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Influences of dietary supplementation with *Lepidium meyenii* (Maca) on stallion sperm production and on preservation of sperm quality during storage at 5 °C

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SUMMARY

Stallion semen is damaged by oxidative stress during cooling and transport. Semen processing and extenders have been tested to improve the fertilizing capacity of semen and to preserve semen during transport. Dietary supplementation with natural antioxidants has been proposed to prevent oxidative damages. In this study, for the first time, the effect of dietary supplementation with *Lepidium meyenii* (Maca) on the characteristics of fresh and chilled stallion semen was evaluated. Maca is a traditional Andean crop used as a nutraceutical for the fertility-enhancing properties that are linked with antioxidant activity. The diet of five stallions was supplemented with 20 g of Maca powder daily for a total of 60 days. A control group of five stallions received the same diet without Maca. Semen was collected once before the administration of Maca (D0), twice during the administration at 30 and 60 days (D30 and D60), and finally twice at 30 and 60 days after the end of the administration (D90 and D120). Ejaculates were processed for cooled shipping at 5 °C and evaluated in the laboratory for total and progressive motility, acrosome integrity, and lipid peroxidation after collection and after 24, 48, and 72 h of storage. Dietary supplementation with Maca improved sperm concentration (from 213 ± 80.4 to 447 ± 73.1 × 10^6 spz/mL) and total sperm count (from 10,880 ± 4377 to 24,783 ± 4419 × 10^6 spz). The beneficial effects of Maca supplementation on motility and acrosome integrity in the raw semen were detected from the end of treatment with Maca (D60) until the end of the study (D120). Furthermore, during cooling storage, total motility, progressive motility, and acrosome integrity declined more slowly in the Maca-treated group than in the control group. Lipid peroxidation did not change during cooling storage in either group and did not show a significant difference between the two groups. In this study, the dietary supplementation with Maca increased sperm production and stabilized semen quality during chilled storage.

INTRODUCTION

Over the past decades, artificial insemination (AI) has become a primary breeding tool in many horse breeds (Aurich & Aurich, 2006). Cooled–transported semen is used worldwide, allowing breeders to benefit from the best stallions of most breeds while avoiding transportation of animals (Aurich, 2008). However, cooling and storage of spermatozoa are associated with a reduction in cell viability, motility, and fertilizing capacity (Yoshida, 2000). One of underlying mechanisms maybe related to an imbalance in the production and scavenging of Reactive Oxygen species (ROS; reviewed by Pagl *et al.*, 2006).

ROS in the male genital tract and in spermatozoa are not per se a negative phenomenon, and in fact, ROS are produced in the testes during normal spermatogenesis and steroidogenesis (Makker *et al.*, 2009; Mathur & D'Cruz, 2011). Spermatogenesis and Leydig cell steroidogenesis are sensitive to excessive concentration of ROS in the testis (Hales *et al.*, 2005), with the consequent production of immature and defective spermatozoa. To avoid an excessive concentration of ROS, the testes have enzymatic and non-enzymatic antioxidant systems, although these systems are not always sufficient to counteract an increase in ROS concentration. Additionally, during cold storage of equine semen, ROS production increases (Aurich, 2005), causing damage to all biomolecules of sperm cells, including lipids and proteins of membranes and DNA (Ball, 2008; Aitken *et al.*, 2010). Low concentrations of ROS are indispensable for several important functions of spermatozoa, such as maturation, capacitation, hyperactivation, acrosome reaction, and spermatozoa–oocyte fusion (reviewed by Pagl *et al.*, 2006). However, an inefficient antioxidant concentration, an excessive production of ROS, or

both by immature and damaged sperm cells provoke oxidative stress (OS), thereby damaging the other sperm cells (Aitken *et al.*, 2012; Roca *et al.*, 2013).

To maintain the quality and fertility of stallion semen for much longer periods, semen processing has been optimized, and supplementation of extenders with antioxidants has been tested with varying results (Aurich *et al.*, 1997; Bruemmer *et al.*, 2002; Kankofer *et al.*, 2005; Aurich, 2008; Brogan *et al.*, 2015). An alternative strategy to prevent oxidative damage to spermatozoa in the testes and epididymis and to improve the antioxidative capacity of spermatozoa during storage may be found in food supplementation with antioxidants and/or polyunsaturated fatty acids (PUFAs; Brinsko *et al.*, 2005; Deichsel *et al.*, 2008; Contri *et al.*, 2011; Schmid-Lausigk & Aurich, 2014; Freitas *et al.*, 2016).

Studies in humans (Hunt *et al.*, 1992; Ziegler & Filer, 1996; Eskenazi *et al.*, 2005) suggest that dietary supplementation with antioxidants reduces seminal oxidative stress and improves semen quality, particularly in subfertile males (Wong *et al.*, 2000; Ross *et al.*, 2010). Furthermore, Yue *et al.* (2010) demonstrated that supplementation with a natural antioxidant (Vitamin E) improves shipped semen quality via protecting testicular cell membranes and mitochondria from oxidative stress. The mechanism underlying this beneficial effect on oxidative status of semen is incompletely known. Most likely, the antioxidants in diets improve testicular concentrations of antioxidants, contributing to balance the testicular oxidative status. A correlation between diet consumption and semen concentration of PUFAs is reported in different species (Blesbois *et al.*, 1997; Drokin *et al.*, 1998; Conquer *et al.*, 2000). Dietary intake with PUFAs increases quality of fresh and cooled stallion semen (Brinsko *et al.*, 2005; Contri *et al.*, 2011), most likely due to a change in lipid composition such as that observed in other species (Cerolini *et al.*, 2006; Zaniboni *et al.*, 2006; Mourvaki *et al.*, 2010; Radomil *et al.*, 2011). Changes in lipid composition of essential membrane components must improve membrane stability and also reduce the vulnerability to oxidative stress during storage.

Lepidium meyenii (Maca) is an Andean crop that grows at an altitude between 3800 and 4500 m. Maca has many traditional therapeutic uses, including the treatment of rheumatism, respiratory problems, premenstrual and menopausal disorders, and as a laxative (Kilham, 2000). However, the interest in this plant is based on the positive effects on the reproductive tract and semen quantity and quality of mammals (reviewed by Clément *et al.*, 2012; and by Gonzales, 2011). Clinical trials show that Maca administration increases the number and the motility of spermatozoa and increases sexual function in humans (Gonzales *et al.*, 2001, 2002). Several studies conducted in rats demonstrated the beneficial effect of Maca administration on spermatogenesis, improving sperm count and motility (Gonzales *et al.*, 2003a, 2004, 2006, 2013b; Chung *et al.*, 2005; Gasco *et al.*, 2007; Yucra *et al.*, 2008). Dietary supplementation with Maca flour improved sperm count and motility and reduced the percentage of DNA fragmentation index in bulls (Clément *et al.*, 2010).

The effect of Maca on the reproductive system has been related to the lipid fraction of the plant, which contains primarily fatty acids and macamides (Zheng *et al.*, 2000; Hudson, 2008). Macamides and macaenes are secondary metabolites and represent a novel group of long-chained saturated fatty acids and PUFAs, and their amides are characteristic for this plant (Zheng *et al.*, 2000). In addition to a range of other components from Maca (reviewed by Wang *et al.*, 2007), several authors demonstrate that the plant has antioxidant properties (Valentova *et al.*, 2001; Sandoval *et al.*, 2002; Lee *et al.*, 2005). The mechanisms of action remain unknown for Maca that increases semen parameters. Macamides and the lipid-extractable fraction of Maca may act directly on the reproductive tract by affecting the antioxidant–oxidant balance (Gasco *et al.*, 2007; Melnikovova *et al.*, 2015).

The aim of this study was to evaluate the effects of dietary supplementation with *Lepidium meyenii* on the quantity of fresh stallion sperm and the quality, that is, motility, viability, acrosome integrity, and lipid peroxidation, of diluted stallion semen during storage at 5 °C up to 72 h.

MATERIALS AND METHODS

Animals

Ten active breeding stallions (9 to 16 years of age, median: 14) weighing 400 to 600 kg were included in the study. The stallions were clinically healthy and had a moderate body condition score of 3 (scale 1 to 5) at the start of the experiment. Stallions were housed in box stalls in the same local stud farm in Sala Consilina (Salerno, Italy) and were fed hay twice daily and concentrate twice daily at 2% of the daily body weight. Water was available *ad libitum*.

The experiment was conducted in accordance with the code of ethics (D.lgs. 26 - 04/03/2014),

and the Ethics Committee of the Department of Veterinary Medicine and Animal Productions at the University of Naples Federico II, Italy (prot. no. 0003909), approved the protocol and procedures.

Source and supplementation of Maca

Yellow Maca hypocotyls used for this experiment were harvested in the district of Junin in the Andean highlands of Peru (4100 m above sea level) and milled to a flour with a particle size of 0.8 mm. Following the traditional open-field drying, Maca flour was obtained from hypocotyls exposed for two months at the extreme temperature cycles, strong light conditions, and atmospheric pressure typical of the high-altitude environment (>3500 m). After such drying outdoors, hypocotyls were selected, washed, powdered, and packaged for use.

Animals of both the C and M groups received an identical diet based on hay and concentrate pellets. In the M group, the concentrate was supplemented daily with 20 g of Yellow Maca powder resulting in an average dose of 4 g Maca/100 kg body weight (minimum: 3 g Maca/100 kg; maximum: 5 g Maca/100 kg). The Maca powder was supplemented for a period of 60 days, starting after the first basal semen collection. The dose was based on human studies (Gonzales *et al.*, 2001, 2002) and is consistent with minimum effective doses reported for rats (Zheng *et al.*, 2000; Cicero *et al.*, 2001, 2002; Gonzales *et al.*, 2004).

Experimental design

The animals were divided into a control group (C; $n = 5$) and a treatment group (M; $n = 5$). All subjects were randomly assigned to groups before initiation of the study, and the sequence of assignments was unknown to any of the investigators. All the animals were in good health and were kept under the same

physical and environmental conditions. After the first basal sperm collection (day 0 = D0), Maca was administered for 60 days, because in the horse, a full spermatogenic cycle (spermatocytogenesis, meiosis, and spermiogenesis) requires 57 days (Johnson *et al.*, 1997). Semen was collected with an artificial vagina three times a week from all stallions starting one month before the start of the study period and during the entire study period. In the course of the experiment, we collected semen once a month (D0, the day the Maca diet supplementation began, and D30, D60, D90, and D120) for a total of five ejaculates/stallion and 50 total samples. Each day of semen collection, before semen collection, blood samples were collected at 5:00 a.m. and were used to evaluate testosterone concentrations.

Blood processing

Blood samples were obtained by venipuncture from the jugular vein of each treated stallion. A total of five samples were obtained for each animal. After incubation, blood samples were centrifuged at 330 ***g*** for 10 min, and sera were placed in 1-mL Eppendorf tubes and stored at −20 °C for subsequent analysis.

At the end of the study, all sera were sent to a reference laboratory (Di.Lab. Veterinary Service, Naples, Italy) for evaluation. Testosterone concentration was determined by chemiluminescence assay (Leme *et al.*, 2012).

Semen processing

Immediately after collection, the gel fraction was removed by nylon semen filter (Minitube, Berlin, Germany), and the semen was filtered through a semen filter pouch (Minitube, Berlin Germany). The sperm concentration was determined with a Burker chamber. The volume of ejaculate was determined for each semen sample by graduated laboratory bottle (Sigma, Milan, Italy). Total sperm count (TSC) was calculated by multiplying sperm concentration by semen volume.

The collected semen underwent routine processing procedures (Nunes *et al.*, 2008) using a Kenney semen extender with antibiotics (EZ-Mixin®) as described below. Semen was centrifuged at 1200 ***g*** for 10 min, and 75% of the supernatant seminal plasma was removed to optimize storage at +5 °C. The remaining pellet was diluted to obtain a final concentration of 500×10^6 sperm/mL in Kenney semen extender. The samples (T0) were immediately refrigerated and brought to the laboratory within 3 h for +5 °C storage and evaluation. The total motility and progressive motility were measured at 3 (T0), 24 (T24), 48 (T48), and 72 h (T72). At each experimental time, an aliquot of each sample was pelleted and frozen at −20 °C and then processed within two months to evaluate semen lipid peroxidation. For each experimental time, a second aliquot was pelleted and fixed in 4% paraformaldehyde overnight, then washed and transferred to 70% ethanol, stored at −20 °C, and processed within two months for immunohistochemical PNA-FITC staining.

Assessment of motility

Progressive motility was evaluated using a phase contrast microscope with a heated stage at 37 °C at 100× magnification. Each sperm sample was pre-incubated before assessment at 37 °C for 10 min. The proportion of total and progressive motile spermatozoa from eight randomly selected fields in each sample was evaluated subjectively in a Makler chamber at 37 °C.

Acrosome status

Acrosome integrity was evaluated with a fluorescent-labeled peanut agglutinin (PNA-FITC, Vector Laboratories, FL, USA; Tamuli & Watson, 1994). An aliquot of a fixed sperm sample (as previously described) was spread on a microscope slide and air-dried. The spermatozoa were then permeabilized with methanol for 15 min at room temperature, washed once with 25 mM Tris-buffered saline, pH 7.6, for 5 min and then twice with H_2O at 5-min intervals. The slides were then air-dried, incubated with PNA-FITC (60 μg/mL) for 1 h, washed twice with H_2O at 5-min intervals, and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA). For each sample, at least 100 cells per slide were evaluated. Cells with green staining over the acrosomal cap were considered acrosome intact (AI); cells with equatorial green staining or no staining were considered acrosome reacted (AR) (Cocchia *et al.*, 2011).

DNA fragmentation

The APO-BrdU terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was used to detect single- and double-strand breaks in the DNA. For recognition of DNA fragmentation in apoptotic cells, an APO-BrdU™ TUNEL Assay Kit with an Alexa Fluor 488® anti-brdU antibody (Invitrogen, Molecular Probes, Carlsbad, CA, USA) was used. This kit is used to detect 30-hydroxyl ends that serve as starting points for TdT, which adds deoxyribonucleotides in a template-independent fashion. The break sites are labeled by the addition of the deoxythymidine analog BrdUTP to the TdT reaction. Once incorporated into the DNA, BrdUTP is detected by an anti-BrdUTP antibody, which is conjugated to Alexa Fluor 488 (excitation: 495 nm; emission: 519 nm). The DNA is visualized by propidium iodide staining (PI; excitation: 535 nm; emission: 617 nm). The spermatozoa were washed twice in a wash buffer and resuspended in the DNA-labeling solution for 60 min at 37 °C in a water bath. The suspensions were washed twice in rinse buffer, and the Alexa Fluor 488-labeled anti-BrdU antibody was added to each suspension. The samples were incubated for 30 min at RT in the dark. Finally, 0.5 mL of PI RNase staining buffer was added. After 30 min of additional incubation in the dark at RT, the sperm suspension was placed on three microscope slides and mounted with Vectashield (Vector Laboratories). At least 100 sperm cells were scored per slide under a Nikon 90 upright fluorescence microscope equipped with a mercury lamp (100 W) using a 488-nm excitation filter and a 530-nm long-pass filter. The heads of all sperm cells were stained red by PI, and only spermatozoa with fragmented DNA were stained also in green, as shown in Fig. 1. The mean of the results for the three slides was considered the final value of TUNEL-positive spermatozoa.

Lipid peroxidation

Lipid peroxidation (LPO) was evaluated using a commercially available assay kit (LP Sperm Test, Diacron International, Grosseto, Italy). The assay is based on the ability of peroxides to promote the oxidation of Fe^{2+} to Fe^{3+}. The product of peroxidation (Fe^{3+}) binds to thiocyanate, developing a colored complex measured photometrically at 505 nm. The increase in absorbance is directly proportional to the concentration of peroxides in the sample. LPO was expressed as millimoles LPO/10^6 spermatozoa.

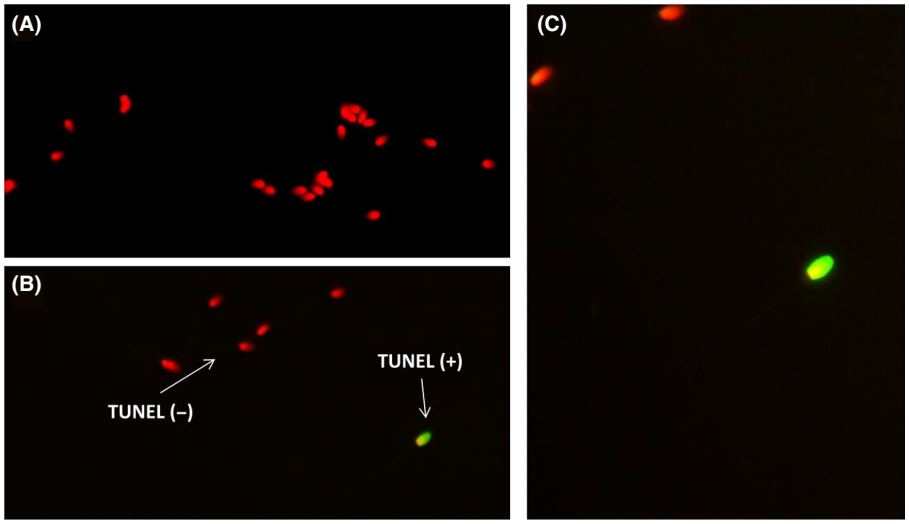


Figure 1 A representative image of equine spermatozoa stained with an APO-BrdU™ TUNEL Assay Kit with anti-brdU Alexa Fluor 488® using a fluorescence microscope. Propidium iodide (PI) stains the nucleus of all sperm cells red, and anti-brdU Alexa Fluor 488® stains the spermatozoa with fragmented DNA green. (A) A negative control; (B) TUNEL-negative (red) and TUNEL-positive (green) sperm cells at 100× magnification; (C) TUNEL-positive (green) sperm cell at 400× magnification.

Briefly (Tafuri *et al.*, 2015), an aliquot of the sperm pellet stored at −80 °C was washed three times in distilled water and then diluted 1 : 1 in PBS and vortexed for 5 min to homogenize the sample. The reagent 1 (1 mL) was transferred into a cuvette, and the sample (40 μL) was added after three drops of reagent 2. The mixture was shaken after each addition and incubated for 5 min at 37 °C. Then, the cuvette was positioned in the reading chamber for the absorbance measurement.

Statistical analyses

First, data were recorded using a computerized spreadsheet (Microsoft® Excel® 2011) and then imported into the SPSS statistical software package (IBM® SPSS® Statistics version 22.0, IBM Corporation, Armonk, NY, USA) for statistical analysis of the following semen parameters: sperm volume, concentration and total sperm count (TSC), total and progressive motility, acrosome integrity, DNA fragmentation, and lipid peroxidation. We assumed non-normality of data, because the sample size of both groups was too small to verify this assumption with a statistical test.

Sperm volume and concentration and Total Sperm Count (TSC) of raw semen at each day were compared between groups with the Mann–Whitney test. A Mann–Whitney U-test was conducted to identify significant differences between medians of Maca and control groups for each semen parameter at both initial (T0) and final (T72) storage times. This procedure was repeated for each sperm collection day (D0 to D120).

Friedman's ANOVA was used to measure the significance of the decrease in each semen parameter during storage times from T0 to T72 for each sperm collection day (D0 to D120) in each group.

To compare the decrease/increase in each semen parameter over the entire time of dietary supplementation, for each sperm collection day (D0 to D120), we calculated the percentage difference at each storage time point with respect to the previous one. Subsequently, the average of percentage differences was calculated for each sperm collection day (D0 to D120).

The medians of the daily percentage difference for each semen parameter were compared between the two groups by Mann–Whitney tests and within each group by Friedman's ANOVAs. Significance was set at $p \leq 0.05$ and $p < 0.01$.

RESULTS

Serum testosterone levels

Serum testosterone concentrations of each treated stallion throughout the experiment are reported in Fig. 2. Serum testosterone concentrations did not change significantly during the experimental period (mean±SD, ng/mL; D0 = 0.52 ± 0.29; D30 = 0.49 ± 0.21; D60 = 0.60 ± 0.23; D90 = 0.77 ± 0.26; D120 = 0.47 ± 0.34).

Quantitative parameters

The ejaculate volume was similar in both groups at the start of the experiment and did not change throughout the study, as shown in Fig. 3A. Sperm concentration and TSC also did not differ between the two groups at the start of the experiment (D0; $p > 0.05$; Fig. 3B,C). Over time, the sperm concentration and TSC increased for stallions treated with Maca from 213 ± 80.4 to 447 ± 73.1 × 10^6 spz/mL and from 10,880 ± 4377 to 24,783 ± 4419 × 10^6 spz, respectively. In the control group, sperm concentration and TSC did not change throughout the experiment (166.7 ± 15.3 vs. 193.3 ± 51.3 × 10^6 spz/mL and 8300 ± 2914 vs. 9933 ± 4317 × 10^6 spz, respectively; $p > 0.05$). The sperm concentration and TSC were higher in the M group than in the C group at 30 days (D90; 377 ± 62.3 vs. 180 ± 20 × 10^6 spz/mL and 20,090 ± 3534 vs. 9307 ± 3675 × 10^6 spz, respectively; both $p < 0.05$) and at 60 days (D120; 447 ± 73.1 vs. 193.3 ± 51.3 × 10^6 spz/mL and 24,783 ± 4419 vs. 9933 ± 4317 × 10^6 spz, respectively; both $p < 0.05$) after the end of diet supplementation (Fig. 3).

Motility

Total and progressive motility of spermatozoa in fresh diluted semen (T0) were not significantly different on any collection day (D0 to D120) between the Maca and the control groups. Total and progressive motility decreased significantly during cold storage in both groups on each day. At T72, no differences were detected between groups at D0, D30, and D60. As shown in Fig. 4A,B, total and progressive motility were higher in the M than in the C group at D90 and D120 (both $p < 0.05$).

The decrease in daily percentage difference of total and progressive motility did not differ between groups at D0, D30, and

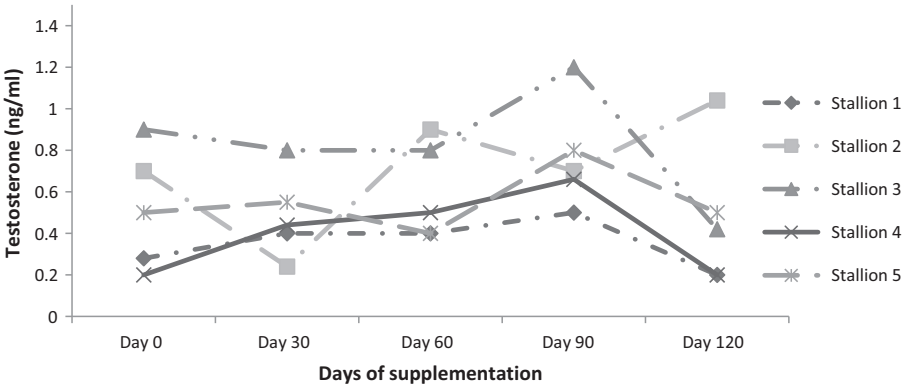


Figure 2 Blood testosterone concentration in treated stallions during the experimental period, from D0 to D120.

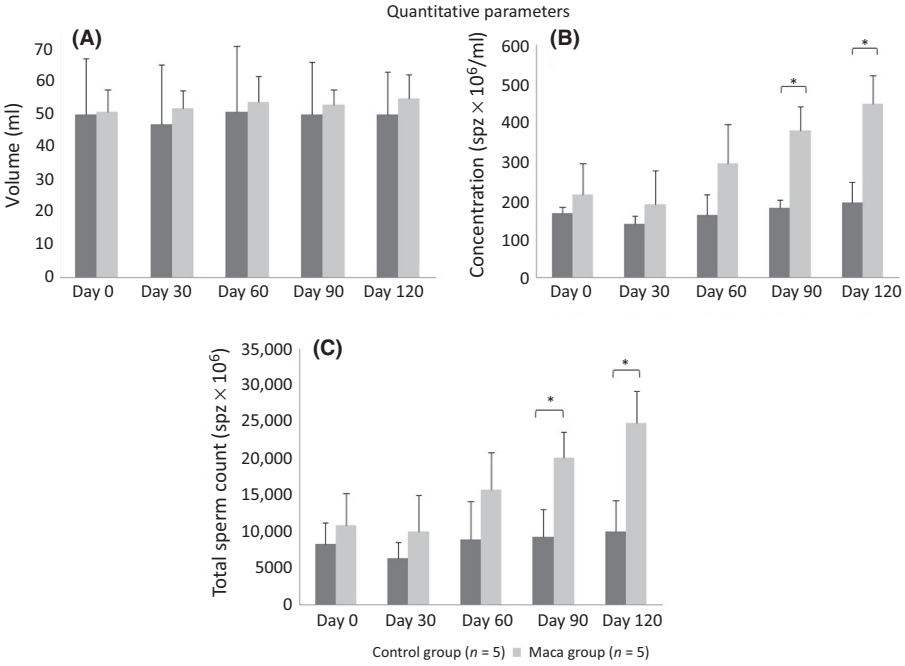


Figure 3 Quantitative parameters (mean ± SD) of semen collected every 30 days during the experiment (from D0 to D120): ejaculate volume (A), sperm concentration (B), and total sperm count (TSC; C); in control ($n = 5$) and treated ($n = 5$) groups (*$p < 0.05$).

D60. However, at D90 and D120, these differences were significantly different between groups ($p < 0.05$; Fig. 5A,B).

As shown in Fig. 5A,B, in the C group, the decrease in daily percentage difference of progressive motility between storage times (T72–T0) did not differ, whereas, in the M group, these differences were significantly lower ($p < 0.05$).

The percentage decrease in total and progressive motility over the entire time of dietary supplementation did not differ significantly in the control group. By contrast, in the Maca group, the percentage decrease in total and progressive motility was significantly lower at the end than at the beginning of dietary supplementation ($p < 0.05$, $p < 0.01$, respectively; Fig. 5A,B).

Acrosome integrity

The percentage of spermatozoa with intact acrosomes did not differ between groups in fresh diluted semen (T0) at any day (D0 to D120). Storage of the stallion semen resulted in a significant decrease in acrosome integrity in both groups ($p < 0.01$). At T72, no significant differences were observed at D0 and D30. Compared with the C group, in the M group at D60, D90, and D120, the percentage of acrosome integrity was significantly higher ($p < 0.01$; Fig. 4C).

As shown in Fig. 5C, the decrease in daily percentage difference of acrosome integrity did not differ between groups at D0 and D30. Starting from D60 until the end of experiment, these decreases were lower in the M group ($p < 0.01$).

Furthermore, in the C group, the percentage decrease in acrosome integrity over the entire time of dietary supplementation did not differ significantly. By contrast, in the Maca group, the percentage decrease in acrosome integrity was significantly lower at the end than at the beginning of dietary supplementation ($p < 0.01$; Fig. 5C).

DNA fragmentation

DNA fragmentation of spermatozoa in fresh diluted semen (T0) collected at D0, D30, D60, and D90 did not show any significant differences between the C and M groups. At D120, DNA fragmentation was significantly higher in the C (10.4 ± 0.6%) than in the M group (5.2 ± 0.6%) $p < 0.01$).

DNA fragmentation increased significantly during cooling time in both groups on each day ($p < 0.01$). As shown in Fig. 4D, at T72, a significantly higher percentage of spermatozoa with fragmented DNA was observed in the C group than in the M group at D30, D60, D90, and D120 ($p < 0.01$).

Figure 4 Parameters of semen quality: total (A) and progressive motility (B), acrosome integrity (C), and DNA fragmentation (D), at 72 h of storage at 5 °C in control (C; $n = 5$) and treated (M; $n = 5$) groups at different days of the experiment (D0, D30, D60, D90, and D120). (*$p < 0.05$; **$p < 0.01$).

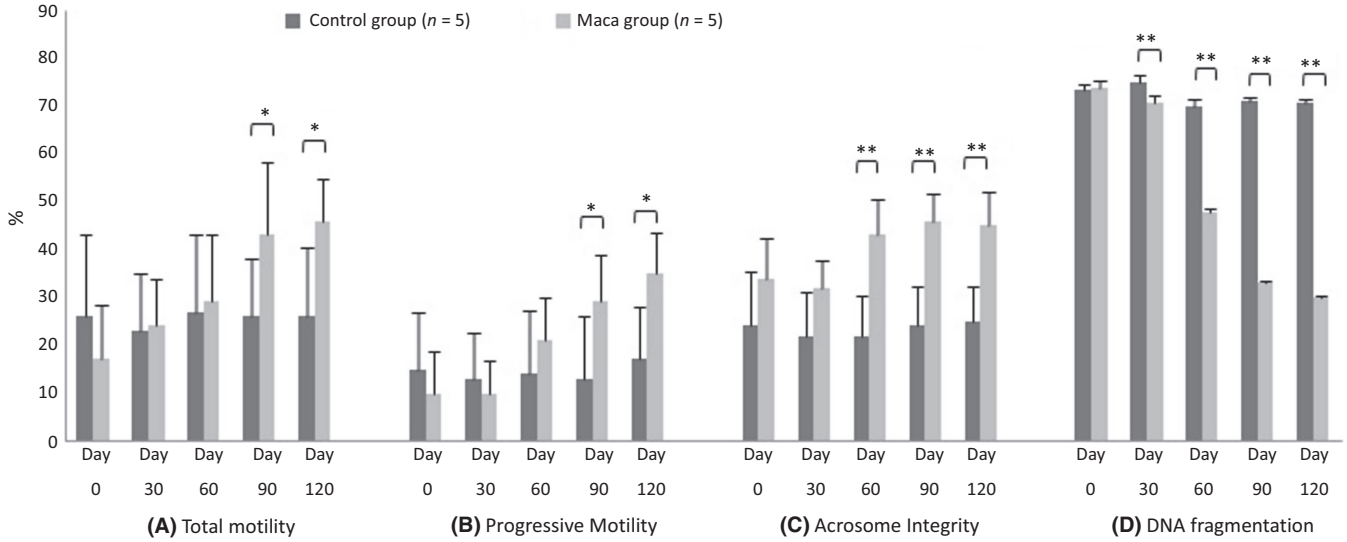
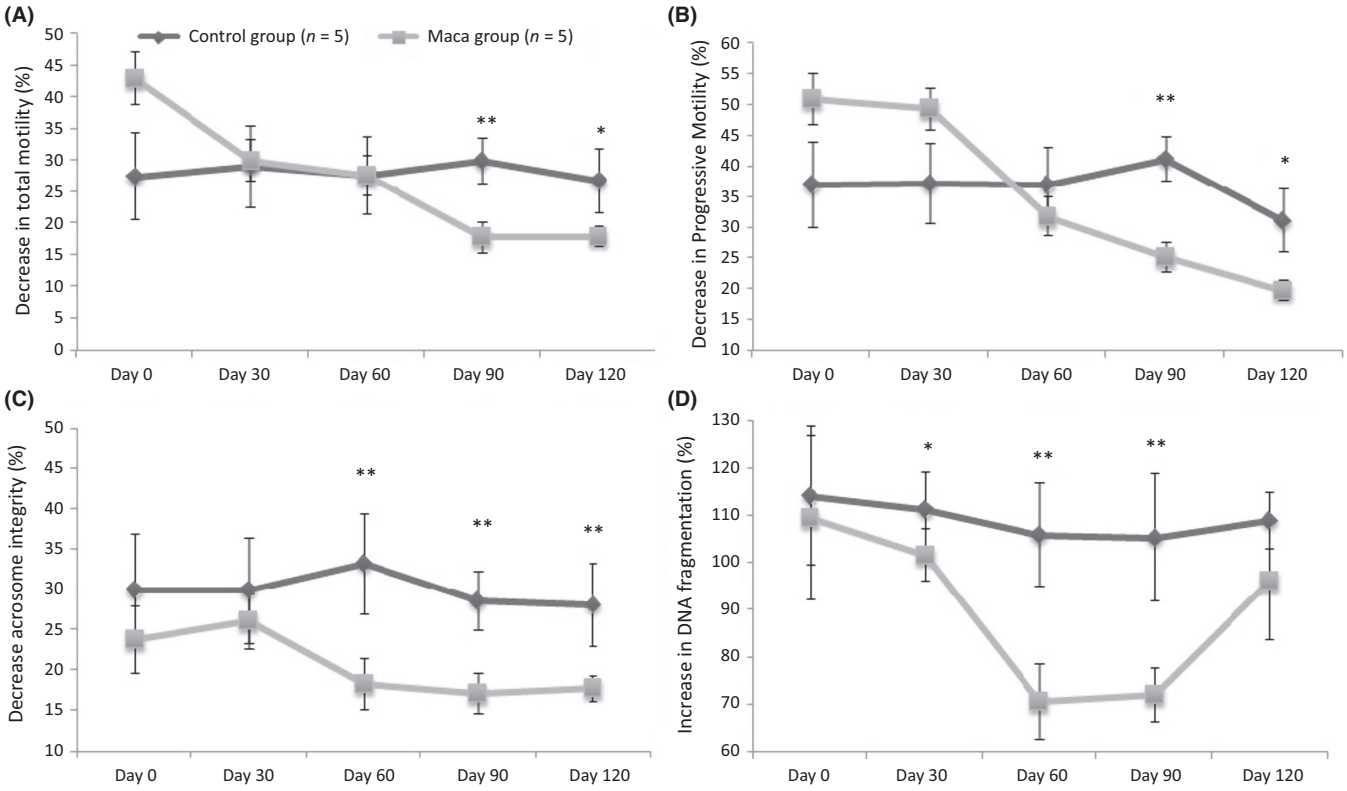


Figure 5 The daily percentage difference in semen quality parameters for both groups (C and M; $n = 5$) during cooled storage time for each collection day: total (A) and progressive (B) motility, acrosome integrity (C), and DNA fragmentation (D).(*$p < 0.05$; **$p < 0.01$).



The increase in daily percentage difference at each sperm collection day from T0 to T72 between the two groups was significantly lower in the M than in the C group at D30 ($p < 0.05$), D60, and D90 ($p < 0.01$). As highlighted by Friedman's ANOVA test results, in the control group, the percentage increase in DNA fragmentation over the entire time of dietary supplementation did not differ significantly. By contrast, in the Maca group, the percentage increase in DNA fragmentation was significantly lower at the end than at the beginning of dietary supplementation (from D0 to D120; $p < 0.01$).

Lipid peroxidation

As shown in Table 1, lipid peroxidation of spermatozoa did not show a consistent trend in either group during the supplementation (from D0 to D120) or during the cooling time (from T0 to T72). Comparison of these data between the groups (C, M)

Table 1 Lipid peroxidation values measured within three hours (T0) after collection and after 72 h (T72) of cooled storage in the control group ($n = 5$) and the Maca-treated group ($n = 5$) throughout the experimental period. Differences between groups and differences within each group were not detected between storage times or collection days

Lipid peroxidation (Mm LPO/10^6)	Fresh semen (T0)					72 h of refrigeration (T72)				
Day after feed supplementation	Day 0	Day 30	Day 60	Day 90	Day 120	Day 0	Day 30	Day 60	Day 90	Day 120
Control group ($n = 5$)	382 ± 99.3	247 ± 74.9	427 ± 100.9	374 ± 79.5	254 ± 85.6	382 ± 89.6	308 ± 98.7	448 ± 110.9	413 ± 110.7	444 ± 118.5
Maca group ($n = 5$)	368 ± 78.2	311 ± 110.1	403 ± 79.6	374 ± 84.8	247 ± 98.8	277 ± 99.5	442 ± 98.6	467 ± 83.6	293 ± 75.5	501 ± 112.1

did not reveal any statistically significant results for either collection day or storage time.

DISCUSSION

This study is the first to demonstrate that diet supplementation with Yellow Maca powder increases semen quantity and quality of stallions. Beneficial effects on raw semen and subsequently chilled diluted semen began as early as one spermatogenic cycle after supplementation.

Sperm quantitative parameters and Maca androgenic activities

During the experimental period, ejaculate volume was constant. Simultaneously, the concentration of spermatozoa and total sperm count in the raw semen increased, starting from the end of treatment with Maca (D60) and continuing to the end of the experiment (D120). Total sperm output was approximately twofold higher at the end than at the start of the experiment. A similar increase in the total sperm count was reported in adult rats (Gonzales *et al.*, 2004, 2006), peripubertal bulls (Clément *et al.*, 2010), and men (Gonzales *et al.*, 2001). However, in contrast to studies in animals, studies involving men also noted increased ejaculate volumes (Gonzales *et al.*, 2001; Melnikovova *et al.*, 2015), which may be explained, in part, by an increase in sexual desire observed for men (Gonzales *et al.*, 2002) that could not be observed in bulls (Clément *et al.*, 2010). Previous studies report that Yellow and Black Maca increase sperm count and sperm motility (Gonzales *et al.*, 2013a,b) and spermatogenesis (Clément *et al.*, 2010, 2012) without influencing hormone levels (Balick & Lee, 2002; Melnikovova *et al.*, 2015). However, Ohta *et al.* (2016) recently found that serum testosterone concentration of male rats increases probably related to the enhanced ability of testosterone production by Leydig cells, after treatment with a hydroalcoholic extract of different ecotypes of Maca. The mechanism of action of Yellow Maca, and those of the other ecotypes, is not clear yet, but some activities of Maca are related to the lipidic fraction that contains fatty acids and macamides (Melnikovova *et al.*, 2012).

To clarify whether Maca increased sperm production by increasing testosterone levels, serum concentrations of testosterone were measured in the treated stallions. Consistent with previous results in humans and in mice (Gonzales *et al.*, 2002, 2003b; Zenico *et al.*, 2009; Leme *et al.*, 2012), dietary supplementation with Maca did not alter blood testosterone concentrations in the stallions. However, in contrast to these results, increased levels of testosterone are reported from in vivo studies of rats after dietary supplementation with a hydroalcoholic extract powder of Maca (Ohta *et al.*, 2016; Yoshida *et al.*, 2017).

Maca hypocotyls are found in different colors or phenotypes in nature. Several studies show differences in the biological response to the three primary ecotypes of *Lepidium meyenii* (Yellow, Red, and Black Maca). Previous reports suggest that different phenotypes are associated with different chemical compositions of the hypocotyls. Simultaneously, different ecotypes are associated with different biological effects and medical targets. For example, the Red Maca variety reverses benign prostatic hyperplasia in mice and experimentally induced osteoporosis (Gonzales *et al.*, 2014) and shows usefulness in stimulating sperm count (Gonzales *et al.*, 2006). Black and Yellow Maca show the best results on spermatogenesis, memory, and fatigue, with increases in memory and learning in mice (Rubio *et al.*, 2007). Additionally, Black Maca reduces glucose levels, and consumption of this variety is associated with lower blood pressure and an improved health score (Gonzales *et al.*, 2013b). In the present study, the powder of the Yellow Maca ecotype was used.

Studies investigating the in vitro and in vivo effects of Maca on different cultured cells, for example, macrophages, hepatocytes, and neurons, confirm cytoprotective effects (Sandoval *et al.*, 2002; Valentova *et al.*, 2006; Pino-Figueroa *et al.*, 2010, 2011). Sandoval *et al.* (2002) investigated the antioxidant activity of Maca and showed that Maca scavenges free radicals and provides cytoprotection during oxidative stress (Sandoval *et al.*, 2002). Recent investigations showed that methanol extracts of Maca have an antioxidative effect on neurons, thereby increasing cell viability (Rodríguez-Huamán *et al.*, 2017). To explain this protective role of Maca, a mechanism is proposed by which the oxidation–reduction balance of the enzyme mechanism increases by increasing the activity of the enzyme superoxide dismutase (Rodríguez-Huamán *et al.*, 2017). In a previous study, Večeřa *et al.* (2007) demonstrated a positive effect of Maca on systemic antioxidant status, with improved activity of enzymatic ROS scavengers (superoxide dismutase, glutathione peroxidase, and glutathione). An improvement in systemic antioxidant capacity after Maca supplementation might explain the beneficial effect on the quality of fresh semen. Further evaluation of the systemic antioxidant capacity of stallions after dietary supplementation with Maca may confirm this effect. The antioxidant status of seminal plasma after Maca supplementation should also be investigated, because improved antioxidant capacity of the seminal plasma could explain the improved preservation of semen quality during cooled semen storage.

Effect of Maca on stored semen: possible mechanisms and perspectives for frozen semen

The obvious benefit of Maca is that supplementation directly increased the amount of total motile spermatozoa for production of insemination doses. Additionally, the costs for production and importation of Maca may be offset by more efficient use of stallions.

Moreover, in semen from stallions fed for 60 days with Maca, the reduction in total and progressive motility and in spermatozoa with acrosome integrity and DNA integrity was lower than that in the control group during cooled storage at 5 °C.

Similar effects on preservation of quality of stallion semen are noted in studies with diet supplementation of polyunsaturated fatty acids (PUFA) from fish oil (Brinsko *et al.*, 2005). Indeed, macamides and macaenes in Maca powder represent a diverse group of saturated and polyunsaturated fatty acids (Wang *et al.*, 2007; Wu *et al.*, 2013). Spermatozoa require high PUFA content to provide the plasma membrane with the fluidity essential for activation of signal transduction pathways necessary to the fertilization processes (Wathes *et al.*, 2007). Oxidative stress-induced changes in the PUFA composition of spermatozoa, particularly in the n-6 to n-3 ratio, cause changes in the membrane architecture that alter membrane permeability and the fluidity of its functions (Cross, 2003; Maldjian *et al.*, 2005). Diet supplementation with PUFA added with Vitamin C or E, as antioxidants in the food, increases sperm quality of rabbits (Castellini *et al.*, 2004), chickens (Surai, 2000), cockerels (Cerolini *et al.*, 2005), boars (Liu *et al.*, 2015), Japanese quail (Al-Daraji *et al.*, 2010), rams (Alizadeh *et al.*, 2014; Jafaroghli *et al.*, 2014), goats (Dolatpanah *et al.*, 2008), and bulls (Kaka *et al.*, 2015). Without antioxidants added to preserve food from oxidation, PUFA administration produces contrasting results (De Graaf *et al.*, 2007; Grady *et al.*, 2009; Fair *et al.*, 2014), most likely due to peroxidation of food lipid content (Cerolini *et al.*, 2006).

In the present study, the refrigeration and storage of the semen in both control and Maca groups caused a progressive and significant reduction in semen quality. Refrigeration is associated with damage to sperm functions due to various factors, including excessive ROS production (Wang *et al.*, 1997; Ball, 2008; Thomson *et al.*, 2009) and/or alteration of antioxidant defense systems in semen. An imbalance between oxidant and antioxidant systems with a prevalence of ROS can induce changes in membrane structure and consequently changes in membrane fluidity (Martínez-Soto *et al.*, 2013; Tafuri *et al.*, 2015).

The fluidity of the sperm plasma membrane is influenced by the lipidomic composition. Consumption of PUFAs modifies membrane composition of sperm cells (Moallem *et al.*, 2015). According to Martínez-Soto *et al.* (2013), the level of saturated fatty acids in stallion spermatozoa is correlated with the incidence of membrane-damaged spermatozoa. By contrast, the percentage of highly unsaturated fatty acids is positively correlated with intact membranes post-thaw after freezing and thawing. In our study, dietary supplementation of Maca alleviated the decrease in sperm quality caused by refrigeration of stallion spermatozoa, particularly based on the spermatozoa with intact acrosomes. Maca, containing macamides and macaenes, is composed of PUFAs; thus, Maca may improve the resistance of spermatozoa to cold shock (Giraud *et al.*, 2000; Clément *et al.*, 2012).

One initial hypothesis was that Maca supplementation might increase the intrinsic resistance of the ejaculated spermatozoa to lipid peroxidation; however, this hypothesis was rejected, because no significant difference in lipid peroxidation was detected between experimental groups at any time during the experiment. The absence of an increase in lipid peroxidation during storage of equine semen is consistent with previous findings shows that equine spermatozoa appear relatively more resistant to membrane peroxidation than spermatozoa of other domestic animals during storage at 5 °C (Baumber *et al.*, 2000; Kankofer *et al.*, 2005; Neild *et al.*, 2005). According to these authors, lipid peroxidation did not increase during cold storage of equine semen despite a decrease in sperm motility and acrosome integrity.

In this study, we could not exclude insufficient sensitivity of the method used for detecting lipid peroxidation. Alternatively, lipid peroxidation and consequently, oxidative stress levels in equine semen can be investigated based on lipid peroxidation end products such as malondialdehyde (Stradaioli *et al.*, 2001; Ball & Vo, 2002), which can be determined measuring the amount of thiobarbituric acid reactive species (TBARS). The labeling of spermatozoa with the fluorescent probe C11-BODIPY is another option for detecting lipid peroxidation, and such lipid peroxidation has been related to apoptotic-like changes in stallion spermatozoa (Ball & Vo, 2002; Neild *et al.*, 2005; Ortega-Ferrusola *et al.*, 2009).

The results of our study further suggest that molecules other than lipids were primary targets of ROS in chilled and stored stallion semen. Our results on DNA fragmentation support the assumptions that oxidative stress degrades DNA, in addition to damaging cell membranes. ROS are the primary cause of DNA damage in spermatozoa (Aitken & De Iuliis, 2007). Wnuk *et al.* (2010) found a negative correlation between total antioxidant capacity of stallions and sperm DNA damage, postulating that the redox status of seminal plasma may be an additional important parameter for evaluation of equine semen quality. Furthermore, after ROS exposure, a dose-dependent increase in sperm DNA damage was observed in equine spermatozoa (Baumber *et al.*, 2003). In our study, the damage to DNA integrity was significantly reduced at 72 h of storage after Maca dietary supplementation. The only reasonable hypothesis is that Maca influences the enzymatic antioxidant levels in seminal plasma and systemic antioxidant status, protecting spermatozoa against oxidative stress. To clarify this potential effect of Maca on systemic antioxidant capacity, further analysis is required.

Mitochondria or proteins involved in the acrosome reactions could be other potential targets for ROS in spermatozoa. The detection of activated apoptosis markers such as caspase 3, 7, and 9 by flow cytometer analysis or Western blotting may help to provide more information on potential ROS actions and protective mechanisms of Maca at the level of mitochondria (Brum *et al.*, 2008; Ortega-Ferrusola *et al.*, 2008). The preservation of acrosome integrity maybe associated with the ability of Maca to alleviate the effect of ROS on phosphorylation of proteins involved in the acrosome reaction mechanism (Cocchia *et al.*, 2011). Irrespective of the underlying mechanism, our findings support the possibility for using Maca as a food supplement for stallions to produce semen in greater quantity and of better quality. Future research will determine whether similar positive effects on semen quality are obtained with cryopreserved spermatozoa.

CONCLUSIONS

Although the number of animals was limited in the present study, we showed for the first time that supplementation with Maca in the diet of stallions improves semen production, resulting in more artificial insemination (AI) doses per ejaculate. Furthermore, the semen from Maca-treated stallions was more resistant to cooling and storage, preserving acrosome and DNA integrity and total and progressive sperm motility. The underlying mechanisms that promote the effect of *Lepidium meyenii* (Maca) on stallion semen remain unclear. Further research is required to verify and quantify oral absorption of Maca in horses, to elucidate the mechanisms of *Lepidium meyenii* effects on semen production and to explore the potential effect of Maca on the systemic antioxidant capacity in horses.

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None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the article.

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