after insemination, and percentage of cleaved oocytes at 36 h after insemination were significantly lower in group A than group B.

Discussion

We created a hypercholesterolaemia model similar to that described by Girerd and coworkers (1990) to illustrate the influence of cholesterol-enriched diets on testicular endocrine and exocrine function. Such a study is of clinical importance because hypercholesterolaemia is a social problem in many developed countries and high-cholesterol products are often advertised for commercial reasons.

Peripheral serum basal testosterone levels between hypercholesterolaemic and control rabbits showed no significant differences. Of course a serum basal testosterone profile is a good indicator of Leydig cell function, but it often fails to indicate small changes in the rate of testicular testosterone synthesis (Steinberger et al., 1973). Therefore, evaluation of the possible potency of Leydig cell function caused by

Table 3. Epididymal caudal sperm content, motility and fertilizing capacity in hypercholesterolaemic and control rabbits

	Hypercholesterolaemic group (Group A)	Control group (Group B)
Sperm content (× 10/mL medium)	413 ± 151*	734 ± 106
Motile sperm (%)	61 ± 7*	79 ± 6
Motility grade	2.3 ± 0.4*	3.3 ± 0.3
Oocytes with two pronuclei (%)†	27 ± 4.3*	53 ± 7.6
Cleaved oocytes (%)†	23 ± 5.1*	47 ± 7.3

Values are expressed as mean ± SD.

* $P < 0.05$ compared with the control group.

† Sperm samples for IVF in groups A and B were used after preparation via the swim-up and Sperm Prep methods, and the swim-up method, respectively. There were no significant differences in the percentage of motile sperm between groups A and B after above preparations.

hCG stimulation should be important. In fact, the present study reveals secretory dysfunction of stimulated Leydig cells in a hypercholesterolaemic testicular environment. We evaluated testosterone responses to hCG 3 h after stimulation because we has previously demonstrated that the peak testosterone response to hCG stimulation in rabbits occurs 3 h after injection (Sofikitis and Miyagawa, 1994). Optimal Leydig cell function and testosterone secretion are known to be prerequisites for i) normal activation of spermatogenesis (Steinberger et al., 1973) and ii) maintenance of epididymal sperm maturation process and development of sperm capacity to exhibit a vigorous forward progression (Turner, 1985). Thus, the significantly lower epididymal caudal sperm counts and motility profiles in animals with hypercholesterolaemia may be attributable to a defect in the secretory function of the Leydig cells resulting in impaired spermatogenesis and an impaired epididymal sperm maturation process.

In this study the proportion of cleaved oocytes to fertilized oocytes was significantly lower in rabbits in the hypercholesterol group compared with the control group. Because there were no significant differences in the percentage of sperm motility between the groups after preparation of the sperm by Sperm Prep and the swim-up method, these results may not be attributable to the decreased sperm motility in rabbits with hypercholesterolaemia.

These findings may suggest that hypercholesterolaemia results in quantitative or qualitative alterations in sperm membrane lipids leading to an impaired sperm capacity for capacitation and acrosome reaction. In addition, hypercholesterolaemia may have a harmful effect on the final event of the sperm fertilizing process [i.e., Zona binding penetration of the zona pellucida, fusion with the vitelimembrane, and so on (Yamamoto et al., 1997)] and subsequently on the potential for transformation of the sperm head to male pronuclei. Furthermore, the significantly lower percentage of cleaved oocytes in rabbits treated with cholesterol diets clearly indicated that early embryonic development is impaired.

The absence of a significant difference in testicular tissue cholesterol content between the two groups is consistent with the results of previous reports (Diaz-Fontdevilla and Buston-Obregon,1992; Diaz-Fontdevilla et al., 1993) showing that cholesterol content does not increase in the seminal plasma of animals treated with cholesterol-enriched diets. Our results and the above previous studies taken together suggest the existence of a blood-testis or a blood-male reproductive tract barrier for cholesterol.

It is difficult to explain why Leydig cell function is impaired in hypercholesterolaemic animals without an elevation of testicular tissue cholesterol concentration. We measured testicular temperatures known to be regulated by a

counter-current heat exchange testicular vasculature to evaluate whether vascular consequences of hypercholesterolaemia on the counter-current heat exchange system are responsible for the testicular damage (Rubenstein et al., 1995). However, the functionality of the counter-current heat exchange system in hypercholesterolaemic animals appears to be intact.

Our hypotesis is that hypercholesterolaemia may not exert a direct influence on cells in seminiferous tubules, but other substances induced by hypercholesterolaemia have a detrimental effect on spermatogenesis.

The present study is the first report showing an effect of cholesterol-enriched diets on testicular endocrine and exocrine function, the epididymal sperm maturation process, and the overall sperm fertilizing capacity. The concept that there is a blood-testis barrier for cholesterol is supported by the current findings and deserves further investigation.

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