REPRODUCTIVE BIOLOGY

ORIGINAL RESEARCH

Effects of dietary supplementation with polyunsaturated fatty acids and antioxidants on biochemical characteristics of boar semen

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SUMMARY

The aim of this study was to investigate the effect of providing a supplement containing polyunsaturated fatty acids and antioxidants (PROSPERM®) on the biochemical characteristics of boar semen. Two sexually mature boars were fed a standard diet with PROSPERM® (250 g daily) for a 24-week period. Ejaculates collected prior to supplementation were used as the control. Semen quality and biochemical parameters were analyzed. The dietary supplementation enhanced sperm characteristics, including the percentage of spermatozoa with intact plasma membrane and osmotic resistance of the acrosomal membrane. Higher production of malondialdehyde was concurrent with increased activity of superoxide dismutase in the seminal plasma and spermatozoa after 8 weeks of supplementation. These changes were accompanied by a high content

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of total protein and low-molecular antioxidants of the seminal plasma. It was observed that PROSPERM supplementation enhanced the survivability of boar spermatozoa during storage in a standard semen extender supplemented with lipoprotein fractions, isolated from hen egg yolk or ostrich egg yolk, at 5°C and 16°C. These results indicate that PROSPERM supplementation of boars had a beneficial effect on the biological characteristics of the spermatozoa, which could be useful for semen preservation at different temperatures.
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INTRODUCTION

In cell membranes, fatty acids are both structural elements and bioactive compounds. The testis has been shown to contain a high content of 20 (C_{20}) and 22 (C_{22}) carbon atoms of polyunsaturated fatty acids (PUFAs; [15]). Phospholipids of mammalian sperm cell membranes characteristically contain very high proportions of long-chain (C_{22}) polyunsaturated fatty acids, particularly n-3 series. In most mammals, docosahexaenoic acid (DHA, 22:6n-3) is the dominant polyunsaturated fatty acid, although, in several species docosapentaenoic acid (DPA, 22:5n-6) is also a major component of the sperm cell membranes [5, 14].

The importance of C_{22} polyunsaturates in relation to male fertility has been illustrated by studies in humans demonstrating that the amount of docosahexaenoic acid in spermatozoa is positively correlated with sperm motility [27]. Speculation about the function of PUFA in testis has been related to their possible effect on the fluidity of the sperm plasma membrane and the packing of membrane-bound receptors. A previous study showed that a reduction in motility and the number of spermatozoa in ejaculates from ageing bulls was accompanied by a decrease in DHA proportion in the sperm phospholipid [10]. Moreover, PUFAs which are concentrated in the head and tail membrane regions of spermatozoa have been shown to play an important role in both sperm capacitation [21] and the interaction between spermatozoa and uterine surface environment [25].

Sperm plasma membranes are susceptible to lipid peroxidation because of the high content of PUFA and their inadequate defensive mechanisms [1]. Recent evidence has shown that oxidative stress significantly impairs sperm function, and plays a major role in the etiology of defective sperm function. Both spermatozoa and seminal plasma possess antioxidant systems, including glutathione and ergothioneine, capable of counteracting the harmful effects of reactive oxygen species [19, 24]. The antioxidants that are present in the seminal plasma compensate for the deficiency in cytoplasmic enzymes in the spermatozoa.

Porcine commercial diets commonly contain large amounts of cereals, with most supplementary protein being added in different forms, including soybean and rapeseed. The fatty acids of these diets typically have a n-6 PUFA to n-3 PUFA ratio of greater than 6:1 and contain no long chain polyunsaturated (n-3) fatty acids [16]. The present study represents an attempt to remedy this dietary imbalance of n-3 to n-6 PUFAs by feeding mature boars with a diet supplemented with DHA plus antioxidants (PROSPERM®). Previous studies showed that PROSPERM supplementation affected the fatty acid composition of boar spermatozoa by increasing the n-3 PUFAs at the expense of n-6 PUFAs in the sperm plasma membranes [12, 13]. The results of these studies demonstrated that n-3 PUFAs had a beneficial effect on the characteristic of boar spermatozoa. However, there is no information regarding the effect of PUFA supplementation on the biochemical parameters of boar semen. Therefore, the aim of this study was to investigate the effect of dietary PROSPERM supplementation on the biochemical characteristics of boar semen. Also, the effect of this supplementation on the survivability of preserved boar spermatozoa at 5°C and 16°C was analyzed.

MATERIAL AND METHODS

Animals

Two sexually mature Polish Large White boars were used in this experiment. The average age of the boars was 12 months at the start of PROSPERM

supplementation. The boars were housed under the same conditions and were given a commercial porcine ration (basal diet). Water was available ad libitum.

PUFA dietary supplementation

The boars received the standard diet together with nutritional supplement with antioxidants (PROSPERM®, JSR Clover Corporation Ltd., UK) for a 24-week period, starting from July 2002 through February 2003. The animals were given 250 grams daily together with the standard diet. The main ingredients of PROSPERM® were wheat, fish oil and soybean meal. The composition of the supplemented diet is shown in tab. 1. The supplementation was implemented for a period of 24 weeks in order to provide a continuous source of n-3 PUFA throughout the duration of the experiment. The purpose of Vitamin E supplementation was to prevent any peroxidation of the 22:6n-3 polyunsaturates, which could potentially reduce the level of the fatty acid in the sperm lipids. PROSPERM® was kindly provided by Minitüb GmhH&Co. KG (Tiefenbach, Germany).

Semen quality assessment

Prior to dietary supplementation, ejaculates collected once every 7-10 days from each boar were used as the controls (unsupplemented). At the start of the supplementation period, semen was collected once every 9-10 days from the boars. The duration of the experiment was divided into 2 phases, Period 1 and Period 2. Period 1 represented semen collected during the first 8 weeks, whereas Period 2 represented semen collected during the next16 weeks of feeding (9-24 weeks). We assumed that a period of 8 weeks will be convenient for maximum incorporation of PUFA since spermatogenesis and epidymidal transport take 34 and 10 days, respectively, in the boar [22]. At the time of collection, the semen volume was filtered through sterile gauze (gel-free semen volume).

Table 1. Supplementary feed for boars (PROSPERM®, JSR Clover, Ltd, UK)

Ingredients	
Crude protein	22.0%
Lysine	0.8%
Crude fat	25.0%
Raw ash	2.0%
Moisture level	10.0%
Phosphorus	0.3%
Additives per kg:	1000 mg Vitamin E 750 mg Vitamin C 2.75 mg Selenium

Sperm motility was analyzed by placing a sample on a pre-warmed (37°C) microscopic slide covered with a coverslip, and examined under a light microscope at a magnification x 200. Sperm concentration was determined using a haemocytometer. The total number of spermatozoa per ejaculate was calculated by multiplication of the gel-free semen volume with sperm concentration. This study was approved by the local Ethics Committee.

Sperm morphology was evaluated using the Giemsa staining method. The osmotic resistance of the acrosomal membrane (ORT) was conducted as described in a previous study [20]. The percentage of viable fluorescent sperm cells (intact-membrane) was observed by fluorometric evaluation (dry preparations) using DNA-specific fluorochrome Hoechst 33258 [6].

Biochemical characteristics of semen
Protein measurements

Following centrifugation (1 000 × *g*, 5 min) of the semen, the seminal plasma was aspirated and recentrifuged (10 000 × *g*, 10 min). The resulting supernatant was used for protein measurements. Total protein content was measured using the biuret method [26].

Antiperoxidant activity and lipid peroxidation measurements

The antiperoxidant properties of seminal plasma were determined on washed spermatozoa [8]. Aliquots of sperm suspensions (1 ml) were incubated (60 min, 37°C) in the presence of 0.2 ml homologous seminal plasma; 0.01M ferrous sulphate ($FeSO_4$); 0.1M sodium ascorbate and 0.7 ml Tris buffer (pH 8.0). The lipid peroxidation (LPO) was measured as the production of malondialdehyde (MDA), with and without the seminal plasma, using the thiobarbituric acid (TBA) reaction [23], with some modifications [18]. The absorbance was measured at a wavelength of 530 nm using malondialdehyde dimethylacetal (Merck kGaA, Darmstadt, Germany) as a standard. Antiperoxidant activity was expressed as the percentage inhibition of MDA production. The lipid peroxidation level was expressed as nanomoles of MDA generated by 10^8 spermatozoa during 60-min incubation at 37°C.

Superoxide dismutase (SOD) activity in seminal plasma and spermatozoa

The SOD activity was measured in the seminal plasma obtained after centrifugations. The sperm pellets obtained after centrifugation were resuspended at a concentration of 3 x 10^8 in 0.85% NaCl, washed twice by centrifugation and finally suspended in 0.85% NaCl. The sperm extracts were prepared by homogenization (IKA, Ultra Turbox, T8) in an ice-bath for 5 min followed by centrifugation at 10 000 x *g* for five minutes. The supernatant was aspirated and stored at -80°C, until required for SOD analysis.

The SOD activity in the seminal plasma and spermatozoa was measured according to the method of Beauchamp and Fridovich [3]. The SOD activity was measured spectrophotometrically (560 nm) by the inhibition of nitroblue tetrazolium (NBT) reduction due to superoxide anion radical ($O_2^{\cdot -}$) generated by the xanthine and xanthine oxidase system. One unit of SOD activity represented the amount of enzyme that caused a 50% inhibition of this reaction at 25°C (pH 10.2).

Antioxidant properties of seminal plasma

The content of ascorbate and glutathione was measured as described previously [19]. The ergothioneine content was measured in seminal plasma following deproteinization with ethanol as described by Mann and Leone [11].

Statistical analysis

All results are presented as means ± standard error of means (SEM). Means were compared using one-way analysis of variance (ANOVA) followed by Duncan multiple comparison test. Calculations were carried out using the STATISTICA computer package (STATISTICA for Windows, StatSoft Incorporation, Tulsa, OK., USA). Differences between means were evaluated at 5% level ($p \leq 0.05$) for statistical significance.

RESULTS

The effect of PROSPERM supplementation on the quality of boar semen is shown in table. 2. Higher gel-free semen volume was concurrent with an increased ($p \leq 0.05$) number of spermatozoa per ejaculate during Period 2 of the dietary supplementation. There were no statistically significant changes ($p \geq 0.05$) in the percentage of sperm motility and morphologically abnormal spermatozoa. Throughout the entire period of PROSPERM supplementation, higher ($p \leq 0.05$) membrane resistance to changes in osmotic conditions (ORT) was concomitant with an increased percentage of membrane-intact spermatozoa, assessed by the DNA-binding fluorochrome, Hoechst 33258.

Compared with the control, a high level of MDA production was paralleled by a corresponding increase ($p \leq 0.05$) in SOD activity in the seminal plasma and spermatozoa in Period 2 (tab. 3). During the 8-week period of supplementation, even though MDA production and SOD activity were higher than the control, the changes were not significantly different ($p \geq 0.05$).

The significant increase ($p \leq 0.05$) in ascorbate content in the seminal plasma throughout the entire period of PROSPERM supplementation was

Table 2. Effect of PROSPERM supplementation on boar semen quality (mean ± SEM)

PROSPERM		No. of ejaculate	Gel-free semen volume (cm^3)	Sperm motility (%)	Sperm concentration ($x\ 10^6/cm^3$)	No. of sperm per ejaculate ($x\ 10^9$)	Total abnormal spermatozoa (%)	Membrane-intact spermatozoa (%)	ORT
Control (unsupplemented)		24	169.00^a ±8.78	70.75 ±1.10	282.05^a ±16.49	44.38^a ±2.33	6.35 ±0.59	84.59^a ±0.73	73.16^a ±0.76
Supplemented	Period 1 1st-8th week (8 weeks)	11	137.27^b ±9.35	73.00 ±1.11	461.36^b ±43.83	60.74^b ±4.43	7.01 ±1.36	90.07^b ±0.84	77.25^b ±1.41
	Period 2 9th-24th week (16 weeks)	19	248.95^c ±12.45	71.18 ±0.68	322.24^c ±21.13	76.08^b ±4.95	6.84 ±0.94	89.42^b ±0.86	79.61^b ±0.72

^{abc}means with different letters in the same column are significantly different ($p \le 0.05$); ORT – osmotic resistance test

Table 3. Malondialdehyde (MDA) production and superoxide dismutase (SOD) activity of boar spermatozoa during PROSPERM supplementation (mean ± SEM)

PROSPERM		No. of ejaculate	MDA production (nmol/10^8pz./h)	SOD activity	
				Seminal plasma (U/cm^3)	Spermatozoa (U/10^9spz)
Control (unsupplemented)		24	26.83[a] ±0.78	96.63[a] ±6.67	328.61[a] ±49.39
Suplemented	Period 1 1st-8th week (8 weeks)	11	27.65[a] ±1.04	105.43[ab] ±11.09	363.76[a] ±25.86
	Period 2 9th-24th week (16 weeks)	19	30.56[b] ±0.81	119.88[b] ±6.92	429.89[b] ±18.66

[ab]means with different letters in the same column are significantly different ($p \le 0.05$)

more pronounced in Period 2 compared with the control and Period 1 (tab. 4). Furthermore, a significant increase ($p \le 0.05$) in glutathione content and antiperoxidant activity was evident only in Period 2. Even though, there were fluctuations in protein and ergothioneine content during the dietary supplementation, the changes were not significantly different (tab. 4).

In Period 2, spermatozoa preserved in Kortowo (K3) extender supplemented with lipoprotein fractions, extracted from hen egg yolk (LPFh) or ostrich egg yolk (LPFo), exhibited higher ($p \le 0.05$) motility on Day 2, 3 and 4 during storage at 5°C and 16°C, when compared with the control (tabs. 5 and 6). Furthermore, in Period 2, spermatozoa preserved in K-3/LPFh or K-3/LPFo extender had significantly higher ($p \le 0.05$) motility than those in Period 1 on Day 3 of storage at 5°C or 16°C. In this study, the enhancement in motility was not evident when spermatozoa were preserved in K-3 extender, without lipoprotein supplementation, irrespective of the storage temperature. Most of the changes in motility of spermatozoa preserved in K-3 extender were not significant ($p \ge 0.05$), particularly during storage at

Table 4. Total protein content, antioxidant properties and antiperoxidant activity of boar seminal plasma during PROSPERM supplementation (mean ± SEM)

PROSPERM		No. of ejaculate	Total protein (mg/cm^3)	Ascorbate (mg%)	Ergothioneine (mg%)	Glutathione (mg%)	Antiperoxidant activity (%)
Control (unsupplemented)		24	44.14 ±4.15	0.34[a] ±0.03	7.90 ±0.64	4.67[a] ±0.59	23.13[a] ±3.28
Supplemented	Period 1 1st-8th week (8 weeks)	11	35.03 ±4.54	0.53[b] ±0.10	7.51 ±1.07	5.48[a] ±0.64	20.97[a] ±3.21
	Period 2 9th-24th week (16 weeks)	19	47.13 ±5.42	1.69[c] ±0.30	9.73 ±1.85	14.76[b] ±3.46	37.41[b] ±5.58

[abc] means with different letters in the same column are significantly different ($p \leq 0.05$)

Table 5. Effect of PROSPERM supplementation on motility (%) of boar spermatozoa stored in K3 extender, supplemented with or without egg yolk lipoproteins at 5°C (means ± SEM)

PROSPERM		Storage days											
		Day 1			Day 2			Day 3			Day 4		
		K-3	K-3 /LPFh	K-3 /LPFo	K-3	K-3 /LPFh	K-3 /LPFo	K-3	K-3 /LPFh	K-3 /LPFo	K-3	K-3 /LPFh	K-3 /LPFo
Control (unsupplemented)	*(n=16)*	64.00[a] ±1.90	70.00 ±1.38	70.35 ±1.14	44.28[a] ±3.38	62.14[a] ±2.37	61.43[a] ±2.37	29.00 ±1.63	42.67[a] ±5.41	43.67[a] ±4.06	19.29 ±1.73	28.57[a] ±2.94	24.64[a] ±1.52
Supplemented	*(n=11)* Period 1 1st-8th wk (8 wks)	58.50[ab] ±5.68	68.00 ±5.28	65.50 ±5.02	40.00[a] ±5.02	68.13[ab] ±3.13	68.88[ab] ±2.82	24.29 ±2.97	46.25[a] ±5.40	43.13[a] ±5.74	20.25 ±3.76	30.00[ab] ±2.89	28.57[ab] ±4.04
	(n=19) Period 2 9th-24th wk (16 wks)	43.23[b] ±4.72	76.94 ±0.92	73.67 ±1.33	26.25[b] ±5.07	72.22[b] ±1.29	70.33[b] ±1.58	18.71 ±5.53	59.69[b] ±4.86	61.15[b] ±5.16	9.29 ±2.67	40.36[b] ±5.66	35.45[b] ±4.74

[ab]means in the same column with different letters are significantly different ($p \leq 0.05$); K-3: Kortowo 3 extender; K-3/LPFh: Kortowo 3 extender supplemented with lipoprotein fractions extracted from hen egg yolk; K-3/LPFo: Kortowo 3 extender supplemented with lipoprotein fractions extracted from ostrich egg yolk.

Table 6. Effect of PROSPERM supplementation on motility (%) of boar spermatozoa stored in K3 extender, supplemented with or without egg yolk ipoproteins at 16°C (means ± SEM)

PROSPERM		Storage days											
		Day 1			Day 2			Day 3			Day 4		
		K-3	K-3 /LPFh	K-3 /LPFo	K-3	K-3 /LPFh	K-3 /LPFo	K-3	K-3 /LPFh	K-3 /LPFo	K-3	K-3 /LPFh	K-3 /LPFo
Control (unsupplemented)	*(n=16)*	66.33 ±1.58	71.67 ±1.16	72.50 ±0.87	50.00 ±3.14	62.50[a] ±2.71	61.07[a] ±2.58	30.33 ±2.56	45.33[a] ±4.96	42.33[a] ±4.28	22.32 ±1.97	27.87[a] ±1.77	22.86[a] ±1.15
Supplemented	*(n=11)* Period 1 1st-8th wk (8 wks)	62.50 ±4.79	70.50 ±5.45	68.00 ±5.64	41.25 ±6.10	66.25[ab] ±3.10	66.20[ab] ±3.11	28.57 ±4.72	46.88[a] ±5.42	42.50[a] ±5.82	25.00 ±5.00	28.57[ab] ±1.43	31.42[ab] ±2.61
	(n=19) Period 2 9th-24th wk (16 wks)	55.59 ±4.20	76.94 ±0.92	75.00 ±0.98	43.43 ±5.49	73.06[b] ±0.59	71.67[b] ±1.26	27.86 ±5.34	63.75[b] ±3.49	66.92[b] ±2.00	13.79 ±2.51	35.71[b] ±3.74	34.55[b] ±3.66

[ab]means in the same column with different letters are significantly different ($p \leq 0.05$).

16°C. However, a decline in motility of spermatozoa stored in K-3 extender was more marked in Period 2 on Day 1 and 2 during storage at 5°C, when compared with the control.

DISCUSSION

In this study we assumed that an 8-week period for the time-related improvements in semen quality is necessary for maximum incorporation of PUFA into the sperm plasma membranes, since spermatogenesis and epididymal transport take about 44 days in the boar. We observed that the maximum effect of PUFA supplementation, as shown in the sperm characteristics, was more noticeably after this 8-week period. Evidence has shown that differences in the phospholipids of polyunsaturated fatty acid composition may affect the flexibility and compressibility of sperm membranes [9, 16]. It is conceivable that such properties may influence the ability of the plasma membrane to accommodate the characteristic flagellar motion of the spermatozoa.

Because of the seriously damaging potential of reactive oxygen species (ROS), sperm cells depend on elaborate defense mechanisms to effectively neutralize or metabolize the toxic intermediates. One of the by-products of lipid peroxide decomposition is MDA production, which has been used in biochemical assay to monitor the degree of peroxidative damage sustained by spermatozoa. However, the concurrent increase in MDA production and SOD activity might indicate that SOD makes a significant contribution to the protection of boar spermatozoa against peroxidative damages. Our studies are consistent with previous studies indicating that high MDA levels were accompanied by high SOD activity in human spermatozoa [1, 2]. It has been shown that SOD dismutates the superoxide anion radical into hydrogen peroxide, which is scavenged by glutathione peroxidase [1]. Moreover, SOD is the primary enzymatic defense against lipid peroxidation and plays a central role in the defense against oxidative insult. According to Taylor [24], extracellular SOD binds to the neck region of subgroup of spermatozoa which retain motility longer than those without bound SOD. Moreover, Cerolini et al. [4] reported that semen samples with the highest viability after freez-

ing and thawing were also characterized by high SOD activity. Our findings demonstrate that PUFA supplementation induced an increase in SOD activity, which was also associated with high sperm viability.

In the present study, changes in the antioxidant defense mechanisms of the seminal plasma were related to lipid peroxidation mediated by increased levels of n-3 PUFA. Since dietary manipulation caused an increase in lipid peroxidation, it appears that the antioxidants were able to provide a practical protection from lipid peroxidation and might also be speculated to promote stability of the spermatozoa. Moreover, the maintenance of sperm membrane phospholipids together with the susceptibility to peroxidation depends on adequate antioxidant properties, which reduce the risk of damage to spermatozoa and probably their lack of survival during storage. Previous studies showed that antioxidants that are present in boar seminal plasma are derived from low molecular weight fractions [17, 19]. Thus, a deficiency of these fractions can affect the overall protection of the spermatozoa from oxidative damage, which can have a negative effect on sperm motility and fertilization. Ascorbate and thiol group may contribute to the chain-breaking antioxidant capacity [19]. Furthermore, the high level of ascorbate might be attributed to its biological role as a key chain-breaking antioxidant combating free radicals, thus protecting spermatozoa from oxidative assault. Sulphydryl constituents of boar semen, such as glutathione and ergothioneine, have an important role in protecting boar spermatozoa against oxidative damage [17]. Under normal circumstances a balance is maintained between the extent of free-radical production and antioxidant capacity of the semen. However, a change in the antioxidant defense status of the spermatozoa and surrounding seminal plasma may affect the sperm fertilizing ability.

The results of this study show that alteration in dietary PUFAs enhanced the antioxidant status of boar seminal plasma after 8 weeks of supplementation. We observed that this period of PUFA supplementation was also associated with increased antiperoxidant activity of the seminal plasma. It has been reported the antiperoxidant activity is dependent on the presence of antiperoxidant factors, which may attach to the spermatozoa at ejaculation and can protect them from lipid peroxidative attacks during their passage through the female genital tract [20].

Alteration in dietary n-3 and n-6 PUFAs also enhanced the motility of boar spermatozoa during storage in K3 extender supplemented with lipoprotein fractions of egg yolk. It can be suggested that PUFA supplementation improved the protective action of egg yolk lipoproteins, which was manifested in reduced sperm cold shock susceptibility and storage-dependent ageing process, particularly during storage at 5°C. The beneficial effect of egg yolk lipoproteins on maintenance of sperm survivability during liquid preservation has been reported in a previous study [7].

In summary, supplementing the diet of boars with n-3 PUFA changed the semen characteristics favorably. From the current data, the enhanced lipid peroxidation was compensated with high levels of antioxidants, which are essential for spermatozoa to maintain equilibrium in an oxidative environment. The results of this study also show that PUFA supplementation enhances the biochemical characteristics of boar semen, which have a beneficial effect on the survivability of preserved spermatozoa during storage in an extender containing egg yolk lipoproteins. Finally, a further understanding of the functional implications of the enhanced polyunsaturated spermatozoa and antioxidant capacity of boar semen is needed to improve semen preservation since phospholipid composition of the sperm plasma membranes plays an important role in either liquid storage or freeze- thawing process.

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