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Effect of a dietary antioxidant supplementation on semen quality in pony stallions

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Abstract

Lipid peroxidation contributes to the damage of the sperm plasma membrane. In different species, dietary supplementation with antioxidants has been shown to improve semen quality. Therefore, we tested effects of dietary supplementation with antioxidants and L-carnitin on semen quality in Shetland pony stallions ($n = 6$). Semen was collected twice a week over a time period of 16 weeks. From weeks 5 to 12, a special diet for stallions containing a variety of antioxidants (STALLION®, Pavo Pferdenahrung GmbH, Goch, Germany; tocopherol 300 mg/day; ascorbic acid 300 mg/day; L-carnitin 4000 mg/day; folic acid 12 mg/day) was added to the basal diet (hay, mineral supplements, water). Ejaculates were evaluated for total sperm count, semen motility (percentage of totally and progressively motile spermatozoa, longevity for 24 h at 5 °C) and membrane integrity (SYBR-14/PI staining): All values given are means ± S.E.M. No changes in motility, progressive motility and membrane integrity or semen longevity for 24 h were detected. A slight but significant reduction of morphologically abnormal spermatozoa was found (weeks 1–4: 43.7 ± 7.1%; weeks 13–16: 39.4 ± 7.2%, $p < 0.05$). Results show that a supplementary diet with antioxidants in the given concentration and duration does not result in pronounced effects on semen quality of stallions. It is therefore questionable to support stallions with dietary antioxidants as long as they receive an adequately balanced basal diet.

Keywords: Stallion; Semen; Dietary supplementation; Vitamin E; Vitamin C

1. Introduction

Mammalian sperm plasma membranes contain very high concentration of long-chain (C_{22}) polyunsaturated fatty acids. Therefore and because of their inadequate defensive mechanisms they are highly susceptible to lipid peroxidation [1,2]. Spermatozoa have the ability to produce reactive oxygen species (ROS) which have physiological functions in signalling events controlling sperm capacitation, acrosome reaction and sperm-oocyte fusion as long as they are produced in a controlled manner [3]. An imbalance in the production or degradation of ROS may have serious adverse effects on sperm function [4], for example on sperm motility which declines prior to detectable changes in membrane integrity [5]. Oxidative stress appears as a consequence of the extreme ROS production and results in a decrease of intracellular ATP levels which initiates lipid peroxidation in the sperm plasma membrane [6]. To

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avoid such a ROS overproduction, organisms have developed several defence mechanisms that include an enzymatic defence system (superoxide dismutase, catalase, glutathione transferase, and glutathione peroxidase) and antioxidants (ascorbate, reduced glutathione, urate, vitamin E and β-carotene) [7]. Vitamin E (α-tocopherol) is believed to be the primary component of the antioxidant system of spermatozoa [8] and is one of the major membrane protectants against ROS and lipid peroxidation [9]. It is not synthesized by mammalian cells and once membrane tocopherol is consumed during periods of oxidative stress, cellular lipids are subject to peroxidation which can result in toxic damages [10]. Vitamin C is most effective as an antioxidant in the aqueous phase, and it seems to scavenge free radicals and positive interactions with vitamin E have been suggested [11].

Dietary supplementation with vitamin E, vitamin C, selenium and other antioxidative substances is claimed to be associated with improved antioxidant defense mechanism and prevention of free radical-associated damages in testes and epididymides. Recently an improvement in semen quality (semen motility, longevity, morphology as well as total sperm count) after dietary intake of antioxidants alone or in combination with polyunsaturated fatty acids has been reported in a variety of species [12–18]. It has therefore become popular to add antioxidants to the diet of breeding animals of various species. For the stallion, so far no controlled studies on the effects of dietary supplementation of antioxidants on semen quality have been performed. The objective of this study was thus to evaluate the effects of dietary supplementation with antioxidants on quality of equine semen immediately after collection and on longevity of semen cooled-stored for 24 h.

2. Materials and methods

2.1. Animals

A total of six fertile Shetland pony stallions were used in this study. These animals belonged to the Centre for Artificial Insemination and Embryo Transfer for several years (two animals were born from mares of our experimental herd, four were bought as 3-year-old stallions). At the start of the experiment, the animals were aged between 8 and 16 years (11.0 ± 1.3) and weighed between 150 and 190 kg. From these stallions, ejaculates are collected and examined on a regular basis for breeding or teaching purposes throughout the year (e.g. two to three times per week). The experimental stallions were kept as a group in a spacious stable with access to an outdoor paddock from 7:00 a.m. to 6:00 p.m. As all ponies from our experimental herd, they were kept on a diet of hay (fed twice daily), water and mineral supplements were freely available. Experiments were carried out from September to the end of December.

2.2. Experimental design

Semen was collected twice a week from each stallion over 16 weeks in total. For the first 4 weeks of the experiment feeding of the basal diet (hay, mineral supplements, water) was continued without any supplementation (weeks 0–4). From weeks 5 to 12 a special diet for stallions containing a variety of antioxidants and L-carnitin (STALLION®, Pavo Pferdenahrung GmbH, Goch, Germany) was added to the standard diet. The animals received 15 g daily. The composition of the supplemented diet is shown in Table 1. The last 4 weeks of the experiment, the stallions were again fed their basal diet without any supplementation (weeks 13–16). Ejaculates were evaluated for total sperm count, semen motility (percentage of totally and progressively motile spermatozoa, longevity for 24 h at 5 °C with a CASA system), morphological aberrations of spermatozoa and membrane integrity (SYBR-14/PI staining).

2.3. Semen collection and semen analysis

Semen was collected with an artificial vagina (Hannover model, Minitüb, Tiefenbach, Germany) after exposure of the stallion to a stimulus mare until erection and readiness to mount, followed by mounting of a dummy. Immediately after collection, the gel fraction of the ejaculate was removed and semen was filtered through sterile gauze and volume and colour were determined. The sperm concentration was measured photometrically (SpermaCue, Minitüb). Total sperm count per ejaculate was calculated from volume and

Table 1
Composition of supplementary diet for stallions (STALLION®, Pavo Pferdenahrung GmbH, Goch, Germany) in mg/(stallion day)

Vitamin E	300 mg
Vitamin C	300 mg
L-Carnitin	4000 mg
Folic acid	12 mg
Copper	–
Zinc	–
Manganese	–
Selenium	–

sperm concentration. The pH was determined with test strips (Merck, Darmstadt, Germany) and sperm morphology was determined after Hancock fixation. The percentage of totally and progressively motile spermatozoa (immediately after collection and 24 h after collection in semen diluted with EquiPro semen extender (Minitüb), final dilution 50×10^6 spermatozoa per ml, storage temperature 5 °C) as well as membrane integrity (viability) was determined with a computer assisted sperm analyzer (CASA; SpermVision®, Minitüb) as described [19,20]. For the assessment of spermatozoa viability, 100 μl of semen were placed in a vial with 2 μl of SYBR-14/PI and incubated for 10 min at room temperature in darkness. On a glass slide, covered with a glass coverslip, one droplet was placed and evaluated by fluorescence microscopy at magnification 400× (Olympus AX70, Olympus Optical Co. Ltd., Japan; U-MWB filter block, BP420-480 excitation filter, BA515 suppressor filter, dichromatic mirror: DM500). At least 15 fields were evaluated and the average value was calculated by the CASA system. Results were given as percent of membrane intact cells.

2.4. Statistical analysis

Statistical analysis was performed with the SPSS/PC+ statistics package [21]. Because data were not always normally distributed, non-parametrical tests were used throughout. For the whole experimental period (weeks 1–16), all parameters in the same animals were compared by Friedman's test. Mean values for the periods before (weeks 1–4) and after (weeks 13–16) supplementation were calculated and compared by Wilcoxon's test. A p-value < 0.05 was considered significant. All values given are means ± standard error of mean (S.E.M.).

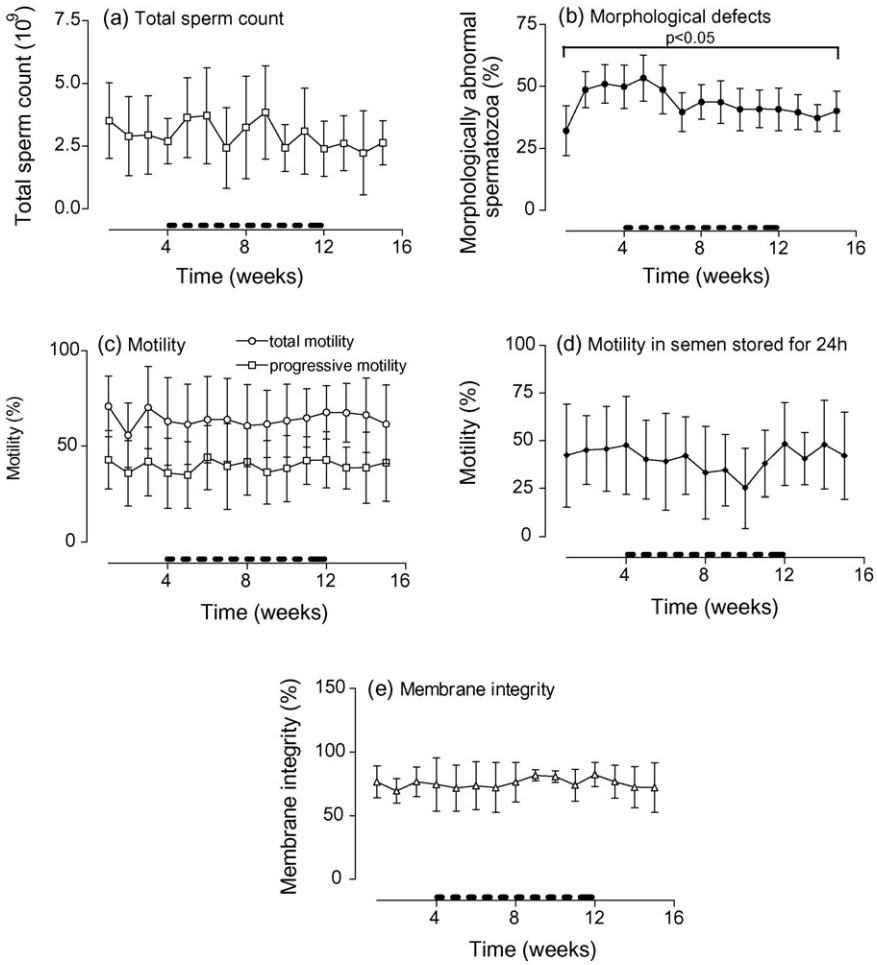

Fig. 1. Semen traits ((a) total sperm count, (b) morphological defects, (c) total and progressive motility, (d) motility after storage for 24 h and (e) membrane integrity) of pony stallions treated with a supplementary diet for 8 weeks (weeks 4–12, indicated by dotted bar).

Table 2
Morphological semen defects (%) in ejaculates from pony stallions treated with a supplementary diet for 8 weeks (weeks 4–12) for the periods before (weeks 1–4) and after treatment (weeks 13–16)

	Before treatment	After treatment	p-Value
Acrosomal defects	5.4 ± 1.3	5.0 ± 1.5	n.s.
Head defects	1.9 ± 0.2	1.9 ± 0.2	n.s.
Neckpiece defects	13.0 ± 2.3	14.8 ± 2.8	<0.05
Proximal droplets	13.4 ± 1.5	11.4 ± 1.2	n.s.
Main piece defects	11.2 ± 2.8	7.4 ± 1.5	0.08
Tailpiece defects	7.0 ± 1.5	5.7 ± 1.7	<0.05
Distal droplets	1.1 ± 0.7	0.4 ± 0.3	<0.05
Paired spermatozoa	0.9 ± 0.1	0.8 ± 0.1	n.s.
Immature germ cells	1.5 ± 0.4	1.1 ± 0.4	n.s.
Total	43.7 ± 7.1	39.4 ± 7.2	<0.05

3. Results

Mean total sperm count decreased from $3.2 \pm 0.4 \times 10^9$ spermatozoa in the period before supplementation (weeks 1–4) to $2.5 \pm 0.4 \times 10^9$ spermatozoa in the period after supplementation (weeks 13–16; $p < 0.05$). As shown in Fig. 1, no changes for motility, progressive motility and membrane integrity between these periods could be found and mean corresponding values were 64 ± 6.7 and 65.8 ± 6.9% for motility, 38.6 ± 5.5 and 40.4 ± 6.1% for progressive motility and 72.9 ± 3.5 and 75.9 ± 5.4% for membrane integrity (n.s.). Longevity of spermatozoa for 24 h was not affected by supplementation of feeding and was 43.3 ± 7.7% motile spermatozoa after 24 h of cooled-storage in the pre-treatment period and 44.7 ± 7.1% in the post-treatment period (n.s.). A slight but significant reduction in the percentage of morphologically abnormal spermatozoa could be detected. The percentage of morphological defects decreased from 43.7 ± 7.1% in the period before treatment to 39.4 ± 7.2% in the period after treatment ($p < 0.05$). However, the reduction of morphological aberrations could not be attributed to a specific morphological defect (Table 2).

4. Discussion

In the present study, we could not find pronounced effects of a dietary supplementation with vitamin E, vitamin C and carnitin on semen quality or total sperm count in stallions. This result is in contrast to findings in humans, boars, rats and poultry where supplementation of food with vitamin E or C improved semen quality [12,15,16,22] and total sperm count [16,23]. In the present study, the duration of treatment (56 days) and further evaluation of semen quality for another 28 days should have been sufficient to find possible effects of the diet. In the stallion, spermatogenesis has a total duration of 55–57 days and is followed by maturation and storage in the epididymidis which lasts approximately 9 days. In conclusion, semen collected during the last 3 weeks of the experimental period contained spermatozoa that underwent complete spermatogenesis and epididymal maturation during the period of dietary supplementation. The absence of pronounced effects of the diet might be related to the fact that oxidative effects in equine semen are less important than in other species (reviewed by [24]). The addition of antioxidative defense systems such as vitamin E or C may therefore be of minor importance. This is in agreement with the finding that addition of antioxidants to semen extenders for cooled-storage of equine semen does not result in pronounced effects on semen motility [4,19,25]. However, stallions used in our study are normally kept on a rather limited diet of hay and mineral supplements to avoid illness such as obesity, laminitis or hyperlipiemia frequently found in overfed Shetland type ponies. The diet was not created to provide an optimal semen quality. It cannot be completely excluded that supplementation of vitamins and carnitin alone was not sufficient for an improvement of semen quality. An effect may not become visible before unsaturated fatty acids are provided simultaneously as has been done in pigs [12]. This assumption is supported by the fact that feeding of unsaturated fatty acids (docosahexaeoic acid) to stallions resulted in improved motility in semen cooled-stored for 48 h. Moreover, in animals with poor semen quality after cooled-storage, improvements in semen motility after 24 h could be seen [26]. However, no effects on semen quality immediately after collection were found. Therefore, beneficial effects of antioxidants or unsaturated fatty acids may not become visible in the absence of ROS imbalance. Cooled-storage of semen is considered a situation were ROS production increases [27], however, this was not sufficient to exert positive effects of the antioxidant diet in the present study.

A significant decrease in total sperm count during the experimental period is probably not related to the diet that in other species resulted in an increase of daily sperm production [13,16]. It can be explained by the time of the year where the study was performed. Horses are long-day breeders and in stallions, spermatogenic activity decreases during the non-breeding season. In less-domesticated breeds as in many pony breeds, seasonal effects on gonadal function are rather pronounced [28]. A comparable decrease in total sperm count at that time of the year was found during previous

and subsequent years in the pony stallions from our research herd (data not shown).

The only positive effect found was a small but significant decrease in the percentage of spermatozoa with morphological defects. This is in agreement with reports from boars [13,16]. Altogether, the stallions of our group have a relatively high number of spermatozoa with primary defects (Table 2). However, all of them have shown to be fertile by producing pregnancies with an average per cycle pregnancy rate. Semen quality is regularly checked and did not show pronounced changes during the last year before the experiment started (data not shown). Before and during the experimental period, semen was collected twice weekly to keep semen quality stable. The decrease in morphological defects can therefore not be attributed to changes in the semen collection protocol in this group. The time of the year (end of the natural breeding season) would also not contribute to a decrease of morphological spermatological defects. It cannot be totally ruled out that the small improvement in semen morphology was related to feeding of the diet. A possible improvement in semen quality by the diet is most probably related to an inhibition of lipid peroxidation of the sperm plasma membrane as was shown in rats [15] and fish [18]. This possibility is supported by the type of sperm abnormalities that showed an improvement (i.e. neckpiece and tailpiece defects, distal droplets), but at least in part is contradicted by the fact that no parallel increase in plasma membrane integrity occurred. In addition, it appears questionable that such a minor improvement of semen quality might lead to any increase in fertility in treated stallions. In conclusion, the alteration of spermatozoa membrane quality and/or antioxidative defense mechanism via dietary supplementation in stallions remains an interesting option; however, more controlled experiments are necessary to provide effective feeding protocols and optimal combinations of supplements.

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