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Changes in Turkey Semen Lipids During Liquid In Vitro Storage[1]

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ABSTRACT

The changes in lipid composition of spermatozoa and seminal plasma and changes in motility, viability, and morphological integrity of spermatozoa were measured in turkey semen diluted in Beltsville poultry semen extender and stored for 48 h (4°C). The total phospholipid content of spermatozoa decreased during storage, while no quantitative decrease was observed in seminal plasma. More precisely, significant decreases in phosphatidylcholine, and to a lesser extent in sphingomyeline, phosphatidylserine, and phosphatidylinositol were observed in spermatozoa. The fatty acid profile of turkey spermatozoa partly reflected diet composition and had a high level of n-9 polyunsaturated fatty acids. Neither fatty acid profile nor free cholesterol were affected by storage. The lipid composition of seminal plasma was quite different from that observed in spermatozoa and was similar to the high density lipoprotein composition of chicken seminal plasma. In vitro storage did not significantly affect lipid classes and only small changes were observed in phospholipid classes of seminal plasma. The motility, viability, and morphological integrity of spermatozoa decreased during storage. These changes in phospholipid content may be explained by membrane phospholipid lysis followed by endogenous metabolism or by a complex combination of lysis, metabolism, and peroxidation. They are likely to affect semen quality and the success of in vitro storage severely.

sperm

INTRODUCTION

Optimization of the management of turkey breeder males includes the need for efficient methods of semen storage. However, the current methods of semen storage are only effective for short periods of time (up to 12 h) and need to be improved [1]. One of the conditions necessary to store semen in vitro is a cool temperature, generally 2–5°C [2]. However, the use of low temperatures in combination with a buffered saline medium containing glycolytic substrates and intermediates of the citric acid cycle are not sufficient to ensure prolonged in vitro survival of turkey spermatozoa.

Improvements in the methods of liquid storage of spermatozoa are limited by the lack of basic knowledge of the biochemical mechanisms regulating spermatozoa functions in vivo and in vitro. Lipids are known to have a major impact on the structure and function of spermatozoa both in vivo and in vitro [3–6]. Lipids are altered in vivo by the successive processes occurring during maturation of spermatozoa in the male tract and, at least in mammals, by capacitation and acrosomal reaction prior to fertilization in the female tract [7–9]. In these species the major lipid changes particularly affect the phospholipid and cholesterol contents of the spermatozoa membranes, with consequences on membrane fluidity, stability, and fusion capacity. In birds lipids are believed to have a significant role in in vivo storage in the female uterovaginal glands [10, 11].

In all species studied to date, lipids appear to be involved in the success of in vitro semen storage: in the case of cooling without freezing, the membrane lipid moieties are in a liquid-crystalline phase that affects the physical and biochemical properties of the cells [4]. In addition, endogenous phospholipids have been found to be metabolized during in vitro storage of mammalian sperm [12, 13]. Polyunsaturated fatty acids (PUFAs) of the membrane phospholipids may also be very sensitive to in vitro lipid peroxidation. The susceptibility of chicken and turkey spermatozoa to in vitro lipid peroxidation and the positive effect of antioxidants on the success of storage have previously been reported [14–18].

A decrease in the lipid content of chicken spermatozoa has been shown to occur after 48 h of in vitro storage [19]. Turkey spermatozoa differ from fowl spermatozoa in many aspects of their metabolism [20–22] and resistance to cold storage [23]. Their fatty acid (FA) profile [24, 25] also markedly differs from that of spermatozoa of other bird species in the higher proportion of n-9 PUFAs and lower proportion of n-6 PUFAs [24]. The antioxidant activity of the turkey seminal plasma is also higher than in other bird species [25]. In contrast, the activities of the antioxidant enzymes of turkey spermatozoa are lower than in the chicken, duck, and gander [25], which could indicate specific regulation of gamete susceptibility to in vitro lipid peroxidation. However, changes in lipid composition of turkey semen during in vitro storage and in relation to semen quality have never been studied. Such basic knowledge will be extremely useful in the development of new improved methods for in vitro storage. The aim of the present study was to evaluate whether and how the lipids of turkey semen are modified by storage.

MATERIALS AND METHODS

Chemicals

Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and lysophosphatidylcholine from bovine brain; phosphatidylserine from bovine liver; sphingomyelin from chicken egg yolk; cardiolipin from bovine heart; phosphatidic acid, cholesterol, cholesterol oleate, and triolein were obtained from Sigma (St. Louis, MO). Standard FA mixtures were obtained from Sigma and Supelco (Bellefonte, PA). Eosin (eosin Y) and nigrosin (water soluble) were from Sigma. SYBR14 and PI (LIVE/DEAD sperm viability kit) were from Molecular Probes (Eugene, OR). The kit for enzymatic determination of cholesterol (Cholesterol RTU) was obtained from BioMerieux (Charbonnières les Bains, France), and solvents used for lipid extrac-

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TABLE 1. Fatty acid (FA) composition of the experimental diet.

FA	% of total FA
C14:0	1.54
C16:0	23.7
C16:1n-9	0.34
C16:1n-7	1.84
C18:0	9.79
C18:1n-9	29.8
C18:1n-7	2.08
C18:2n-6	28.7
C18:3n-3	2.25

tion and analyses were obtained from Prolabo (Fontenay Sous Bois, France).

Animals

Twenty-five males (BUT Big 6, 40 wk of age) divided in five groups of five animals each were used for experimentation. They were raised in floor pens and fed a commercial diet containing 12.4 MJ metabolizable energy/kg, 10% protein, and 3.8% lipid ad libitum. The FA composition of the diet is described in Table 1.

Semen Collection

Semen was routinely collected five times a week by dorsoabdominal massage [26]. Over the following 3 wk, the first two collections of the first 2 wk and the first collection of the third wk were used for the experiments. Consequently five collections for each of five groups were used for the analyses. Care was taken to avoid any contamination of semen with cloacal products.

Semen Treatment

After each collection, pools of semen (each pool originating from five males) were diluted (1:3) in Beltsville poultry semen extender (BPSE) [27], shaken (200 times/min with agitator KS 125basic, Labortechnik, Staufen, Germany), and stored for 48 h at 4°C. The spermatozoa concentration was estimated using a photometer (IMV, L'Aigle, France) at a wavelength of 545 nm [28]. Aliquots were removed at 1 h, 4 h, 24 h, and 48 h after collection for further measurement of semen quality and lipid analysis of whole semen and of spermatozoa and seminal plasma separated by centrifugation (600 × *g*, 15 min, 4°C) just before lipid analysis.

Lipid Analyses

Lipids of whole semen, spermatozoa, and seminal plasma were extracted with chloroform:methanol (v:v, 1:2) by the method of Bligh and Dyer [29]. The phospholipid content of whole semen was evaluated by measuring total phosphorus content of the lipid extracts [30].

The determination of lipid and phospholipid classes was performed on both spermatozoa and seminal plasma. After extraction, 1 ml of the chloroform phase was dried under N_2 and used for determination of total lipid classes and phospholipids, after resolubilization with 20 μl of chloroform. Lipid and phospholipid classes were separated by thin-layer chromatography on silica rods. The migration conditions comprised 20 min in a solvent composed of hexane:ethylether:acetic acid (v:v:v, 87:13:0.75) for the lipid classes and 45 min in a solvent composed of chloroform: methanol:water (v:v:v, 80:35:3) for the phospholipid classes. Detection and quantification were performed on an Iatroscan (Iatron, Tokyo, Japan). Lipid and phospholipid classes were identified using standard mixtures. Total cholesterol content of spermatozoa and seminal plasma was quantified with an enzymatic kit [31] after evaporation of 1 ml of the chloroform phase and resolubilization in 100 μl of isopropanol. Combination of the cholesterol content and the proportions of various lipids and phospholipids allowed calculation of the spermatozoa and seminal plasma content of each lipid and phospholipid.

Fatty acids of spermatozoa were separated and identified from their methyl ester derivatives by gas liquid chromatography on a BPX70 column 50 by 0.32 mm (0.25 μm film thickness) (SGE, Ringwood, Victoria, Australia). Analyses were performed on a gas chromatograph GC 8000 (Fisons Instrumentals spa, Milan, Italy) equipped with a column injector and a flame ionization detector (270°C). Helium was used as carrier. Fatty acids were identified using standard mixtures.

Semen Quality

Motility was assessed under light microscopy (250×) on two scales: massal motility ranged from 0 to 10, giving a general view of the type and intensity of spermatozoa movement and the impact on movement of the number and size of agglutinations [32], and the second ranging from 0 to 100 and representing the proportion of motile spermatozoa.

Viability was assessed by two techniques: SYBR141/PI staining [33, 34] and eosin-nigrosin smears [35]. The latter is the only routine test allowing the observation of morphologically abnormal spermatozoa. The times scheduled did not permit performance of each test at each time, and viability was measured at 4 h and 24 h of semen storage by SYBR14/PI while viability and morphological integrity evaluated with eosin-nigrosin was measured at 1 h, 4 h, 24 h, and 48 h. The slides were examined for SYBR14/PI using fluorescence microscopy (Zeiss MC 100spot) (600×). Membrane-damaged cells showed red fluorescence (PI) and viable spermatozoa green fluorescence (SYBR14). With the eosin-nigrosin method, spermatozoa were considered to be viable and normal when they were not stained and when morphology was normal from head to tail. All other shapes were considered to be abnormal. Magenta-stained spermatozoa were considered to be dead. The proportions of viable and morphologically normal spermatozoa measured with eosin-nigrosin smears were then estimated under light microscopy (Zeiss MC 100spot) (600×); two slides were observed per sample for both techniques and 300 spermatozoa counted per slide. All the quality tests were performed by the same person.

Statistical Analyses

Changes in the lipid components of whole semen, spermatozoa, and seminal plasma and changes in motility, viability, and morphological integrity of spermatozoa were analyzed by analysis of variance, followed by the Fisher protected least significant difference test [36].

RESULTS

Lipid Composition of Fresh Semen

Lipid content of fresh turkey spermatozoa. The major lipid components in fresh spermatozoa were phospholipids and free cholesterol (Table 2). Only traces of cholesteryl

TABLE 2. Lipid content of fresh turkey semen.[a]

Variable	Spermatozoa	Seminal plasma
Total lipids (μg/10^9 spermatozoa)	677 ± 42	124 ± 17
% of total lipids		
Free cholesterol	16.2 ± 0.3	31.1 ± 2.3
Cholesteryl esters	<1%	12.3 ± 1.6
Triglycerides	ND	6.8 ± 0.8
Free FA	ND	ND
Phospholipids	83.7 ± 0.6	50.7 ± 2.24
% of total phospholipids		
PC	39.0 ± 0.9	4.6 ± 0.3
PE	29.5 ± 0.9	31.5 ± 2.4
SM	14.2 ± 0.4	20.3 ± 1.5
PI + PS	9.1 ± 0.7	14.9 ± 2.4
DPG + PA	5.2 ± 0.3	8.2 ± 1.0
LPC	3.1 ± 0.3	20.2 ± 5.2

[a] Results are expressed as mean ± SEM, n = 5; ND, not detected.

esters (CE) and triglycerides (TG) were detected. The major phospholipids were phosphatidylcholine (PC, 39%), phosphatidylethanolamine (PE, 29.5%), and sphingomyelin (SM, 14.2%). Phosphatidylinositol plus phosphatidylserine (PI + PS) and phosphatidic acid plus diphosphatidylglycerol (PA + DPG) represented 9.1% and 5.2% of the total phospholipids, respectively. Lysophosphatidylcholine (LPC) was a minor component (3.1%).

Saturated FA (C18:0 and C16:0) accounted for half of the total FA (Table 3). Monounsaturated FA (MUFAs) accounted for 17.9% and PUFAs for 32.7% of total FA. The n-9 FA (C18:1n-9 and C20:1n-9) were predominant in the MUFAs, whereas PUFAs were predominantly represented by n-6 FA (72% of PUFAs) and n-9 FA (27% of PUFAs).

Seminal plasma of fresh turkey semen. Seminal plasma (Table 2) differed from spermatozoa by a far lower lipid content, a lower proportion of phospholipids in total lipids, a lower proportion of PC, and a higher proportion of LPC in phospholipids. By contrast to spermatozoa, there were significant amounts of CE and TG.

Evolution of Lipid Composition of Semen During 48 h of Storage at 4°C

Whole semen. The total phospholipid content of whole semen decreased by about 30% during the first 24 h of in vitro storage (from 1170 to 867 nmol/10^9 spermatozoa) and then remained constant during the following 24 h.

Spermatozoa. The phospholipid content of spermatozoa decreased by 30% between 1 h and 24 h of storage. Most of the reduction occurred between 1 and 4 h (20%). After 24 h of storage, the phospholipid content remained constant (Table 4). The amount of cholesterol did not change throughout the 48 h of storage. Consequently, the cholesterol:phospholipid ratio increased from 0.19 to 0.28 between 1 h and 48 h storage. The loss of phospholipids originated from a decrease in PC (without an increase in LPC), SM, and PI + PS. The PC:PE ratio therefore seriously decreased during storage. For all these phospholipids, the major changes occurred during the first hours of storage.

Despite considerable changes in phospholipid content and proportion of phospholipid classes, the FA profile of spermatozoa was not significantly affected by in vitro storage (Table 3).

Seminal plasma. The storage effects were less severe in seminal plasma than in spermatozoa (Table 5). The various lipid classes did not change significantly during in vitro storage. Changes in phospholipids were small but significant: PI + PS and LPC decreased between 1 and 48 h while PC increased. However, the increase in PC in seminal plasma represented a small proportion (about 6%) of the reduction in PC in spermatozoa.

Evolution of Semen Quality over 48 h of In Vitro Storage

The massal motility of spermatozoa (Fig. 1) decreased over 48 h ($P < 0.05$) corresponding to a reduction in the intensity of movement of the spermatozoa and to an increase in the number and size of agglutinations. The proportion of motile spermatozoa was 40% lower after 48 h of storage (Fig. 2). As measured by eosin-nigrosin smears,

TABLE 3. Spermatozoa FA composition (as a percentage of total fatty acids).[a]

	Time of storage (h)			
FA	1	4	24	48
C14	<1	<1	<1	<1
C16	21.6 ± 1.6	20.4 ± 1.0	19.0 ± 1.7	22.3 ± 1.7
C18	26.9 ± 1.4	26.9 ± 0.9	25.7 ± 1.3	27.9 ± 1.3
Saturated	48.9 ± 1.9	47.4 ± 1.3	44.7 ± 2.1	50.3 ± 1.8
C16:1n-9	<1	<1	<1	<1
C16:1n-7	<1	<1	<1	<1
C18:1n-9	5.5 ± 0.3	5.6 ± 0.1	5.3 ± 0.2	4.9 ± 0.1
C18:1n-7	2.1 ± 0.2	2.4 ± 0.1	2.4 ± 0.2	2.1 ± 0.2
C20:1n-9	8.1 ± 0.5	9.0 ± 0.3	8.4 ± 0.5	7.0 ± 0.4
C22:1n-11	1.1 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	<1
C24:1	<1	<1	<1	<1
Monounsaturated	17.9 ± 0.6	19.4 ± 0.5	18.3 ± 0.9	15.8 ± 0.4
C18:2n-6	3.0 ± 0.1	3.0 ± 0.1	3.3 ± 0.2	3.3 ± 0.1
C20:2n-6	2.9 ± 0.2	3.0 ± 0.2	3.4 ± 0.3	3.3 ± 0.2
C20:4n-6	7.4 ± 0.5	7.3 ± 0.5	8.8 ± 0.6	8.1 ± 0.5
C22:4n-6	9.9 ± 0.6	10.0 ± 0.5	11.2 ± 0.7	9.9 ± 0.5
Total n-6	23.2 ± 0.7	23.3 ± 0.6	26.8 ± 0.7	24.6 ± 0.6
C22:3n-9	8.6 ± 1.1	8.9 ± 0.5	9.5 ± 0.6	8.6 ± 0.6
C22:5n-3	<1	<1	<1	<1
C22:6n-3	<1	<1	<1	<1
Total n-3	0.9 ± 0.1	0.7 ± 0.08	1.15 ± 0.1	0.9 ± 0.09
Total PUFAs	32.7 ± 1.3	33.1 ± 0.8	37.4 ± 0.9	34.1 ± 1.2
Total unsaturated	50.6 ± 1.3	52.5 ± 1.2	55.7 ± 1.8	49.9 ± 1.6

[a] Results are expressed as mean ± SEM; n = 5.

TABLE 4. Evolution in the lipid content (μg/10^9 spermatozoa) of spermatozoa during in vitro storage.[a]

Variable	Time of storage (h)			
	1	4	24	48
Free cholesterol	107 ± 5[b]	96 ± 7[b]	91 ± 6[b]	98 ± 6[b]
Cholesteryl esters	ND	ND	ND	ND
Triglycerides	ND	ND	ND	ND
Free fatty acids	ND	ND	ND	ND
Total phospholipids	570 ± 39[b]	469 ± 41[b,c]	413 ± 33[c,d]	412 ± 46[d,e]
Cholesterol/phospholipids	0.197 ± 0.005[b]	0.213 ± 0.008[b,c]	0.232 ± 0.011[c,d]	0.288 ± 0.032[d]
PC	222 ± 13[b]	186 ± 14[c]	141 ± 9[d]	137 ± 11[d]
PE	168 ± 12[b]	144 ± 14[b]	137 ± 10[b]	150 ± 14[b]
SM	81 ± 5[b]	68 ± 5[c]	52 ± 4[c]	57 ± 5[c]
PI + PS	52 ± 4[b]	28 ± 3[c,d]	31 ± 5[c]	23 ± 2[d]
DPG + PA	30 ± 3	26 ± 4	34 ± 5	30 ± 3
LPC	17 ± 1	14 ± 1	19 ± 2	16 ± 2
PC/PE	1.32 ± 0.07[b]	1.30 ± 0.1[b]	1.02 ± 0.07[c]	0.96 ± 0.04[d]

[a] Results are expressed as mean ± SEM; n = 5; ND, not detected. Different letters indicate significant difference ($P < 0.05$) between storage durations.

there were few dead cells and the numbers rose and were significant only after 48 h of storage (Table 6). Using SYBR14/PI, the number of dead cells significantly increased ($P < 0.05$) between 4 h and 24 h of storage (Table 6). The proportion of abnormal cells was considerably increased during the first 24 h (especially between 1 and 4 h) and subsequently remained constant (Table 6). The major morphological abnormalities observed were bent intermediate piece (increasing from 14% to 30% of total spermatozoa over 48 h) and plasma membrane swelling (increasing from 7% to 20% over 48 h).

DISCUSSION

The importance of lipids in gamete biology is now well documented. They act in vivo in various physiological [6, 37] and metabolic [38] processes, and are biological precursors of steroids, prostaglandins, and second messengers [7]. In addition, they actively participate in the processes leading to gamete fusion [7]. In vitro they are involved in mechanisms of cell resistance to cold shock [39] and aerobic peroxidation [40] and are believed to be metabolized actively [12, 13].

The composition of total lipid classes of fresh turkey spermatozoa reported here is close to that described by Cerolini et al. [24]. However, free FA were not detectable in our study, indicating that the experimental procedure for semen collection and spermatozoa separation did not result in significant hydrolysis of cellular lipids. The overall phospholipid composition of fresh turkey spermatozoa is fairly similar to that previously described in the chicken [4, 19, 24] and the turkey [24], with a predominance of choline-containing phospholipids (PC, SM, LPC). However, the phospholipids in turkey spermatozoa in the present study differed from those described by Cerolini et al. [24] in a lower proportion of PE and a higher proportion of SM and cardiolipin (DPG + PA). In the present study, the high proportion of n-9 FA (25%) and particularly of long-chain n-9 PUFAs (8.1% 20:1n-9 and 8.6% 22:3n-9) may result from both the diet (containing 30% of 18:1) and hepatic lipogenesis, which is paralleled by very active Δ9 desaturation in birds [41]. These long-chain FA derive from elongation and desaturation of the precursor, 18:1n-9. Because the affinity of elongase and desaturases is lower for n-9 than for n-3 and n-6 FA, the presence of long-chain n-9 FA is usually indicative of a dietary deficiency in essential FA (n-3 and n-6 series) [42]. Dietary deficiency cannot be excluded in the present study, because 18:2n-6 and 18:3n-3 accounted for 29 and 2–3% of dietary FA, respectively, which is the lower limit of nutritional needs. However, in the rat, epididymal spermatozoa have been shown to contain large amounts of 22:4n-9 PUFAs [43], which indicates that desaturase and elongase activities may also exist in the testis and may use n-9 FA as substrates independently of any nutritional deficiency.

Seminal plasma contains significant amounts of lipids (Table 2). The predominance of SM and the very low proportion of PC show that seminal plasma lipids do not just originate from spermatozoa membrane degradation. Lipid concentration and composition is similar to that of high- and very-high-density lipoproteins that are normally found

TABLE 5. Evolution of the lipid contents (μg/10^9 spermatozoa) of seminal plasma during in vitro storage.[a]

Variable	Time of storage (h)			
	1	4	24	48
Free cholesterol	32 ± 3	31 ± 3	32 ± 2	31 ± 2
Cholesteryl esters	15 ± 3	16 ± 4	12 ± 2	9 ± 1
Triglycerides	8 ± 1	9 ± 2	7 ± 1	6 ± 1
Free FA	ND	ND	ND	ND
Total phospholipids	69 ± 15	57 ± 11	60 ± 6	58 ± 7
PC	4 ± 1[c]	4 ± 0.6[c]	5 ± 0.8[c]	9 ± 1.4[b]
PE	22 ± 3	19 ± 2	25 ± 3	24 ± 4
SM	14 ± 3	17 ± 2	15 ± 3	14 ± 2
PI + PS	10 ± 3[b]	6 ± 1[c]	5 ± 1[c]	2 ± 0.3[c]
DPG + PA	6 ± 2	5 ± 1	4 ± 1	5 ± 1
LPC	14 ± 3[b]	5 ± 1[c]	5 ± 1[c]	4 ± 1[c]

[a] Results are expressed as mean ± SEM; n = 5; ND, not detected. Different letters indicate significant difference ($P < 0.05$) between storage durations.

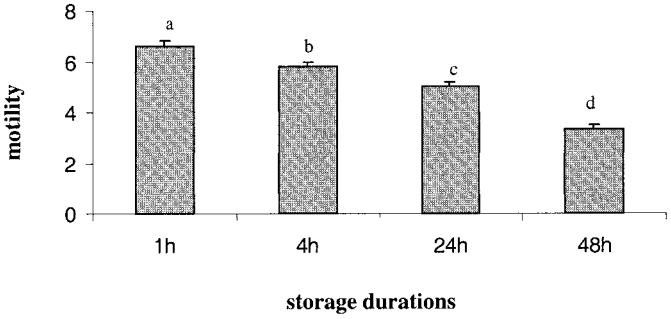

FIG. 1. Evolution of motility during in vitro storage. Results are expressed as mean ± SEM, n = 5; a–d indicate significant difference ($P < 0.01$) between storage durations.

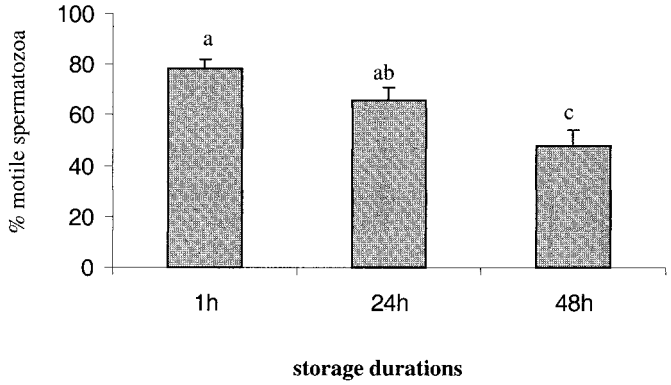

FIG. 2. Evolution of proportion of motile spermatozoa during in vitro storage. Results are expressed as mean ± SEM, n = 5; a–c indicate significant difference ($P < 0.05$) between storage durations.

TABLE 6. Evolution of viability and morphological integrity of viable spermatozoa during in vitro storage.[a]

Storage duration (h)	Viable spermatozoa (% total spermatozoa)		Morphology of viable spermatozoa (% of total spermatozoa)	
	SYBR14/PI	Eosin-nigrosin	Normal	Abnormal
1		91.8 ± 0.4[b]	58 ± 2.7[g]	33 ± 2.5[j]
4	68.2 ± 1.8[d]	90.9 ± 0.8[b]	41 ± 2.4[i]	49 ± 2.4[h]
24	64.5 ± 1.2[e]	90.9 ± 0.9[b]	26 ± 1.8[k]	64 ± 1.7[e]
48		87.2 ± 1.3[c]	25 ± 1.6[k]	61 ± 1.1[f]

[a] Results are expressed as mean ± SEM; n = 5. Different letters indicate significant difference ($P < 0.05$) between storage durations.

in chicken seminal plasma [44]. It is likely that these lipoproteins also contribute to plasma seminal lipids in the turkey.

The present study is the first to show the decrease in turkey spermatozoa phospholipids without corresponding increase in seminal plasma and the parallel decrease in the quality of spermatozoa. Previous studies on chicken semen showed either no significant difference in lipids for unstored spermatozoa and spermatozoa incubated in very stressful conditions [45, 46] or a decrease in the spermatozoa phospholipids [19].

The decrease in spermatozoa phospholipids in our study mostly affects PC and PI + PS and to a lesser extend SM. Phosphatidylcholine is the major spermatozoa phospholipid, and its decrease was not paralleled by a corresponding increase in PC in seminal plasma or by a substantial increase in LPC in either seminal plasma or spermatozoa. Moreover, FA composition is not affected by in vitro storage. This indicates that 1) the decrease in phospholipids is not due to a simple shift from spermatozoa membranes to seminal plasma and 2) both FA chains of PC are involved in the process.

Therefore, the considerable and specific PL loss during storage may result from phospholipid hydrolysis followed by FA peroxidation and/or endogenous metabolism.

Phospholipid degradation may start with phospholipase actions. In mammalian semen, a wide variety of phospholipases (A, C, D, LP) actively participate in the preparation of spermatozoa for fertilization [47]. This is the first paper suggesting the activity of such enzymes in turkey semen and their probable action during in vitro storage. The degradation of acyl chains following phospholipase action depends primarily on the type of active phospholipases. Phospholipase A1 or A2 will induce release of only one of the two acyl chains of a phospholipid. If this had been the case in our results, we would have observed an increase in LPC. This indicates that lysophospholipases are active in our system, liberating the second acyl chain. The two liberated acyl chains may further be degraded by beta-oxidation.

This endogenous metabolism of FA could explain why FA are not detected on the lipid profile of spermatozoa after storage. Endogenous phospholipid metabolism has been previously described in mammalian spermatozoa [12] but not in birds [45, 46]. Bird spermatozoa are cells without intracytoplasmic energy reserves, and the preferential substrates for energy metabolism of turkey spermatozoa are still unknown. In vitro, the major substrate present in the storage medium (BPSE) is fructose and probably unknown components of seminal plasma. In addition, turkey spermatozoa have low glycolytic activity [20], which could suggest the need for another metabolic pathway especially metabolism of FA.

Alternatively, the loss of phospholipids might result from FA peroxidation. Indeed, although far lower than in chicken, the fairly high PUFA content (33%) in turkey spermatozoa predisposes them to lipid peroxidation. Oxygen is involved in lipid peroxidation but is also necessary for in vitro turkey survival [22]. Addition of antioxidants to the diluent increases semen quality [17]. However, significant lipid peroxidation in our study would have involved the preferential degradation of PUFAs and a corresponding decrease in the proportion of PUFAs in the FA profile. This was not the case, and consequently lipid peroxidation is not a preferential hypothesis to explain the phospholipid loss in the present study.

In birds, especially in turkeys, semen quality and fertility are generally decreased when semen is stored for more than 6 h or 24 h in vitro [48–50], leading to unsuccessful rational management of adult males. In the present study, clearly the decrease in quality and lipid content of spermatozoa are parallel. However, the different quality parameters measured do not give the same information. Motility tests (massal motility and the proportion of motile spermatozoa) and the proportion of morphologically normal living spermatozoa decrease considerably in 48 h. Viability, as measured by eosin-nigrosin is also altered but to a lesser extent. Eosin-nigrosin is known to show fewer dead spermatozoa than SYBR14/PI [34]. Measurements performed on other samples with SYBR14/PI indicate 15% loss of viable spermatozoa over 48 h (unpublished observations), suggested by the evolution presented in the present paper between 4 and 24 h. This decrease in viability is still far lower than the decreases in morphological and motility semen characteristics.

In addition, the major abnormal forms are separated into two categories: The must numerous (30% of total spermatozoa at 48 h) concerns bent intermediate pieces that could be related to motility alterations and indicate that the region of spermatozoon involved in energy pathways is more susceptible to injury during storage than acrosomal and nuclear regions. The second relevant category of abnormal living spermatozoa is the swollen cells (20% of total spermatozoa at 48 h) corresponding to tension on the cytoplasmic membrane that might represent weakening and partial changes in permeability. These two categories of abnormality are preliminary stages before disruption of the membrane and may be related to the lipid modifications observed. The spermatozoa phospholipids affected by in vitro storage are PC, PS + PI, and SM. Loss of PC is greater, which could

mean that a PC-enriched region of the spermatozoon is preferentially affected by storage. However, the decreases in the proportions of PS + PI and SM are also greater than the decrease in PE. These phospholipids are mostly believed to remain preferentially on the outer part of the plasma membrane [51, 52].

Variations in the PL equilibrium of the outer and inner part of the membrane, especially in the PC:PE ratio, are known to affect membrane stability [53]. The PC:PE ratio was so affected by storage in the present study that it was inferior to 1 after 24 h, with a consequent decrease in the curvature of the plasma membrane. Thus, the membrane may be unable to ensure its cohesion after storage and possibly disrupt. This may be one factor increasing the number of abnormal and, to a lesser extent, the number of dead spermatozoa during storage. Most probably the abnormal forms (especially those affecting the intermediate piece) are related to the low motility results. Loss of morphologic integrity and motility will limit the capacity of spermatozoa to migrate in the female tract up to the uterovaginal glands (UVG) and further to the site of fertilization. In addition, damaged spermatozoa will probably have limited capacity to survive in the UVG.

In conclusion, the phospholipid profile and content of turkey spermatozoa are severely affected by in vitro storage, and the evolution of phospholipids is parallel to the decrease in semen quality. This could preferentially originate from the endogenous metabolism of the FA of the membrane phospholipids and induce membrane destabilization. However, it is not possible to exclude a combination of many complex factors, including phospholipid lysis, endogenous metabolism, and lipid peroxidation. In order to improve storage methods, our results may lead to the supplementation by more appropriate metabolic substrates and phospholipase inhibitors among the diluents.

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