Effects of high-density lipoproteins on storage at 4°C of fowl spermatozoa

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Summary. Qualitative and quantitative characterization of lipoproteins found in seminal plasma from domestic cocks was performed after isolation by density gradient ultracentrifugation. Triglyceride-rich lipoproteins (very low, intermediate- and low density lipoproteins) were not detectable in seminal plasma. High-density lipoproteins (HDL), identified on the basis of size, chemical composition and protein moiety, were present at a concentration of 66 µg/ml. A fraction possibly corresponding to VHDL (very high density lipoproteins, 77% protein, 23% lipid) was also detected but appeared contaminated by a protein-rich opalescent material. Since HDL contains mostly phospholipid and cholesterol, the physiological role of these lipoproteins on the storage of fowl spermatozoa was studied. Replacing seminal plasma with a solution containing chicken HDL at physiological concentration (66 µg/ml) had no effect on fertilizing ability of spermatozoa stored at 4°C for 24 h. However, higher concentrations of HDL (560 µg/ml) had deleterious effects on spermatozoa stored *in vitro*.

Keywords: lipoproteins; chicken; seminal plasma; sperm storage; *in vitro*

Introduction

The role of lipoproteins as lipid transporters in biological fluids such as blood plasma, lymph and interstitial fluid is well known. Certain lipoproteins, notably LDL (low-density lipoproteins) and HDL (high-density lipoproteins) are implicated in the exchange of cholesterol between cells and extracellular fluids and these interactions with male gametes may be of importance in the processes leading to fertilization, i.e. maturation in the male, capacitation in the female and resistance to cold shock during in-vitro storage. For storage of fowl spermatozoa at 4°C, a high degree of membrane stability and high cholesterol content may be important for the resistance of cells to cold shock (Darin-Bennett & White, 1977). Nevertheless, Ansah & Buckland (1982) demonstrated that a strain of cock exhibiting seminal plasma and spermatozoa with a low cholesterol content had a higher resistance to cold shock than did a strain with a high cholesterol content. Similarly, Hess & Thurston (1984) showed that male turkeys from subfertile lines had more cholesterol in their seminal plasma than did birds from lines with normal fertility. The components of seminal plasma are implicated in the success of artificial insemination with fresh semen (Lake & Ravie, 1987) or stored semen. In the latter case, toxic effects of seminal plasma have been suggested (Blesbois, 1986; Blesbois & Mauger, 1987; Sexton, 1988).

It would therefore be beneficial to determine the form in which cholesterol and other related lipids are present in seminal plasma and whether these components may be responsible, at least in part, for the reported toxicity after semen storage. The cholesterol and phospholipid content of bird seminal plasma and spermatozoa is well documented (Ansah & Buckland, 1982; Hess &

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Thurston, 1984; Ressequie & Hughes, 1984), but the presence and role of their biological transporters, the lipoproteins, have been poorly investigated. Their existence in seminal plasma has been suggested in mammals (Lewin *et al.*, 1974; Clavert *et al.*, 1980; Polakoski & Kopta, 1982) and in birds (Kichev & Danov, 1972) on the basis on electrophoretic data. To reinforce our knowledge of fowl seminal plasma (Lake, 1971, 1984) and its role on the metabolism of spermatozoa, the present study was undertaken to determine (1) whether lipoproteins are present in the seminal plasma of domestic cocks and (2) the influence of seminal plasma lipoproteins on the viability of spermatozoa stored at 4°C for 24 h.

Materials and Methods

Animals. The males were 48 heavy male fowl from Line 199 which was originally derived from a Cornish strain at the Poultry Research Station (INRA, Nouzilly, France). The females were 300 dwarf 'Vedette JV15' birds. At the start of experiment, all birds were 32 weeks of age. All animals were housed in individual battery cages. The lighting regimen was 14 h light:10 h dark (lights on between 03:00 and 17:00 h each day). The males received 110 g/day and the females 120 g/day of a diet containing 150 g crude protein/kg and 11.7 MJ/kg energy.

Semen collection and treatment. Semen was collected by massage (Burrows & Quinn, 1936). Care was taken to avoid any contamination of semen with the cloacal products and particularly with the transparent fluid excreted from the lymph folds of the cloaca during ejaculation. The presence of glucose in the ejaculate is indicative of a contamination of spermatozoa by transparent fluid (Lake, 1966). Glucose determination was therefore systematically performed for all samples (Glucose analyser 2, Beckman, Palo Alto, CA, USA) so that any samples containing transparent fluid could be discarded. To limit the time between semen collection and treatment, a maximum of 12 birds was used at the same time for semen collection (5 min). Seminal plasma was separated from spermatozoa by successive centrifugations. To ensure separation without the release of intracellular components into seminal plasma, the centrifugations were carried out as follows: the first centrifugation of whole semen was performed no more than 10 min after the start of the semen collection session, and the second and third centrifugations concerned the respective supernatant from the preceding centrifugations. The first two centrifugations lasted for 10 min at 500 *g* and 20°C. The last centrifugation was performed at 3000 *g* and 4°C for 30 min. After separation, seminal plasma was considered to be 'crude seminal plasma'. 'Centrifuged seminal plasma' was defined as part of the crude seminal plasma submitted to a 4th centrifugation for 1 h at 10 000 *g* in an attempt to eliminate cellular fragments. Both crude and centrifuged seminal plasma were frozen at -20°C and stored for subsequent analysis.

Lipoprotein analysis. Mixed seminal plasma from 100 ejaculates was equally divided into 'crude' and 'centrifuged' seminal plasma. Lipoprotein subfractions were isolated by density gradient ultracentrifugation, according to the method of Chapman *et al.* (1981) and modified by Hermier *et al.* (1985). Ultracentrifugation was performed in a Beckman SW41 rotor for 48 h at 40 000 r.p.m. (56×10^7 g/min) and 15°C. At the end of centrifugation, 7 fractions of 1 ml each were collected successively from the meniscus of the gradient, and then 10 fractions of 0.5 ml each. This fractionation did not take into account the appearance of visible bands in the gradient, but the presence of these bands was noted according to the fractions in which they were collected. Lipoprotein fractions were dialysed in Spectrapor tubing (molecular weight cut-off 12 000) for 2×48 h at 4°C against a solution containing 0.05 M-NaCl, 0.005 M-NH₄HCO₃, 0.04% EDTA and 0.01% sodium azide.

The following components were determined in each lipoprotein fraction: protein by the method of Lowry *et al.* (1951); free cholesterol, total cholesterol and phospholipids by enzymic methods (Richmond, 1973; Takayama *et al.*, 1977), using kits from Bio-Mérieux (Charbonnières-les-Bains, France); and triacylglycerol by the colorimetric method of Biggs *et al.* (1975). The amount of cholesteryl ester was calculated using the formula: cholesteryl ester = (total cholesterol - free cholesterol) $\times$ 1.67. The concentration of material present in each fraction was obtained by addition of the concentrations of the lipid and protein components.

Estimation of the size of the native lipoprotein particles, determination of apolipoprotein molecular weight and apolipoprotein detection by dot immunobinding were performed as described by Hermier *et al.* (1988).

Fertilizing ability test. Semen from 10-12 birds was pooled and the concentration of spermatozoa estimated immediately using a photometer at a wavelength of 540 nm. Samples were then divided into 1-ml fractions containing an average of 6×10^9 spermatozoa. For each experiment, pooled semen was used within each treatment group. Samples destined for storage without seminal plasma (reconstituted semen) were centrifuged for 10 min at 500 *g* (Sexton, 1973). The resulting supernatant (seminal plasma) which represented half of the initial volume of semen was discarded and replaced by Beltsville poultry semen extender (BPSE) (Sexton, 1977) or a lipoprotein solution. Since the lipoproteins present in seminal plasma were identified as HDL (see 'Results'), this solution contained HDL prepared from chicken blood plasma (Hermier *et al.*, 1984).

Thus, in some experimental lots, seminal plasma was replaced by a solution of HDL diluent (0.05 M-NaCl, 0.005 M-NH₄HCO₃) alone or containing either 66 µg HDL/ml (corresponding to the normal concentration of HDL in seminal plasma) or 560 µg HDL/ml (a concentration 8-fold higher than normal). Before storage, all samples (whole

semen or reconstituted semen) were diluted 1:1 in BPSE, and then stored for 24 h at 4°C in aerobic conditions (Wishart, 1981). The oxygen concentration of semen samples was approximately 400 nmol/ml (Blesbois, 1985), as measured with a Clark electrode. The pH of all samples was adjusted to 7.2 and osmolarity to 350 mosmol. The fertilizing ability of spermatozoa was estimated from the results of intravaginal inseminations of 200×10^6 spermatozoa/female/week over 5–8 consecutive weeks. Two fertility tests were applied, one during the first half of the reproductive period ('young' animals, 35–43 weeks old), and the second at the end of the reproductive period ('old' animals, 55–60 weeks old). In each case males and females were of the same age. For each treatment 24 females were inseminated and eggs were collected on Days 2–8 inclusive after each insemination (500–1200 eggs per treatment). Fertilization rates (fertile eggs/incubated eggs $\times$ 100) were determined by candling 7 days after setting incubation. Hatchability rates (hatched eggs/fertile eggs $\times$ 100) were determined after 21 days of incubation.

Statistical analysis. For the fertilizing rates, heterogeneity of variance and non-normal distribution of data made analysis of variance inappropriate (Dagnelie, 1975). Consequently, non-parametric tests (Kruskal–Wallis one-way analysis of variance, Friedmann two-ways analysis of variance, and χ^2 tests) were used (Siegel, 1956). With none of these tests could the interaction between time and treatment be analysed. However, all tests gave the same hierarchy of treatments.

Results

Identification of lipoproteins in seminal plasma of cockerels by ultracentrifugation of 'crude' or 'centrifuged' seminal plasma

'Crude' seminal plasma. The banding pattern of the density gradient of crude seminal plasma (Fig. 1a) differed markedly from that of a blood plasma density gradient. No pigmented band corresponding to the position of chicken lipoproteins was seen in the crude seminal plasma gradient. However, two close but distinct opalescent bands, with density limits corresponding to 1.130–1.190 g/ml (Band A, fractions 9–12), and to 1.190–1.250 g/ml (Band B, fractions 13–16), were visible.

Chemical analysis of the successive subfractions revealed the presence of lipids and proteins in the density range greater than 1.075 g/ml (i.e. from fraction 6). Three different fractions could be detected (Fig. 1a) with density limits corresponding approximately to: Peak I, 1.075–1.130 g/ml (fractions 6–8); Peak II, 1.130–1.190 g/ml (fractions 9–12); and Peak III, 1.190–1.250 g/ml (fractions 12–16). Peak I did not correspond to any visible band on the density gradient, whereas Peaks II and III corresponded to Bands A and B respectively. The concentrations of these 3 peaks are shown in Table 1. Peak III was the major fraction because of its higher protein content.

Fractions 6–16 contained triglyceride, free and esterified cholesterol and phospholipid, but the presence of considerable amounts of protein prevented accurate determination of their chemical composition, and necessitated a supplementary treatment of seminal plasma before ultracentrifugation.

The triglyceride-rich apolipoprotein B-containing lipoproteins, such as very low-, intermediate-, and low-density lipoproteins (VLDL, IDL, LDL), were either absent, or present at levels too low to be detected.

'Centrifuged' seminal plasma. Since the occurrence of opalescent bands on the gradient could be attributed to protein-rich cellular fragments, the 'crude' seminal plasma was centrifuged for 1 h at 10 000 *g* before density gradient ultracentrifugation (see 'Methods'). The resulting gradient was different from that obtained from 'crude' seminal plasma (Fig. 1b). Centrifugation resulted in the disappearance of the opalescent Band B (> 1.190 g/ml) and in the loss of the corresponding lipids and of a considerable proportion of its protein content. The opalescent Band A (1.130–1.220 g/ml) was still present after centrifugation but had lost approximately half of its protein, phospholipid and cholesterol content (Fig. 1b). Peak II (1.130–1.220 g/ml, Fractions 9–13) resembled a lipoprotein peak with a chemical composition possibly corresponding to that of VHDL (very high density lipoproteins) (Table 2).

The amounts of protein and lipids in Peak I were not markedly modified by centrifugation, which indicates that this material was not of cellular origin. The density limits of Peak I

Fig. 1. Density profiles of fowl seminal plasma lipoproteins isolated by density gradient ultracentrifugation. (a) Crude seminal plasma; (b) centrifuged seminal plasma (see 'Methods'). The vertical lines correspond to the visible limits of the opalescent band.

(1.075–1.130 g/ml, fractions 6–8), and its chemical composition (50% protein, 25% phospholipids, 25% cholesterol, 1–2% triglyceride approximately) (Table 2), were consistent with the features of chicken HDL.

Polyacrylamide gradient gel (from 4 to 30% acrylamide) electrophoresis of material present in Peak I demonstrated the presence of a single particle population, with a size typical of HDL (8.3–9.2 nm, Fig. 2). The protein moiety of the material present in Peak I contained essentially one protein species which displayed a molecular weight of ~30 000 and probably corresponded to

Table 1. Concentrations of successive lipid-containing fractions isolated from chicken seminal plasma by density gradient ultracentrifugation

Peak*	Density (g/ml)	Concentration of peaks in seminal plasma (mg/ml)	
		Crude	Centrifuged
I	1.075–1.130	0.082	0.066
II (Band A)	1.130–1.190	0.785	0.541
III (Band B)	1.190	1.814	—

*I, II and III correspond to the three peaks characterized on the density profile after analysis of the different lipid and protein components in the successive density fraction (Fig. 1).

Table 2. Chemical composition (mean weight %) of the two lipoprotein fractions corresponding to Peaks I and II separated by density gradient ultracentrifugation from centrifuged cock seminal plasma

Peak	Density (g/ml)	Free cholesterol	Cholesteryl esters	Triglyceride	Phospholipid	Protein
I	1.075–1.130	5.3	15.4	4.0	23.0	52.3
II	1.130–1.220	6.7	1.2	3.3	11.5	77.2

apolipoprotein A-I, the major apolipoprotein of HDL. This protein was confirmed to be apolipoprotein A after dot-immunobinding with specific antibodies against chicken apolipoprotein A-I. Apolipoprotein A was also detected in Peak II, together with albumin and high molecular weight proteins.

Effects of HDL on the fertilizing ability of fowl spermatozoa stored for 24 h at 4°C

HDL effects. In young animals (35–43 weeks old; Fig. 3, Table 3), no significant difference was observed between treatments of spermatozoa stored with 66 µg HDL/ml or without HDL. However, the solution at 560 µg HDL/ml impaired the maintenance of fertilizing ability (90 vs 94%). In older animals (55–60 weeks old; Fig. 3, Table 3), fertilization rates were lower and differences between treatments became more evident. There were significant differences between treatments of stored spermatozoa. The lowest results were obtained with spermatozoa stored with seminal plasma (35%), spermatozoa stored with the solution used to dilute HDL (45%) and spermatozoa stored with 560 µg HDL/ml (50%). The best results were obtained with spermatozoa stored without seminal plasma in BPSE diluent (59%) or when seminal plasma was replaced by 66 µg HDL/ml (57%).

Effects of age on fertilization and hatchability rates. Higher fertilization rates were obtained with young animals than with old ones. The difference existed with fresh semen (97% versus 90%), and was significantly greater with stored semen (90–94% versus 35–59%). In young animals, stored semen gave fertilization rates (94%) similar to those of fresh unstored semen (97%) except for spermatozoa stored with 560 µg HDL/ml when fertilization rates (90%) were significantly lower ($P < 0.01$) than those obtained with fresh undiluted semen (97%). In old animals, stored spermatozoa gave lower fertilization rates (35 compared with 59%) than fresh unstored semen (90%). Hatchability was also higher for young animals (94–96% eggs hatched) than for old animals (90–93%). No difference between treatments was observed for hatchability whatever the age of the animals.

Fig. 2. Electrophoresis of native lipoprotein gradient subfractions on polyacrylamide gel slab containing a gradient of 4–30% acrylamide. Lanes 6–8 = Peak I; lanes 9–12 = Peak II; S = standard protein markers, with their respective Stokes' diameters on the right. The arrows at left indicate the position of the HDL particles.

Discussion

As in mammalian semen (Lewin *et al.*, 1974; Polakoski & Kopta, 1982), the existence of lipoproteins in cock semen (Kichev & Danov, 1972) has been suggested on the basis of electrophoretic data only. This paper is the first to describe and quantify the lipoproteins present in domestic cock seminal plasma.

From our results, the only lipoproteins present in fowl seminal plasma were particles containing apolipoprotein A-I, i.e. HDL and possibly VHDL. The seminal plasma HDL have the same qualitative characteristics as those of chicken blood plasma HDL, as defined by Hermier *et al.* (1985). They were identifiable on the basis of their density limits (1.075–1.130 g/ml compared to 1.052–1.130 g/ml in chicken blood plasma HDL), chemical composition, size (8–9 nm) and protein moiety (apolipoprotein A-I being the major component). The concentration of HDL in seminal plasma was only 66 µg/ml whereas the plasma HDL concentration in male chickens is 50–100-fold higher (Yu *et al.*, 1976; Hermier *et al.*, 1984). The origin of seminal HDL is unknown. HDL may be present in the transparent fluid which is a 'lymph'-like liquid exuded from the cloaca at ejaculation. However, the conditions of semen collection and control measures (see 'Materials and Methods') prevented the contamination of experimental samples with this fluid. Consequently, the hypothesis that seminal HDL originate from transparent fluid is very unlikely. Despite the fact that the

Table 3. Effects of HDL on fertilizing ability (fertilization rate, %) of fowl spermatozoa stored for 24 h at 4°C in young (35–43 weeks old) and old (55–60 weeks old) animals

	Young animals (1300–1500 eggs per treatment)	Old animals (550–600 eggs per treatment)
Fresh undiluted semen	97 ^a	90 ^c
Spermatozoa stored for 24 h at 4°C		
Whole semen diluted in BPSE	94 ^{ab}	35 ^f
Spermatozoa alone resuspended in BPSE	94 ^{ab}	59 ^d
Spermatozoa alone resuspended in BPSE + HDL dilution solution*	94 ^{ab}	45 ^c
Spermatozoa alone resuspended in BPSE + 66 µg HDL/ml	94 ^{ab}	57 ^d
Spermatozoa alone resuspended in BPSE + 560 µg HDL/ml	90 ^c	50 ^c

Fertilization rates were defined as in 'Materials and Methods'. Values were the mean of 8 successive inseminations for 'young animals'; 5 successive inseminations for 'old animals'.

*NaCl/NH₄ HCO₃.

Within columns, values with the same superscript do not differ significantly (χ^2 , $P > 0.01$).

Fig. 3. Effects of HDL on the fertilizing ability of fowl spermatozoa stored for 24 h at 4°C ('young' animals; 35–43 weeks old). The standard 'fresh semen' is represented by: ●—●. The results of the 4 following treatments are mixed: whole semen diluted in BPSE, spermatozoa diluted in BPSE with seminal plasma replaced by BPSE alone or by dilution solution for HDL or by 66 µg HDL/ml (○—○). Spermatozoa stored diluted in BPSE, seminal plasma replaced by 560 µg HDL/mg are represented by +—+.

synthesis and secretion of chicken HDL has been described in liver only, the possibility of synthesis or secretion in the deferent ducts cannot be excluded. However, the very low concentration of HDL in seminal plasma and the lack of larger lipoproteins is consistent with seminal HDL originating from blood plasma and passing through the blood–testis barrier which exists in fowl (see Lake, 1984, for review). HDL are lipoproteins of small size, and their transfer through the testis and possibly the deferent ducts may be easier than for the larger lipoproteins such as LDL, IDL, and VLDL. This hypothesis is consistent with previous studies reporting the size exclusion of large triglyceride-rich lipoproteins from follicular fluid (Suchanek *et al.*, 1988) or interstitial fluid (Vessby *et al.*, 1987). Similarly, in the laying hen, VLDL release from the thecal capillaries may cross the basal lamina, pass through intercellular gaps in the granulosa cell monolayer, and finally reach the ovary, only because these particles are considerably smaller than the other triglyceride-rich lipoproteins such as portomicrons or VLDL found in immature chickens (Griffin & Perry, 1985).

The ultracentrifugation procedure demonstrated that only one opalescent band persisted after the elimination of cellular fractions (1 h centrifugation at 10 000 *g*). This band was rich in proteins,

Fig. 4. Effects of HDL on the fertilizing ability of fowl spermatozoa stored for 24 h at 4°C ('old' animals; 55–60 weeks). Fresh semen (●—●). All other treatments represent original or 'reconstituted' semen stored 24 h at 4°C before artificial insemination: whole semen (○—○), semen with seminal plasma replaced by BPSE (×—×), by the dilution solution for HDL (◆—◆), by 66 µg HDL/ml (□—□) or by 560 µg HDL/ml (+—+).

including apolipoprotein A-I, and lipids. Its density limits (1.130–1.190 g/ml) and composition (78% protein, 11.5% phospholipid, 7% free cholesterol, 2.3% triglyceride, 1.7% cholesteryl esters) were consistent with those of the VHDL found in chicken plasma (1.130–1.172 g/ml) (Hermier *et al.*, 1985). Similarly, significant amounts of protein-rich lipoproteins with a density higher than that of plasma HDL were detected in human interstitial fluid (Vessby *et al.*, 1987). However, since centrifugation did not eliminate opalescence in Peak II, we cannot exclude the possibility that this VHDL-like material corresponds to a cellular residue. In the cock, a lipoprotein complex possibly responsible for the opalescence of seminal plasma has been described by Servouse *et al.* (1976). This complex was shown to contain a lipid fraction, ATPase and phosphatase acid activities, and could originate from secretions of the deferent ducts. The opalescent components described by Servouse *et al.* (1976) and the one described here could correspond to the same lipid protein complex. Its precise composition, lipoprotein identity and possible role in gamete biology require further investigation.

The effects of two concentrations of HDL on the fertilizing ability of stored spermatozoa were tested. One (66 µg/ml) was equivalent to that found in seminal plasma and had no effect on the fertilization rates under the usual storage conditions. This indicated that HDL are not responsible for the toxic effects of seminal plasma. Furthermore, a possible beneficial effect of 66 µg HDL/ml, which was masked by the toxic action of the solvent used to dilute HDL, cannot be ignored. The other dose of 560 µg HDL/ml impaired the fertilizing ability of fowl spermatozoa when stored at 4°C. HDL are cholesterol carriers and allow cellular cholesterol efflux (Eisenberg, 1984). Consequently, supraphysiological levels of HDL may decrease the stability of the sperm cell membrane, which impairs its resistance to cold shock.

In our experiments, the fertilization rates obtained with spermatozoa stored for 24 h at 4°C over the first 8 weeks of the experiment were as high as the highest levels reported in the literature (Lake & Ravie, 1979; Van Wambeke, 1984). In the young animals, there were no significant differences between fresh unstored semen and most of the treatments of semen stored for 24 h. It is possible

that the number of fertile spermatozoa was so high that it partly masked the effects of the treatments on fertilization rates (Wishart, 1985). The effect of the age of the animals appeared important since, at the end of the reproductive period, fertilization rates were lower, the fall being far more dramatic for stored semen (less 35–50%) than for unstored semen (less 7%). A female factor in this effect of age is likely (Tomhave, 1956) since there is an age-related decrease in the duration of the period over which fertile eggs are laid after one insemination (18 days at 35 weeks old compared with 10 days at 60 weeks; personal observations). Similarly, increasing age may also affect the reproductive performances of the male (de Reviers & Williams, 1984).

In conclusion, the only lipoproteins detected in fowl seminal plasma were HDL, and possibly VLDL. Despite their role as cholesterol and phospholipid carriers, HDL added at the physiological concentration were not responsible for the toxic effect of seminal plasma on in-vitro storage of fowl spermatozoa. However, the supraphysiological dose of HDL was toxic to spermatozoa, especially in older birds. It therefore appears that the studies of factors affecting sperm storage are more discriminatory when the birds are not at the optimum of their reproductive production.

We thank Dr A. D. Mills for help in correcting the English; Dr M. de Reviers for helpful discussions; and I. Mauger, J. Courtemanche and J. M. Meslier for technical assistance.

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Received 9 February 1990