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Original article

Cholesterol/phospholipid ratio in sperm of several domestic species does not directly predict sperm fitness for cryopreservation

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Abstract – Sperm cryopreservation is used for preservation and diffusion of genetic diversity and genetic progress. One approach to improve cryopreservation is to identify and control the cellular parameters responsible for intrinsic sperm fitness for cryopreservation. The aim of this study was to determine if a relationship exists between sperm cholesterol/phospholipid molar ratio (CHO/PL) and sperm fitness for cryopreservation. CHO/PL in goat and boar semen were respectively 0.281 ± 0.048 ($n = 112$) and 0.375 ± 0.043 ($n = 47$). CHO/PL of washed stallion spermatozoa was 0.541 ± 0.072 ($n = 17$). In stallion spermatozoa and goat semen, CHO/PL and sperm motility after thawing were negatively correlated, although the significance of this relation fluctuated between ejaculates (stallion) and between months of collection (goat). In boar, mobility parameters after thawing were not related to CHO/PL, but a positive correlation was noted between CHO/PL and the percentage of spermatozoa alive and normal 120 min after thawing. No relationship was found between blood plasma cholesterol (goat: 1160 $\text{nmol.mL}^{-1} \pm 186$; boar: 1410 $\text{nmol.mL}^{-1} \pm 200$) or seminal plasma cholesterol (boar sperm rich fraction: 104 $\text{nmol.mL}^{-1} \pm 52$) and sperm CHO/PL. To conclude, sperm CHO/PL is not a direct indicator of sperm fitness for cryopreservation. However, as supported by the observed correlation, it could not be ruled out that CHO/PL is involved in the cryopreservation success.

sperm / cryopreservation / cholesterol / lipid / phospholipid

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Résumé – Le rapport cholestérol/phospholipide dans le sperme de diverses espèces domestiques n'est pas un indicateur direct de l'aptitude à la congélation du sperme. La congélation du sperme est utilisée pour la conservation et la diffusion de la diversité et du progrès génétique. Une approche permettant d'améliorer la qualité du sperme congelé est d'identifier et de contrôler les paramètres cellulaires impliqués dans l'aptitude intrinsèque du sperme à la congélation. Le but de la présente étude était de déterminer si une relation existait entre le rapport molaire cholestérol/phospholipide (CHO/PL) du sperme et son aptitude à la congélation. Le rapport CHO/PL de la semence de bouc et de verrat était de 0.281 ± 0.048 ($n = 112$) et 0.375 ± 0.043 ($n = 47$) respectivement. Le rapport CHO/PL des spermatozoïdes lavés d'étalon était 0.541 ± 0.072 ($n = 17$). Pour les spermatozoïdes d'étalon et la semence de bouc, une corrélation négative a été trouvée entre ce rapport et les paramètres de mobilité des spermatozoïdes après décongélation bien que la signification statistique de cette relation soit fluctuante entre éjaculats (étalon) et entre les mois de collecte (bouc). Chez le verrat, les paramètres de mobilité après décongélation n'ont pu être corrélés au rapport CHO/PL, mais une corrélation positive est apparue entre le rapport CHO/PL et le pourcentage de spermatozoïdes vivants normaux 120 min après la décongélation. Aucune relation n'a été trouvée entre le cholestérol du plasma sanguin (bouc : 1160 nmol.mL^{-1} ± 186; verrat : 1410 nmol.mL^{-1} ± 200) ou du plasma séminal de la fraction riche de la semence (verrat : 104 nmol.mL^{-1} ± 52) et le rapport CHO/PL. En conclusion, le rapport CHO/PL n'est pas un indicateur direct de l'aptitude d'une semence à la congélation. Cependant, les corrélations observées ne permettent pas d'exclure que le rapport CHO/PL est impliqué dans le succès de la congélation.

sperme / cryoconservation / cholestérol / lipide / phospholipide

1. INTRODUCTION

In domestic species, methods for sperm preservation at low temperature and sperm cryopreservation are widely used in the sharing of genetic advances. Sperm cryopreservation also allows the *ex situ* preservation of genetic diversity in many mammalian, bird and fish species. Besides, the use of cryopreserved sperm extends the reproductive period of females under photoperiodic control to obtain offspring all year long, which has important economic consequences.

However, almost one third of male goats, pigs and horses shows a marked unsuitability for sperm cryopreservation. Among males selected for their fitness for sperm cryopreservation, many ejaculates have to be removed from the cryopreservation program because of low spermatozoa viability at thawing. These rejections due to intra-male variability are costly and raise the question of a possible loss of genetic diversity, with the risk of selecting or losing genetic traits carried or not by the cryoresistant spermatozoa.

To date, most improvements in sperm cryopreservation methods have been obtained by success-error trials, but further progress will likely require a better knowledge of the parameters involved in sperm cryoresistance. Membrane lipids are often described as having a role in cell cryopreservation, mainly because of

their influence on the more or less heterogeneous phase transition withstood by the plasma membrane during cooling (reviewed by [16]). As proposed by Drobnis *et al.* [7], a high cholesterol content in the membrane could be a key parameter in broadening and flattening the lipid phase transition in sperm cells, thus reducing the damage induced by phase separation. However, once lipids are in the gel phase, the bulkiness of cholesterol induces unpacking of lipids which may lead to membrane destabilization. Although cholesterol is a common component in spermatozoa plasma membrane, it has to be removed during the capacitation process to allow acrosome reaction of spermatozoa and fertilization of the egg.

Such properties of cholesterol complicate the characterization of its role in spermatozoa fitness for cryopreservation. In interspecies comparisons, the high viability of human spermatozoa or turbot spermatozoa after freeze-thawing, when compared to boar or trout, is often proposed as being linked to the highest cellular cholesterol/phospholipid molar ratio (CHO/PL). This is at odds with comparisons within one species such as fowl [1] and rainbow trout [12]. In both cases, a high spermatozoa CHO/PL was correlated with a low fitness for cryopreservation. In ram spermatozoa, Parks and Hammerstedt [13] showed that the molar ratio CHO/PL increases during epididymal maturation and spermatozoa taken from the caput epididymis (lower CHO/PL) are less affected by cold shock than those from the cauda epididymis [17]. Although indirect, these observations suggest that a low CHO/PL favors cold shock resistance in ram spermatozoa.

The present study was conducted on boar, goat and stallion spermatozoa. The hypothesis of a relation between spermatozoa CHO/PL and spermatozoa quality after cryopreservation was investigated in each species.

2. MATERIAL AND METHODS

In the following sections, semen will refer to unwashed ejaculates (spermatozoa + seminal plasma). Spermatozoa will refer to the male gametes only while sperm will be a general designation which will refer equally to spermatozoa in seminal fluid or in extender.

2.1. Collection and treatment of semen

Goat semen was collected twice a week from 86 alpine breed and 89 Saanen breed young bucks during their first sexual season from September to February. Only one randomly chosen ejaculate from each male was used in this study. The cell concentration of the ejaculates was 1.328×10^9 spermatozoa/ml$\pm$0.467 (SD). Semen was also collected from 12 adult bucks (10 alpine and 2 Saanen) from January 1998 to August 1998. Every month, one randomly chosen ejaculate from each male was used for this study. Semen was collected into an

artificial vagina at 37 °C. Ejaculates into collection tubes were then kept in a water bath at 30 °C. The volume was determined and spermatozoa concentration was assessed with a spectrophotometer. The remaining sample was used for lipid content determination of total semen and washing before cryopreservation.

Boar semen was collected by the manual hand-gloved method from 47 boars belonging to eight genotypes. Only the spermatozoa-rich fraction was used in this study. The volume (mL) was determined by weighing the sample and spermatozoa concentration was assessed with a spectrophotometer. The average concentration was 273.9×10^6 spermatozoa/ml $\pm$ 42.9 (SD). The remaining sample was used for lipid content determination of total semen and washing before cryopreservation.

Stallion semen was collected into an artificial vagina at 37 °C from 14 Selle Français and 3 Trait Breton. After filtration on gauze, spermatozoa concentration and spermatozoa quality were determined and the semen was used for washing before lipid content determination of washed spermatozoa and cryopreservation.

2.2. Lipid content determination

For *goat and boar semen*, 150 μL of each ejaculate (unwashed spermatozoa) were inserted into a 0.25 mL straw (IMV, France), or 50 μL were put into a microtube. The straws and microtubes were directly plunged into liquid nitrogen and stored before lipid extraction and assay. Boar seminal plasma was collected from 1 ml of the spermatozoa-rich fraction after centrifugation at 600 g for 5 min at 4 °C and filtration of the supernatant through a 0.45 μm syringe filter. Blood was collected from the same males as those whose sperm was collected, and centrifuged at 3000 g for 20 min at 4 °C. The blood and seminal plasma were frozen before cholesterol content determination.

For *stallion semen*, 400 to 500 $\times$ 10^6 spermatozoa were diluted (v/v) in Hank's salts supplemented with 20 mM Hepes (HH) and centrifuged at room temperature for 5 min (600 g). This washing procedure allowed the removal of prostasome-like particles very rich in cholesterol described by Arienti *et al.* [2]. The cell pellet was resuspended in 1.5 mL HH and injected in 0.5 mL straws. These were then frozen over liquid nitrogen vapor and stored in liquid nitrogen before lipid content determination. CHO/PL was determined on two ejaculates from the same male.

Sperm lipids of the three species were extracted according to Bligh and Dyer [4] and resuspended in isopropanol before phospholipid and cholesterol determination. Phospholipid content was assessed by the phosphorus assay according to Rouser *et al.* [19]. Cholesterol content was assayed with a kit from Boehringer Mannheim (Roche Diagnostics, Meylan, France, ref. 1442341). For the lipid samples in isopropanol, the cholesterol standard curve was determined

in isopropanol while the determination of blood and seminal plasma cholesterol was performed in aqueous solution.

2.3. Sperm freeze-thawing

Goat semen was treated according to the cryopreservation procedure described by Corteel [6]. Briefly, unwashed semen at 30 °C was first diluted in Krebs-Ringer-Glucose solution before centrifugation to remove the seminal plasma. The pellet was resuspended in the skim milk extender to obtain a concentration of 10^9 cells.mL^{-1}, at +4 °C. A final dilution was made using the milk-based extender with 7% of glycerol to obtain 100×10^6 spermatozoa per 0.25 mL straw. The straws were then placed 16 cm above the liquid nitrogen surface for 2 min and at 4 cm for 3 min before they were plunged into liquid nitrogen. Before use, the straws were thawed for 30 s in a 37 °C water bath.

Boar unwashed semen was diluted (1:1) in BTS [15] at 28 °C and cooled for 2 h to 15 °C. Diluted semen was centrifuged at 800 g for 25 min and the cell pellet was resuspended in the cooling diluent (D1: lactose 11%, egg-yolk 20%, Orvus es paste Equex Nova Chemical 1.6%, in water) to obtain a final concentration of 1.7×10^9 spermatozoa.mL^{-1}. Sperm was then cooled for 2 h to 5 °C. One volume of diluted sperm was added to one volume of D1 containing 6% glycerol in order to obtain a 3% glycerol concentration in the final solution. This second dilution (glycerolization) was performed in three steps to prevent osmotic shock. After 90 min, sperm was injected into 0.5 mL PVC straws (IMV France) each containing 0.8×10^9 cells. Straws were placed 4 cm above liquid nitrogen ($t = -140$ °C) for 5 min before being plunged into liquid nitrogen. Straws were thawed for 12 s in a 55 °C water bath. Thawed spermatozoa were then diluted in BTS at 37 °C to obtain 40×10^6 cells.mL^{-1}.

Stallion semen was processed for freezing according to Vidament *et al.* [20]. Briefly, unwashed semen was diluted in INRA82 extender supplemented with 2% egg yolk at 37 °C and cooled to 22 °C (10 min) before centrifugation at room temperature for 10 min at 600 g. The pellet was suspended in INRA82 +2% egg yolk +2.5% glycerol at a concentration of 100×10^6 spermatozoa.mL^{-1} and cooled to 4 °C for 60 to 90 min. The diluted sperm was injected into 0.5 mL straws, frozen with an automated freezer from 4 °C to −140 °C at 60 °C·min^{-1} (freezing rate) and then plunged into LN2. Thawing was performed at 37 °C for 30 s.

2.4. Sperm quality assessment

Goat sperm quality was assessed after thawing using one straw per ejaculate. The percentage of motile spermatozoa and the progressive mobility (scale 0 to 5) were determined at 37 °C under a coverslip using phase-contrast microscopy (×200).

Boar sperm quality was assessed immediately after collection, 10 min and 120 min after thawing, the spermatozoa being kept at 37 °C in a water bath. Spermatozoa motility (% of mobile cells) and mobility score (scale 0 to 5 [3]) were assessed by observation of 10 μl of sperm on a 37 °C glass side at X 100 magnification and phase contrast. Spermatozoa viability and morphology were assessed using eosin-nigrosin staining and observation of 200 cells at X 1000 magnification [8].

Stallion sperm motility after thawing was estimated with three straws per ejaculate using a computer-assisted sperm analyzer (HTM, Hamilton-Thorne, Denvers, USA). The evaluation of motility was performed on two drops per straw after dilution and three fields of observation per drop. From 6 to 27 ejaculates were used to test the males.

3. RESULTS

3.1. Goat

Sixteen bucks were separated into two groups on the basis of their spermatozoa average motility after freeze-thawing assessed during their breeding season. CHO/PL was determined from one random ejaculate collected at the same period on each of these 16 males. As shown in Figure 1, the group with an average motility above 30% after freeze-thawing during the breeding season showed a significantly lower CHO/PL (0.280 ± 0.038 sd) than the group with a motility below 30% after freeze-thawing (0.350 ± 0.054 sd).

The following year, the spermatozoa of more than 100 bucks were assessed for their motility parameters after thawing together with their CHO/PL. CHO/PL was quite variable (Tab. I) and 90% of CHO/PL values ranged between 0.213 and 0.333. As shown in Figure 2A, the percentage of sperm motility after freeze-thawing was not correlated with CHO/PL of the corresponding sperm. The sperm CHO/PL was not correlated with the blood plasma cholesterol (Fig. 2B), suggesting that these two parameters are independent.

To test how the variability of the sperm CHO/PL was distributed over several months, sperm of 12 males was tested from January to August (Fig. 3A). The highest variability was observed in January and February with two males above 0.500. CHO/PL values became less and less variable with time and varied little from one month to another. The blood plasma cholesterol was very low in May, and the highest variability was observed in August (Fig. 3B). Whatever the month of collection, no relation was observed between plasma cholesterol and sperm CHO/PL. Strikingly, although no general correlation was observed between CHO/PL and sperm quality after thawing, such a correlation was significant ($p < 0.05$) when taking into account the ejaculates collected in February and in May. A low CHO/PL was associated with high mobility scores

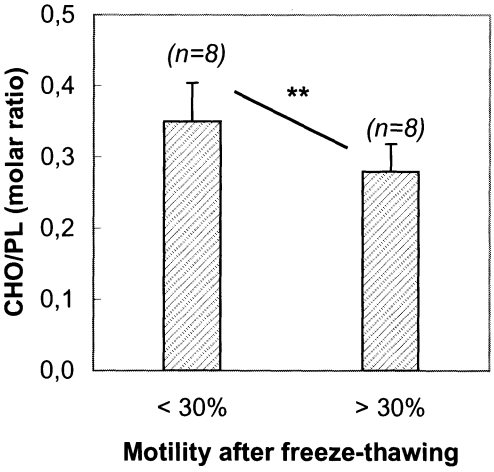

Figure 1. Comparison of the cholesterol/phospholipid molar ratio (CHO/PL) of goat semen taken from two different groups: one with an average motility below 30% after freeze-thawing and the other with an average motility above 30% after freeze-thawing. Each bar is the mean (± standard deviation) of eight ejaculates collected once from eight different goats. ** : $p < 0.01$ (Mann and Whitney U test).

Table I. Cholesterol/phospholipid molar ratio (CHO/PL) of boar semen, goat semen and stallion spermatozoa, and plasma cholesterol content.

	Goat	Boar	Stallion
Sperm CHO/PL (molar ratio)	0.281 ± 0.048 ($n = 112$)	0.375 ± 0.043 ($n = 47$)	0.541 ± 0.072 ($n = 17$)
Blood plasma cholesterol ($nmol.mL^{-1}$)	1160 ± 186 ($n = 113$)	1410 ± 200 ($n = 28$)	ND
Seminal plasma cholesterol (sperm-rich fraction) ($nmol.mL^{-1}$)	ND	104 ± 52 ($n = 28$)	ND

Mean values ± standard deviation.
n: number of males assessed.
ND: not determined.
All the parameters assessed were significantly different between species ($p < 0.01$, student's t test).

after thawing in February ($r = 0.623$, $n = 9$) and with high motility percentage after freeze-thawing in May ($r = 0.622$, $n = 9$). Such a relation between CHO/PL and mobility parameters is summarized in Table II.

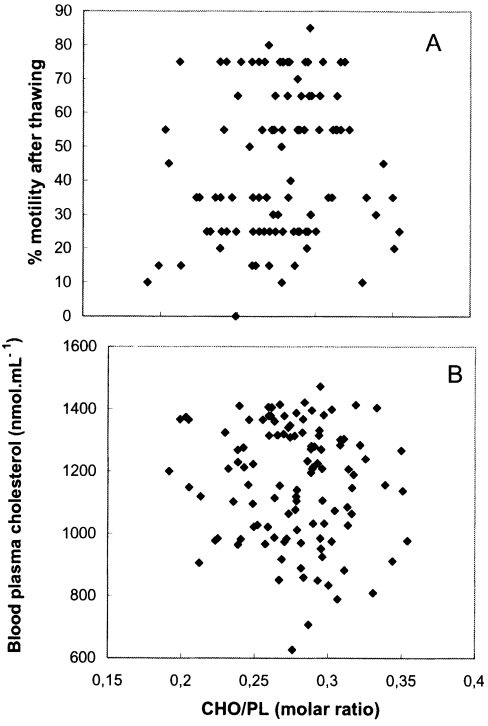

Figure 2. Relationship between goat semen cholesterol/phospholipid molar ratio (CHO/PL) and the percentage of motile spermatozoa after freeze-thawing (A), and the blood plasma cholesterol (B). Each data point represents one ejaculate from one male. No significant correlation were observed between the parameters under study.

Table II. Relationship between sperm cholesterol/phospholipid molar ratio (CHO/PL) and sperm quality after freeze-thawing in the goat, boar and stallion.

	Goat	Boar	Stallion
	CHO/PL (molar ration)		
% motility	$-(p < 0.05)$ (March)	NS	$-(p < 0.02)$
Mobility score	$-(p < 0.05)$ (February)	NS	ND
% alive normal after 120 min	ND	$+(p < 0.05)$	ND

±: positive or negative correlation.
NS: not significant,
ND: not determined.

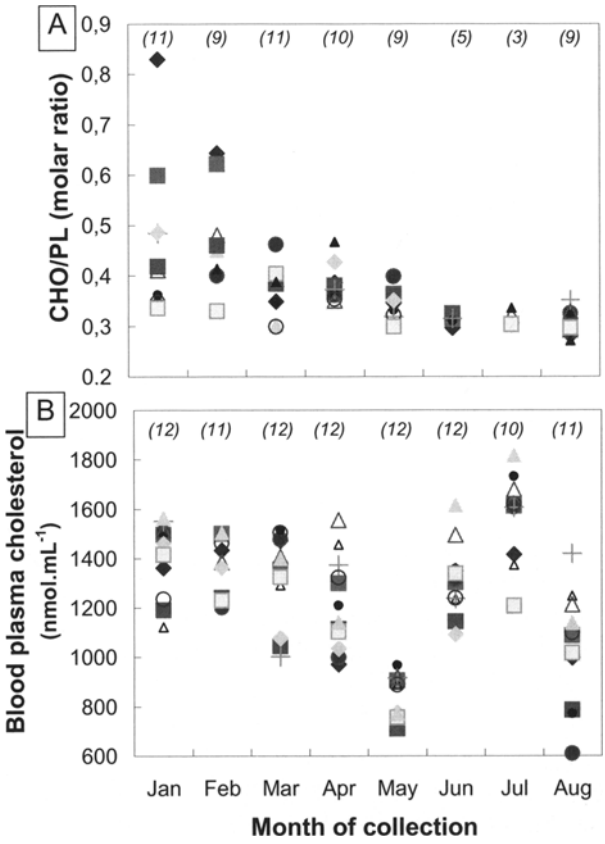

Figure 3. Change in semen cholesterol/phospholipid molar ratio (CHO/PL) (A) and the blood plasma cholesterol content (B) determined each month from 12 different goats. The number of samples is indicated in brackets. A given symbol represents the same male in the whole experiment.

3.2. Boar

The mean CHO/PL determined on 47 ejaculates collected over 2 years was significantly higher than the ratios observed in the goat (Tab. I). Although mobility parameters of boar spermatozoa were not correlated with CHO/PL ($p > 0.05$), a positive correlation was observed between the percentage of spermatozoa alive and normal 120 min after thawing and their corresponding CHO/PL (Fig. 4, summarized in Tab. II). This correlation was not significant when this test was performed only 10 min after thawing ($r = 0.229$, $n = 28$).

Seminal plasma cholesterol was variable between males (Tab. I), with the standard deviation reaching 50% of the mean value. No significant correlation was observed between seminal plasma cholesterol and CHO/PL of the corresponding spermatozoa. Unlike seminal plasma cholesterol, the blood plasma

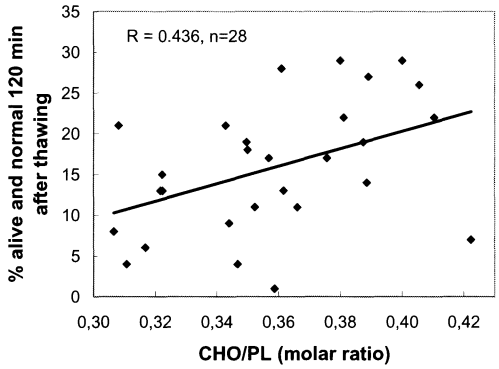

Figure 4. Correlation between cholesterol/phospholipid molar ratio (CHO/PL) in boar semen and the percentage of spermatozoa alive and normal 120 min after thawing ($p < 0.05$).

cholesterol was quite homogenous (Tab. I) with 90% of the samples ranging between 1116 and 1624 nmol.mL^{-1}. The blood plasma cholesterol content was not correlated with CHO/PL of the spermatozoa and the seminal plasma cholesterol. As for the goat, these parameters were independent.

3.3. Stallion

The spermatozoa CHO/PL in the stallion was the highest among the three species under study (Tab. I). It was negatively correlated ($p < 0.02$) with motility after thawing when only the first ejaculate of each male was considered (Fig. 5A, summarized in Tab. II). This correlation was not extremely consistent, as the same tendency was observed with the second ejaculate, but with a relatively low significance ($0.05 < p < 0.10$) (Fig. 5A). This discrepancy is mainly due to the high variability in motility between the two ejaculates. When sperm quality after thawing was rated on the basis of an average motility calculated on 6 to 27 ejaculates per male, this variable was found to be very significantly correlated with the average CHO/PL estimated using the two previous ejaculates ($p < 0.01$) (Fig. 5B).

4. DISCUSSION

This study gave an extensive picture of the sperm CHO/PL molar ratio variability in semen of boar and goat and in washed spermatozoa of stallion. Because of this washing step before lipid determination in stallion spermatozoa, the results cannot be used in comparisons with the two other species. They describe nevertheless a cellular parameter which can be compared with the corresponding freeze-thawing ability of spermatozoa. The mean CHO/PL for goat

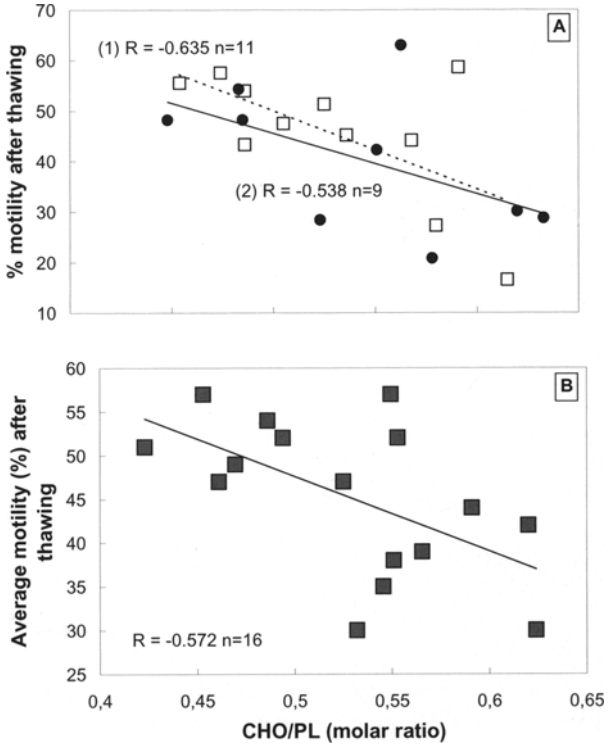

Figure 5. Correlation between cholesterol/phospholipid molar ratio (CHO/PL) in stallion spermatozoa and the motility percentage after thawing. A: Individual values for two ejaculates per male, open symbols and dotted line = ejaculate (1) ($p < 0.02$), full symbols and full line = ejaculate (2) ($0.05 < p < 0.10$). B: Average motility percentage for 6 to 27 ejaculates per male and average CHO/PL for two of these ejaculates ($p < 0.01$).

semen in this study is lower than the molar ratio reported by Iborra *et al.* [10] on washed spermatozoa (0.359) and much lower than the values described by Rana *et al.* [18] on caput epididymal spermatozoa (0.634), but these two data sets would fit in the CHO/PL general distribution of the ejaculates analyzed in the present study. It is hard to hypothesize on the decreasing variability of CHO/PL over the year. The highest variability was observed in winter, when the temperature is the lowest, but the data are too preliminary to postulate a temperature effect on semen CHO/PL. Boar spermatozoa CHO/PL calculated from Komarek *et al.* [11] (0.338) and given by Cerolini *et al.* [5] (0.32) are of the same magnitude as the semen mean ratio in our study. To our knowledge, no data were available on stallion whole spermatozoa, but Parks and Lynch [14] gave a sperm plasma membrane CHO/PL of 0.36.

For the three species investigated in the present study, the relationship between sperm CHO/PL and sperm quality after cryopreservation fluctuated. It

is possible that a given membrane lipid composition favored the integrity of some cellular components, but that the positive consequences of this integrity were more or less hidden by damage occurring randomly at any other cellular level (reviewed by Hammerstedt *et al.* [9]). In goat and stallion sperm, the results tended to show that a low CHO/PL was more favorable to a high motility after thawing, especially when the motility was estimated using several ejaculates. In boar, sperm CHO/PL was not correlated with the mobility parameters, but rather with the integrity of the membrane and of the acrosome as assessed by the eosin-nigrosin staining. All these viability tests are components of the complex multifactorial estimation of cell viability. They do not reflect the same cellular level of investigation. For this reason, the postulating of discrepancies between species with regards to sperm CHO/PL optimal ratio is still preliminary. The absolute determinant of spermatozoa quality is that they are able to fertilize and to give viable embryos, but such an investigation was beyond the scope of this study.

For goat and boar, CHO/PL reflected both the seminal plasma and spermatozoa composition. Goat seminal plasma composition was not assessed in this study, but the results obtained with boar showed how variable seminal plasma cholesterol was, in accordance with the values calculated from [11] (72, 46 and 36 nmol cholesterol/ml in 3 different boars). It is hard to decide whether this variability reflected the inter-male variability or the variability in the collecting procedure of the sperm-rich fraction. Seminal plasma cholesterol did not have a strong influence on CHO/PL since the two parameters were not significantly correlated. To conclude, on the basis of the present data, it is impossible to draw a general picture of the effect of cholesterol on sperm freezing ability that would fit every species. In every case, CHO/PL could not be considered as a direct predictor of the corresponding sperm freezing success. Nevertheless, although variable within each species, the correlation we observed cannot definitely rule out that CHO/PL plays a role in sperm cryoresistance. Determination of the role of cholesterol in sperm freezing, as attempted in this study using CHO/PL, was only a preliminary step. Some experiments are presently under way using spermatozoa of several domestic species to modify the cholesterol membrane content *in vitro* and to compare in same ejaculates the consequences, if any, of increasing and lowering the cholesterol content. Besides the gain expected in freezing fitness of spermatozoa, species by species determination of cholesterol requirements for sperm freezing fitness should help to establish selection criteria for the broodstock.

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