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In vitro supplementation with unsaturated fatty acids improves boar sperm viability after storage at 6 °C

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ABSTRACT

Liquid preservation of the cold-sensitive boar sperm at a lesser temperature than the standard 17 °C would reduce bacterial growth and minimize the use of antibiotics. There was assessment, therefore, of the capacity of individual fatty acids bound to fatty acid free BSA to improve sperm survival at 6 °C because oxidative stress and lipid degradation are prominent detrimental factors. Different effects of the fatty acids were observed. Supplementation with naturally occurring fatty acids (linolenic, linoleic, oleic, palmitoleic acid), which may become metabolically incorporated into sperm lipids, increased the number of motile and progressively motile sperm after 2 days of storage during a thermo-resistance test (5 h at 38 °C) to that of control samples preserved at 17 °C in pure Beltsville Thawing Solution. With the exception of linolenic acid, all naturally occurring fatty acids enhanced the number of sperm with active mitochondria after 3 days of storage. Palmitoleic acid was the most effective supplement with effects already present when sperm were re-warmed for 30 min after 2 and 7 days of storage. The non-endogenous, non-integrated timnodonic acid (20:5) had no effect on sperm variables. Because the application of individual fatty acids attached to BSA had differing effects in preserving boar sperm at 6 °C, the use of combinations of fatty acids could be more efficacious than with use of natural lipid supplements for low temperature preservation of sperm.

1. Introduction

Boar sperm are not only sensitive to freezing but also sensitive to refrigerated preservation. Sperm, therefore, are commonly diluted in Beltsville Thawing Solution (BTS), the most widely used extender for the short-term liquid preservation and stored after supplementation of antibiotics to the extender at 17 °C (Johnson et al., 2000). A storage regimen of 4–6 °C would limit bacterial growth during semen preservation but when this storage regimen is imposed there is a reduction of semen quality.

Liquid preservation of boar sperm in BTS results in increased plasma membrane permeability, decreased mitochondrial membrane potential, and induces lipid peroxidation (Kumaresan et al., 2009). The extent of lipid peroxidation of sperm is negatively correlated with sperm motility, viability and acrosome integrity. Because the mitochondria are the main internal source of reactive oxygen species (ROS), it is assumed that ROS are released from a subpopulation of structurally damaged sperm. The size of this subpopulation of structurally damaged sperm increases as duration of the storage period increases. When ROS concentrations are

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greater than optimal, the number of viable cells continues to decrease because of degradation of the membrane phospholipids (and other compounds) of these cells. The characteristic large amount of polyunsaturated fatty acyl residues in sperm phospholipids makes sperm highly vulnerable to ROS (Wathes et al., 2007), particularly, if the enzymatic defence of sperm and seminal fluid is compromised by the cooling temperature and semen dilution. Furthermore, during re-warming of the semen the action of the intracellular phospholipases released from damaged sperm is increased. These changes in semen characteristics may result in the production of lysophospholipids in combination with free fatty acids (phospholipase A_2) and diacylglycerols (phospholipase C). These compounds represent precursors of the signalling processes which typically occur prior to the acrosome reaction when physiological conditions prevail (Roldan, 1998) and further destabilize the sperm membranes. Associated with the lipid degradation by phospholipases, is a net loss of membrane lipids which may additionally provoke sperm membrane destabilization.

Interestingly, with the use of semen extenders containing BSA, a transport mediator of fatty acids, lysophosphatidylcholines and steroids (Morrisett et al., 1975), there is enabling of long-term liquid preservation of boar semen such as with the use of "Androhep" (Weitze, 1990; Waberski et al., 1994) or Modena (Zhang et al., 2015) extenders at 17 °C. With capacitating conditions at 37 °C, the use of fatty acids attached to BSA leads to improved sperm functions *in vitro* (Hossain et al., 2007b) and the mixture of fatty acids is also effective when conjugated to polyvinyl alcohol and included in semen extenders (Hossain et al., 2007a).

Vazquez and Roldan provided a possible explanation for these findings and reported that there was an active lipid biosynthesis in boar sperm, where palmitic acid (16:0) and arachidonic acid (20:4), classical BSA ligands, are incorporated into phosphatidic acid, diacylglycerols and ultimately phosphatidylcholines (Vazquez and Roldan, 1997b). It was hypothesized that *de novo* phospholipid synthesis is necessary to maintain cellular integrity when sperm are located in sperm reservoirs in the female genital tract. Consistent with these findings, our previous study revealed metabolic incorporation pattern with a final integration into choline-containing phospholipids for a number of naturally occurring unsaturated fatty acids (linolenic, 18:3, linoleic, 18:2, oleic, 18:1, and palmitoleic, 16:1, acid) (Svetlichnyy et al., 2014). It is suggested that both *de novo* synthesis of phosphatidylcholine resulting from activation of the cytidine diphosphate choline pathway and reacylation of lysophosphatidylcholine (LPC) from activation of an acyltransferase, occur to maintain lipid homeostasis and repair/restore catabolized lipid components. Interestingly a non-endogenous fatty acid (timnodonic acid, 20:5) was not metabolized by boar sperm.

Based on the findings in this previous study, there was an investigation in the present study of the effect of these fatty acids on boar sperm survival during long-term (168 h) liquid preservation in BTS at 6 °C. To address differences in the biological effects, individual fatty acids were separately bound to fatty acid free BSA using the linoleic acid albumin concentrations included in commercial cell culture supplements (LAA, Sigma-Aldrich, Taufkirchen, Germany). This supplement was added to the extender for boar sperm before cooling. Semen quality variables such as sperm motility characteristics and mitochondrial activity were assessed because these are still considered reliable indicators of sperm fertilization capacity in recent studies (Ruiz-Sanchez et al., 2006; Schulze et al., 2013; Jung et al.;, 2015; Elmi et al., 2018; Tremoen et al., 2018). Specifically, there was use of sperm motility assessments after long-term storage (Ruiz-Sanchez et al., 2006), or during a thermo-resistance test to simulate conditions in the female genital tract (Schulze et al., 2019). The findings from conducting the present study may explain aspects of sperm viability *in vivo* after insemination and, therefore, values obtained with these assessments may be predictive in developing commercial extenders for boar semen preservation.

2. Materials and methods

2.1. Reagents

Unless otherwise stated, all chemicals and reagents of greatest purity available including hexadecenoic (palmitoleic, 16:1) acid, octadecenoic (oleic, 18:1) acid, octadecadienoic (linoleic, 18:2) acid, octadecatrienoic (linolenic, 18:3) acid, and eicosapentaenoic (timnodonic, 20:5) acid were purchased from Sigma-Aldrich (Taufkirchen, Germany). The fatty acids (3.5 μmol) were dissolved in 10 μL ethanol. Subsequently, 1 mL sterile PBS containing 100 mg fatty acid free BSA (Sigma A-7030) was added to produce the fatty acid stock solution.

2.2. Treatment of semen for preservation

Spermatozoa-rich ejaculate fractions were collected from 16 Pietrain boars (*Sus scrofa domestica*) at a boar stud in Saxony-Anhalt (Germany). All boars were routinely used for semen collection and AI dose processing, received commercial feed (pellets) for AI boars, and were housed in individual pens equipped with nipple drinkers according to the European Commission Directive for Pig Welfare. The collection frequency of ejaculates did not exceed three collections within 2 weeks with at least 3 days elapsing between semen collections. Ejaculates were collected by the gloved-hand method. The day of collection was specified as day 0. The pre-sperm phase of the ejaculate was discarded and the gel fraction of the semen was removed by gauze filtration during collection. Semen preparation was performed using the general guidelines for semen processing of AI studs participating in a quality control audit of the Institute for Reproduction of Farm Animals Schönow e. V. (Riesenbeck et al., 2015).

Spermatozoa concentration was determined using the NucleoCounter SP-100™ (ChemoMetec A/S, Denmark). Spermatozoa motility was analysed using a computer-assisted sperm analysis (CASA) system (SpermVision™, Minitüb, Tiefenbach). Criteria for selection of ejaculates were a sperm motility of at least 70%, and a total amount of $\geq 15 \times 10^9$ spermatozoa per ejaculate. The ejaculates were diluted isothermally in one step to a final concentration of 2.2×10^7 spermatozoa/mL with Beltsville Thawing Solution (BTS, Minitüb®, Tiefenbach, Germany) composed of 10 mM KCl, 20.4 mM trisodium citrate, 15 mM $NaHCO_3$, 3.36 mM

EDTA, 205 mM glucose without antibiotics, pre-warmed to 36 °C (Pursel and Johnson, 1975). In a split sample procedure, 90 mL aliquots of diluted semen (from one ejaculate per boar) containing 2×10^9 were supplemented with 1 mL of one of the fatty acid stock solutions or with pure PBS (two controls). The final concentrations were 39 µM for the fatty acid and 17 µM for BSA in the supplemented samples. Semen was transferred into QuickTip Flexitubes® (Minitüb®, Tiefenbach, Germany). Immediately after filling, samples were placed in a temperature-controlled box at 21 °C for 90 min (controlled room temperature). After 90 min at 21 °C, the temperature was reduced to 17 °C and samples were transported to the laboratory within 2–4 h, where the samples were stored for 7 days at 17 °C (standard preservation in pure BTS, C17 °C) and 6 °C (control in pure BTS, C6 °C, and supplemented samples) during which the samples were subjected to further analyses. Except for days on which analyses occurred, there was no rotation of samples.

2.3. Sperm evaluation

Boar sperm were preserved for as long as 168 h at 6 °C in pure BTS (C6 °C) or supplemented with timnodonic (20:5), linolenic (18:3), linoleic (18:2), oleic (18:1), and palmitoleic (16:1) acid. The standard preservation was performed in pure BTS at 17 °C (C17 °C). Usually sperm are used for insemination within 48 h of storage whereas 72 h is the maximum time period where high quality of diluted semen is guaranteed for AI. For practical reasons, sperm aliquots were evaluated at these different time points for motility (48 h), viability (72 h), and defective acrosomes (72 h). Additionally, a long storage time was used for motility analysis (168 h) so as to have the capacity to ascertain small differences in the samples (Ruiz-Sanchez et al., 2006; Schulze et al., 2019).

In detail, to assess sperm motility and longevity at body temperature, a thermo-resistance test (TRT) was performed (Schulze et al., 2013, 2019). An aliquot of 10 mL stored semen was re-warmed and incubated in a water bath at 38 °C. After 30 and 300 min, motility was determined with use of the CASA system SpermVision™ (Minitüb, Tiefenbach, Germany) equipped with a phase contrast microscope (Olympus CX31), a high-resolution chip camera, and a heating tray (38 °C). Samples of 2.4 µL were transferred into a 38 °C pre-warmed "Leja-4" chamber (Leja, Nieuw-Vennep, The Netherlands) and analysed after complete distribution of the sample in the chamber (approximately 15–30 s). Sperm motion was recorded for 0.5 s at 60 Hz (60 frames/s). All sperm having at least two frames were counted. Analysis was based on the examination of 15 microscope fields with a total of 1000 spermatozoa per sample. The percentage of motile and progressively motile sperm was determined. According to the manufacturer's default setting for boar semen, non-motile sperm were defined by the average orientation change of the head (AOC $< 2.5°$), motile sperm by the straight line distance as sperm cell traversed from the initiation to cessation of the assessment period (DSL < 4.5 µm for locally and DSL ≥ 4.5 µm for progressively motile). Local and progressive sperm were considered motile. For the subpopulation of progressively motile sperm, the velocity of sperm when traversing in a straight line from the initiation to cessation of the assessment period the observed motion (VSL) was calculated.

Sperm viability was evaluated using rhodamine 123 (R123, Invitrogen, Molecular Probes, Eugene, Oregon, USA) and propidium iodide (PI, Invitrogen, Molecular probes, Eugene, Oregon, USA; Schulze et al., 2013). An aliquot of 10 mL stored semen was incubated in a water bath at 38 °C. After 10 min, a 250 µL sample was mixed with 1 µL of a 0.13 mM R123 solution in double-distilled water and with 2.5 µL of the 1.5 mM PI solution. Stained sperm were incubated in the dark for 20 min at 38 °C. The stained sperm suspension (10 µL) were mixed with 2 mL PBS heated to 38 °C for measurement in a flowcytometer (CyFlow space and FlowMax software, Partec, Germany) equipped with a 50-mW solid-state laser (Ex488 nm), a 515–560 nm band-pass for R123 (green), and a 620-nm long-pass filter for PI (red). The system was triggered on the forward light scatter, and 15,000 cells were characterized per sample for the amount of fluorescence at a flow rate of about 200 cells per second. Signal pulse heights were recorded. Sperm with active mitochondria accumulate R123 and have a bright green fluorescence, whereas in dead sperm the PI permeates the cell membrane and enters the nucleus and appears as a red color. The R123-positive sperm did not incorporate PI and were considered to be viable.

For analysis of sperm morphology on day 3 (72 h) of storage, an aliquot of re-warmed semen was diluted at room temperature (23 °C) at a concentration of $50–100 \times 10^4$ sperm per mL PBS containing 1% formaldehyde for sperm fixation. A 4 µL aliquot was transferred to a glass slide and covered with a coverslip. Because with conditions of refrigerated storage, acrosome damage is the predominant abnormality in structure, there was, therefore a focus on this variable. The percentages of sperm with defective acrosomes were evaluated by counting 200 sperm using phase contrast optics at a magnification of 800× (Jenaval, Carl Zeiss Jena, Jena, Germany).

2.4. Statistics

All statistical analyses were performed using IBM SPSS Statistics 24 (SPSS Inc., IBM, Armonk, USA). To address the repeated measures experimental design a mixed linear model (MLM, diagonal covariant matrix type) was applied. The 16 individuals (one boar with exactly one ejaculate allocated to seven storage conditions) were considered to be experimental samples (subjects) with storage condition (7: C6 °C, 20:5, 18:3, 18:2, 18:1, 16:1, or C17 °C), storage time (2: 48 or 168 h) and incubation time (2: 30 or 300 min) being considered as fixed factors. Significant differences as a result of storage conditions were evaluated using the MLM after contrasting for the respective control conditions (6 and 17 °C) at each of the four time points of evaluation (storage × incubation). These differences are depicted in the figures and Table 2. The criterion for statistical significance was an error probability of 0.05. Data were visualized as box plots including mean values (percentage of motile sperm, sperm with active mitochondria, sperm with defective acrosomes) or in Table 2 by mean values and standard deviations (percentage of progressive sperm, VSL).

Table 1
Main effects and interactions between storage conditions (supplementation and temperature), storage time (48 or 168 h) and TRT incubation time at 38 °C (30 or 300 min) on sperm motility variables in a mixed linear model (SPSS, 7 × 2 × 2 factorial, diagonal covariant matrix type, n = 16).

	Motile sperm		Progressive sperm		VSL progressive	
	F	Sig.	*F*	Sig.	*F*	Sig.
Intercept	4004.66	0.000	3276.63	0.000	7572.55	0.000
Storage condition	7.75	0.000	7.53	0.000	2.43	0.033
Storage time	18.99	0.000	13.00	0.000	3.76	0.053
Incubation time	103.22	0.000	85.15	0.000	127.65	0.000
Storage condition * Storage time	0.39	0.886	0.31	0.930	0.26	0.955
Storage condition * Incubation time	1.49	0.190	1.56	0.170	0.39	0.886
Storage time * Incubation time	0.45	0.502	0.48	0.489	0.88	0.350
Storage condition * Storage time * Incubation time	0.15	0.988	0.14	0.991	0.04	1.000

3. Results

3.1. Main effects of storage conditions (supplementation and temperature), storage time (48 or 168 h) and TRT incubation time at 38 °C (30 or 300 min) on values for sperm motility variables

Storage conditions (supplementation and temperature) and storage time as well as TRT incubation time had an effect on the proportion of motile and progressively motile sperm. The straight-line velocity of the progressive sperm was affected by storage conditions and TRT incubation time (Table 1). The interactions between fixed factors were not significant. In general, the proportions of motile and progressively motile sperm decreased with storage time and in the course of TRT incubation time whereas the straight-line velocity (VSL) was greater in the remaining progressive sperm populations at the end of the TRT incubation time. This finding indicates the most viable sperm survive the 5 h incubation at 38 °C.

The effects of storage conditions are addressed in the following paragraphs. Differences between the non-supplemented controls at 17 °C and 6 °C and the effect of fatty acid supplementation at 6 °C were described separately. Because the extent (significance) of storage condition effects was different at the four time points of evaluation, comparisons were depicted (Fig. 1) and data were

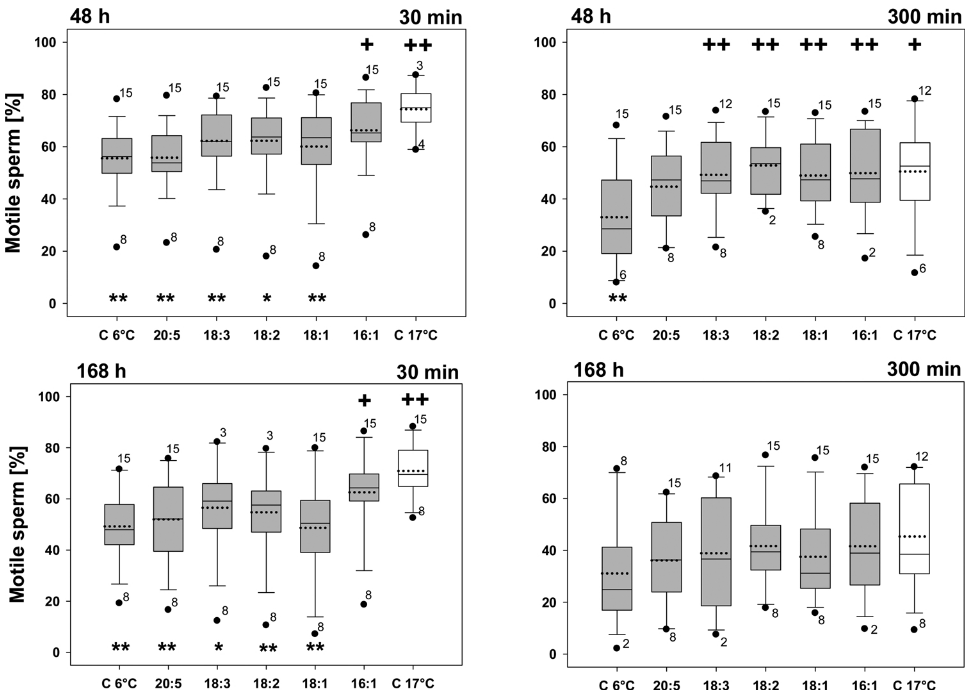

Fig. 1. Effect of semen extender supplementations on sperm motility. Boar sperm were preserved for 48 and 168 h at 6 °C in pure BTS (C6 °C) or supplemented with timnodonic (20:5), linolenic (18:3), linoleic (18:2), oleic (18:1), and palmitoleic (16:1) acid bound to BSA. The standard preservation was performed in pure BTS at 17 °C (C17 °C). Data were recorded after re-warming to 38 °C and incubation for 30 as well as 300 min. Data (n = 16) are displayed by means of box plots. Medians, means (dotted), and the 25 and 75th percentiles are shown. Outliers are denoted by circles with the respective sample numbers. Differences from the C6 °C group are indicated by crosses above boxes (+ $P <$ 0.05, + + $P <$ 0.01). Significant differences from the C17 °C group are indicated by stars below boxes (* $P <$ 0.05, ** $P <$ 0.01).

Table 2
Effect of semen extender supplementation on the proportion of progressively motile sperm and straight-line velocity (VSL). Boar sperm were preserved for 48 and 168 h at 6 °C in pure BTS (C6 °C) or supplemented with timnodonic (20:5), linolenic (18:3), linoleic (18:2), oleic (18:1), and palmitoleic (16:1) acid. The standard preservation was performed in pure BTS at 17 °C (C17 °C). Data were recorded after re-warming to 38 °C and incubation for 30 as well as 300 min. Means, and standard deviations are shown (n = 16). Significant differences for the comparison with C6 °C and C17 °C are indicated on the left and right sides, respectively (+ and *$P <$ 0.05, + + and ** $P <$ 0.01).

	Progressive sperm [%]				VSL progressive [µm/s]			
	Sig.	Mean	SD	Sig.	Sig.	Mean	SD	Sig.
48 h 30 min								
C 6 °C		49.7	13.6	**		43	10	
20:5		50.6	13.3	**		42	11	
18:3		55.7	15.4	**		41	8	*
18:2		56.2	15.2	**		41	8	*
18:1		53.5	17.6	**		40	9	*
16:1		59.6	14.7	*		41	9	*
C 17 °C	+ +	69.6	10.9			48	9	
48 h 300 min								
C 6 °C		29.5	17.1	*		55	11	
20:5		40.2	15.0			53	12	
18:3	+	44.5	14.2			53	11	
18:2	+ +	47.9	11.8			54	11	
18:1	+	43.9	13.7			54	10	
16:1	+	45.7	16.3			54	12	
C 17 °C	+	46.1	18.4			57	11	
168 h 30 min								
C 6 °C		44.6	14.5	**		47	12	
20:5		46.8	17.0	**		44	13	
18:3		52.0	17.4	*		42	11	
18:2		49.7	17.1	**		41	10	*
18:1		44.2	18.9	**		41	12	
16:1	+	57.2	16.2			40	11	*
C 17 °C	+ +	67.2	11.2			50	10	
168 h 300 min								
C 6 °C		28.6	19.9			61	12	
20:5		32.6	16.9			58	13	
18:3		36.0	20.5			54	12	
18:2		38.3	16.4			58	14	
18:1		33.7	16.9			56	13	
16:1		37.8	18.0			56	12	
C 17 °C		41.7	19.4			60	13	

reported (Table 2) for each combination of storage and incubation time.

3.2. Quality differences of boar sperm between standard preservation at 17 °C (C17 °C) and storage at 6 °C (C6 °C) in non-supplemented BTS

The use of the standard protocol for boar semen preservation at 17 °C indicate that at all points of time (except after 168 h storage and 300 min TRT incubation time) there was a greater percentage of motile and progressively motile sperm compared with storage at 6 °C in non-supplemented BTS (Fig. 1 and Table 2). The swimming speed (VSL) of the relatively lesser number of progressively motile sperm in C6 °C group was similar to that of the progressively motile sperm population in the C17 °C group.

Consistent with the proportion of motile and progressively motile sperm, the amount of viable sperm with active mitochondria was greater in the C17 °C compared to C6 °C group after 72 h storage and there were fewer defective acrosomes (Fig. 2).

3.3. Effects of BTS supplementation with individual BSA-bound fatty acids on boar sperm quality after storage at 6 °C

The supplementation with BSA-bound palmitoleic acid resulted in an increase of the percentage of motile and partly progressively motile sperm compared to the C6 °C group (Fig. 1 and Table 1) when there was assessment 30 min after re-warming and there was 48 and 168 h of storage. The values for sperm quality variables when using standard preservation (C17 °C) procedure were similar with there being only one value for a variable and time of assessment (progressive sperm at 48 h 30 min) that was different than those for the C17 °C group. Supplementation with linolenic and linoleic acid tended to result in an improvement in values of sperm motility variables. After 300 min as compared to 30 min for TRT incubation time, there was a decrease in motile and progressively motile sperm in all samples, but this difference was less pronounced in the supplemented samples at day 2 (Fig. 1, Table 2). After 300 min of TRT incubation time, the values for motility and progressive motility in supplemented samples were similar to those of conventionally preserved samples because the values were similar to those of the C17 °C group. Timnodonic acid was the least effective

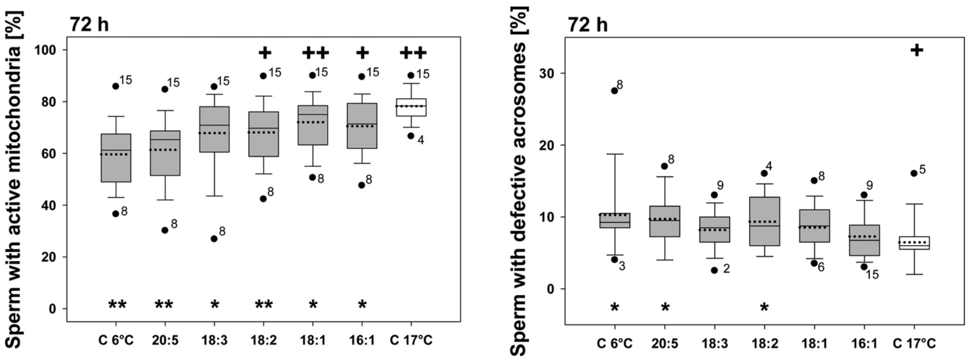

Fig. 2. Effect of semen extender supplementations on sperm mitochondrial function and morphology. Boar sperm were preserved for 72 h at 6 °C in pure BTS (C6 °C) or supplemented with timnodonic (20:5), linolenic (18:3), linoleic (18:2), oleic (18:1), and palmitoleic (16:1) acid bound to BSA. The standard preservation was performed in pure BTS at 17 °C (C17 °C). Data were recorded after re-warming to 38 °C and incubation for 30 min. Data (n = 16) are displayed by means of box plots. Medians, means (dotted), and the 25 and 75th percentiles are shown. Outliers are denoted by circles with the respective sample numbers. Differences from the C6 °C are indicated by crosses above boxes (+ $P <$ 0.05, + + $P <$ 0.01). Differences from the C17°C group are indicated by stars below boxes (* $P <$ 0.05, ** $P <$ 0.01).

supplement in maintaining sperm motility because with this supplementation there was no greater sperm quality as compared with the non-supplemented control group (C6 °C). After long-term storage (168 h) and 300 min of TRT incubation time, there were no motility differences among samples for which different storage conditions were imposed, including differences between non-supplemented control samples (Fig. 1, Table 2).

The swimming speed of progressively motile sperm in some supplemented samples (including palmitoleic acid supplementation) was less at the beginning of the TRT incubation time when compared to the control C17 °C group (Table 2). This finding indicates the average quality of the greater number of progressively motile viable sperm in these supplemented samples was less than when there was storage using the standard conditions.

The increase in the sperm survival rate which resulted in more motile and progressively motile sperm in samples supplemented with individual fatty acids was further confirmed by the greater percentage of boar sperm with active mitochondria after 72 h of storage at 6 °C. Supplementation with all fatty acids, except timnodonic and linolenic acid, improved the percentage of boar sperm with active mitochondria compared to that of the C6 °C group, but values were still less than for the C17 °C group (Fig. 2). Only with supplementation of palmitoleic, oleic and linolenic acid was there (after 72 h of storage) a percentage of sperm with a defective acrosome similar to that of C17 °C group (Fig. 2).

4. Discussion

This study was performed to ascertain the protective effect of BSA or egg yolk based fatty acids on boar sperm survival after refrigerated storage and re-warming of samples. Individual naturally occurring unsaturated fatty acids (linolenic, linoleic, oleic, and palmitoleic) and, for comparison, one non-endogenous fatty acid (timnodonic) were attached to BSA and supplemented in a BTS extender to assess the potential for improving sperm preservation at 6 °C. Effects of individual fatty acids were obvious.

The supplementation with non-endogenous timnodonic acid did not result in an enhancement in values for sperm quality variables as compared with the non-supplemented control group at 6 °C. Only at day 2 of storage when re-warmed samples were incubated for 5 h at 38 °C was there a tendency but non-significant enhancement of motility and progressive motility (Fig. 1, Table 2). The naturally occurring palmitoleic acid proved to be the most potent supplement. It enhanced the proportion of motile and progressively motile sperm after 30 min re-warming to 38 °C on days 2 and 7 to the proportion of control samples preserved at 17 °C (C17 °C). With use of all other naturally occurring fatty acids, the percentages of motile and progressively motile sperm at the end of TRT incubation time on day 2 were similar to the values of standard C17 °C group. Furthermore, with supplementation of all naturally occurring fatty acids, except linolenic acid, there was an increase in the percentage of sperm with active mitochondria on day 3 of storage at 6 °C. With supplementation of palmitoleic, linolenic, and oleic acid during refrigerated storage there was also a similar percentage of sperm with defective acrosomes as compared with those using the standard conditions (C17 °C) for sperm preservation. These results indicate the quality of the diluted and cold stored semen at 6 °C may be improved by supplementation with BSA-bound fatty acids. In some cases, values for quality were similar to those with conventional storage at 17 °C. The industry standard for use of semen for AI is a minimum of 70% motility (Ruiz-Sanchez et al., 2006) and there was a similar percentage of motile sperm after refrigerated storage with the addition of the fatty acids (in case of palmitoleic acid after 48 h storage and 30 min at 38 °C with this occurring in nearly ⅓ of the samples). The addition of fatty acids, therefore, appears to be a promising approach for low-temperature boar sperm preservation. Potential explanations of the beneficial effect of different fatty acid supplements will be discussed in the following paragraphs.

In a previous study, a stable-isotope labelled linoleic acid was incorporated into sperm lipids after incubation for 24 h at 17 °C, primarily into diacylglycerols (DAG), phosphatidic acid (PA) and phosphatidylcholine (PC) (Svetlichnyy et al., 2014). All naturally occurring fatty acids were integrated into DAG. Applying radio-labelled palmitic (16:0) and arachidonic (20:4) acid to boar sperm, Vazquez and Roldan observed an active replenishment of the sperm PC pool as a result of integration of the fatty acids initially into PA and DAG (Vazquez and Roldan, 1997a). The continuous generation of PC during storage in BTS for as long as 72 h at 17 or 25 °C was thought to stabilize the membranes and could also preserve sperm integrity.

It cannot be ascertained whether the applied naturally occurring fatty acids in the present study are metabolized to DAG or PC prior to or during refrigerated storage at 6 °C or predominantly later upon re-warming and TRT incubation time. At least, 30 min of re-warming at 38 °C was sufficient for the beneficial effect of the fatty acids to occur with the supplementation of palmitoleic acid with this effect continuing for at least for 5 h, as more sperm remained motile and progressively motile than with the C6 °C sample.

A continuous synthesis/repair of PC as a result of fatty acid supplementation could also explain previous results that have been reported when there were fatty acid treatments imposed (Hossain et al., 2007b). In this previous study, there was incubation of boar sperm with different fatty acids for 4 h at 37 °C and as a result there was improvement of sperm motility and viability particularly when there was supplementation with BSA-coupled oleic and linoleic acid. Supplementation with oleic and arachidonic acid, but not linoleic acid, however, also resulted in the induction of the acrosome reaction in the reported experiments. In contrast to the present study, by a factor of ∽30, there was supplementation with a greater fatty acid concentration (1.25 mM) and furthermore a capacitating medium was used. There is no indication that fatty acid supplementations stimulated acrosome reactions when there were additions of the fatty acids to the BTS extender, but it is noteworthy that oleic acid was not as effective as palmitoleic acid in stimulation of sperm motility in the present study.

The naturally occurring fatty acids that were used for extender supplementation in the present study might serve as precursors for polyunsaturated fatty acyl residues (PUFAs) and become metabolized in sperm by chain elongation and desaturation (Mayes, 2000; Tran et al., 2017). In contrast to PUFAs the naturally occurring fatty acids used in this study are less sensitive to oxidative stress and, therefore, preferred for use in long-term extenders for liquid preservation of sperm. Studies on lipid changes of boar sperm during cooling and freezing revealed that the relative amount of PUFAs, particularly long-chain PUFAs (22:5 and 22:6), decrease after freezing and thawing and even after cooling of boar sperm to 5 °C (Cerolini et al., 2000, 2001; Maldjian et al., 2005). Waterhouse et al. (2006) determined that the boar sperm survival rate after freezing and thawing is partially related to the amount of endogenous long-chain PUFAs (22:5 and 22:6) in membrane lipids. There have been several studies with boars and other species focused on improving the outcome of low-temperature semen storage by dietary or *in vitro* semen supplementation with PUFAs but the results from these studies are inconsistent (Tran et al., 2017). Because PUFAs are very sensitive to oxidation, the conditions for PUFA supplementation need to be optimized for each species, individual, or extender. The *in vitro* application of PUFAs should ideally be complemented with an adequate supply of antioxidants as ascertained with cryopreservation of epididymal bull sperm (Losano et al., 2018).

There was a positive effect on sperm viability at low temperature preservation when there was supplementation with shorter and less unsaturated fatty acids *in vitro* in other species. Perez-Pe et al. (2001) reported that supplementation with oleic and linoleic acid attached to BSA may protect ram sperm from cold shock (10 min at 5 °C). Ram sperm viability was evaluated after re-warming and incubation at 20 °C. Because in ram sperm, similar to boar sperm, there is incorporation of radio-labelled fatty acids (myristic (14:0), palmitic, stearic, oleic and linoleic acid) primarily into DAG and to a lesser extent into phospholipids (Neill and Masters, 1973), the restoration of sperm phospholipids may result from the positive effect of supplementation with these fatty acids.

The supplementation of BSA-bound palmitoleic acid is also advantageous for maintaining membrane integrity, motility, and viability in ram sperm when there is storage for 72 h at 4 °C (Eslami et al., 2017). Because the extent of lipid peroxidation was reduced and sperm superoxide dismutase was activated, it was suggested that this fatty acid might function as an exogenous antioxidant. This could explain why in the present study palmitoleic acid was the most reliable protective compound although this fatty acid is far less abundant in sperm phospholipids than the other naturally occurring fatty acids evaluated in the present study (Hinkovska-Galcheva et al., 1988; Lessig et al., 2004).

It remains to be assessed whether externally added BSA-bound fatty acids may further promote sperm motility and viability in the female genital tract after artificial insemination. The BSA as fatty acid reservoir could remain attached to the sperm surface although the surrounding seminal fluids are probably separated from the sperm. Naturally, female genital tract secretions can also generate a continuous supply of fatty acids to the sperm *in vivo*. Recently, Drews et al. (2018) reported that oleic and linoleic as well as palmitic and stearic (18:0) acid are the four most prevalent fatty acids in blood plasma as well as uterine and oviduct fluid of mares. Because the stimulation of sperm motility with fatty acids which are potentially incorporated into the sperm membrane lipids is predominant at the end of the TRT incubation period (simulating the sperm persistence in the female), there appears to be a continuous and substantial fatty acid supply at body temperature.

Interestingly, in the present study there was a tendency for stimulation of sperm motility when there was supplementation with the non-endogenous timnodonic acid after 48 h storage and long-term heat stress for 5 h. This fatty acid does not incorporate into sperm lipids, but might have extracellular antioxidant properties during the long incubation period at 38 °C. Furthermore, BSA could contribute in the prevention of a loss of sperm motility. It is not possible to experimentally separate the effects of the pure protein and that of adhered fatty acids because fatty acid free BSA would function as a capacitating agent through removal of cholesterol (Go and Wolf, 1985). The affinity of preloaded BSA to membrane lipids, should be reduced but the acceptor capacity of BSA might still be effective to some extent and remove detrimental lipid degradation products (for example LPL) from the sperm membrane (Kim et al., 2007).

5. Conclusion

Each individual naturally occurring unsaturated fatty acid that was attached to BSA had a differential effect in improving the *in* *vitro* boar sperm quality when there was storage at 6 °C and there was supplementation of a commercial lipid-free extender (BTS). Three modes of action are postulated. Naturally occurring fatty acids which can be metabolically integrated into boar sperm DAGs and phospholipids, can restore the sperm phospholipid pool, which is degraded as a result of the enhanced lipid oxidation during refrigerated storage and re-warming. Because the most consistent effect is mediated by palmitoleic acid, which becomes integrated into DAGs, but is the least abundant natural fatty acid in boar sperm phospholipids, its antioxidative action as a substrate for lipid oxidation has to be considered. In addition, an extracellular antioxidative action or the acceptor function of BSA for lipid degradation products could be beneficial for viability and motility of re-warmed sperm. These results indicate there is a potential advantage of supplementing combinations of selected fatty acids that are attached to BSA rather than native lipid supplements to successfully preserve sperm at refrigerated temperatures.

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Conflict of interest

None of the authors has any conflict of interest to declare.

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References

Cerolini, S., Maldjian, A., Pizzi, F., Gliozzi, T.M., 2001. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Reproduction 121, 395–401.
Cerolini, S., Maldjian, A., Surai, P., Noble, R., 2000. Viability, susceptibility to peroxidation and fatty acid composition of boar semen during liquid storage. Anim. Reprod. Sci. 58, 99–111.
Drews, B., Milojevic, V., Giller, K., Ulbrich, S.E., 2018. Fatty acid profile of blood plasma and oviduct and uterine fluid during early and late luteal phase in the horse. Theriogenology 114, 258–265.
Elmi, A., Banchelli, F., Barone, F., Fantinati, P., Ventrella, D., Forni, M., Bacci, M.L., 2018. Semen evaluation and in vivo fertility in a Northern Italian pig farm: can advanced statistical approaches compensate for low sample size? An observational study. Anim. Reprod. Sci. 192, 61–68.
Eslami, M., Ghasemiyan, H., Zadeh Hashem, E., 2017. Semen supplementation with palmitoleic acid promotes kinematics, microscopic and antioxidative parameters of ram spermatozoa during liquid storage. Reprod. Domest. Anim. 52, 49–59.
Go, K.J., Wolf, D.P., 1985. Albumin-mediated changes in sperm sterol content during capacitation. Biol. Reprod. 32, 145–153.
Hinkovska-Galcheva, V., Peeva, D., Momchilova-Pankova, A., Petkova, D., Koumanov, K., 1988. Phosphatidylcholine and phosphatidylethanolamine derivatives, membrane fluidity and changes in the lipolytic activity of ram spermatozoa plasma membranes during cryoconservation. Int. J. Biochem. 20, 867–871.
Hossain, M.S., Hyeong, L.J., Miah, A.G., Tsujii, H., 2007a. Effect of fatty acids bound to bovine serum albumin-V on acrosome reaction and utilization of glucose in boar spermatozoa. Reprod. Med. Biol. 6, 109–115.
Hossain, M.S., Tareq, K.M.A., Hammano, K.I., Tsujii, H., 2007b. Effect of fatty acids on boar sperm motility, viability and acrosome reaction. Reprod. Med. Biol. 6, 235–239.
Johnson, L.A., Weitze, K.F., Fiser, P., Maxwell, W.M., 2000. Storage of boar semen. Anim. Reprod. Sci. 62, 143–172.
Jung, M., Rudiger, K., Schulze, M., 2015. In vitro measures for assessing boar semen fertility. Reprod. Domest. Anim. 50 (Suppl 2), 20–24.
Kim, Y.L., Im, Y.J., Ha, N.C., Im, D.S., 2007. Albumin inhibits cytotoxic activity of lysophosphatidylcholine by direct binding. Prostaglandins Other Lipid Mediat. 83, 130–138.
Kumaresan, A., Kadirvel, G., Bujarbaruah, K.M., Bardoloi, R.K., Das, A., Kumar, S., Naskar, S., 2009. Preservation of boar semen at 18 degrees C induces lipid peroxidation and apoptosis like changes in spermatozoa. Anim. Reprod. Sci. 110, 162–171.
Lessig, J., Gey, C., Suss, R., Schiller, J., Glander, H.J., Arnhold, J., 2004. Analysis of the lipid composition of human and boar spermatozoa by MALDI-TOF mass spectrometry, thin layer chromatography and 31P NMR spectroscopy. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 137, 265–277.
Losano, J.D.A., Angrimani, D.S.R., Rui, B.R., Bicudo, L.C., Dalmazzo, A., Silva, B.C.S., Viana, C.H.C., Mendes, C.M., Assumpcao, M., Barnabe, V.H., Nichi, M., 2018. The addition of docosahexaenoic acid (DHA) and antioxidants (glutathione peroxidase and superoxide dismutase) in extenders to epididymal sperm cryopreservation in bulls. Zygote 26, 199–206.
Maldjian, A., Pizzi, F., Gliozzi, T., Cerolini, S., Penny, P., Noble, R., 2005. Changes in sperm quality and lipid composition during cryopreservation of boar semen. Theriogenology 63, 411–421.
Mayes, P.A., 2000. Metabolism of unsaturated fatty acids and eicosanoids. In: Barnes, D.A. (Ed.), Harper's Biochemistry. McGraw-Hill, New York.
Morrisett, J.D., Pownall, H.J., Gotto Jr., A.M., 1975. Bovine serum albumin. Study of the fatty acid and steroid binding sites using spin-labeled lipids. J. Biol. Chem. 250, 2487–2494.
Neill, A.R., Masters, C.J., 1973. Metabolism of fatty acids by ovine spermatozoa. J. Reprod. Fertil. 34, 279–287.
Perez-Pe, R., Cebrian-Perez, J.A., Muino-Blanco, T., 2001. Semen plasma proteins prevent cold-shock membrane damage to ram spermatozoa. Theriogenology 56, 425–434.
Pursel, V.G., Johnson, L.A., 1975. Freezing of boar spermatozoa: fertilizing capacity with concentrated semen and a new thawing procedure. J. Anim. Sci. 40, 99–102.
Riesenbeck, A., Schulze, M., Rudiger, K., Henning, H., Waberski, D., 2015. Quality control of boar sperm processing: implications from european AI centres and two spermatology reference laboratories. Reprod. Domest. Anim. 50 (Suppl 2), 1–4.
Roldan, E.R., 1998. Role of phospholipases during sperm acrosomal exocytosis. Front. Biosci. 3, D1109–1119.
Ruiz-Sanchez, A.L., O'Donoghue, R., Novak, S., Dyck, M.K., Cosgrove, J.R., Dixon, W.T., Foxcroft, G.R., 2006. The predictive value of routine semen evaluation and IVF technology for determining relative boar fertility. Theriogenology 66, 736–748.
Schulze, M., Jakop, U., Jung, M., Cabezon, F., 2019. Influences on thermo-resistance of boar spermatozoa. Theriogenology 127, 15–20.
Schulze, M., Ruediger, K., Mueller, K., Jung, M., Well, C., Reissmann, M., 2013. Development of an in vitro index to characterize fertilizing capacity of boar ejaculates. Anim. Reprod. Sci. 140, 70–76.

Svetlichnyy, V., Muller, P., Pomorski, T.G., Schulze, M., Schiller, J., Muller, K., 2014. Metabolic incorporation of unsaturated fatty acids into boar spermatozoa lipids and de novo formation of diacylglycerols. Chem. Phys. Lipids 177, 41–50.

Tran, V.T., Malla, B.A., Kumar, S., Tyagi, A.K., 2017. Polyunsaturated fatty acids in male ruminant reproduction - a review. Asian Austral. J. Anim. 30, 622–637.

Tremoen, N.H., Gaustad, A.H., Andersen-Ranberg, I., van Son, M., Zeremichael, T.T., Frydenlund, K., Grindflek, E., Vage, D.I., Myromslien, F.D., 2018. Relationship between sperm motility characteristics and ATP concentrations, and association with fertility in two different pig breeds. Anim. Reprod. Sci. 193, 226–234.

Vazquez, J.M., Roldan, E.R., 1997a. Diacylglycerol species as messengers and substrates for phosphatidylcholine re-synthesis during Ca2+-dependent exocytosis in boar spermatozoa. Mol. Reprod. Dev. 48, 95–105.

Vazquez, J.M., Roldan, E.R., 1997b. Phospholipid metabolism in boar spermatozoa and role of diacylglycerol species in the de novo formation of phosphatidylcholine. Mol. Reprod. Dev. 47, 105–112.

Waberski, D., Meding, S., Dirksen, G., Weitze, K.F., Leiding, C., Hahn, R., 1994. Fertility of long-term-stored boar semen: influence of extender (Androhep and Kiev), storage time and plasma droplets in the semen. Anim. Reprod. Sci. 36, 145–151.

Waterhouse, K.E., Hofmo, P.O., Tverdal, A., Miller Jr., R.R., 2006. Within and between breed differences in freezing tolerance and plasma membrane fatty acid composition of boar sperm. Reproduction 131, 887–894.

Wathes, D.C., Abayasekara, D.R., Aitken, R.J., 2007. Polyunsaturated fatty acids in male and female reproduction. Biol. Reprod. 77, 190–201.

Weitze, K.F., 1990. Long-term storage of extended boar semen. Proc. 2nd Int. Conf. Boar Semen Preservation 231–253.

Zhang, X.G., Yan, G.J., Hong, J.Y., Su, Z.Z., Yang, G.S., Li, Q.W., Hu, J.H., 2015. Effects of bovine serum albumin on boar sperm quality during liquid storage at 17 degrees C. Reprod. Domest. Anim. 50, 263–269.