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ORALLY ADDED BLACK MACA (*LEPIDIUM MEYENII* WALP.) NANO-EMULSION AMELIORATES FRESH SEMEN QUALITY, TESTOSTERONE LEVEL AND FERTILISING ABILITY OF CHILLED AND CRYOPRESERVED RABBIT BUCK SPERMATOZOA

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Abstract: Maca is an economic crop grown in the highlands of South America that serves as a food source and medication for humans and animals. The goal of this study was to determine the potential beneficial effects of administering the nano-emulsion of black maca (*Lepidium meyenii*) orally to improve fresh, chilled and frozen rabbit semen, as well as its promising effects on fertilising ability and testosterone levels. The study included forty New Zealand mature white rabbit bucks (8 to 10 mo old) and eighty non-lactating, sexually receptive New Zealand white rabbit does. Rabbit bucks were divided into a control group and treatment groups. The control group was given 0.0 mg maca (M0) nano-emulsion. Treatment groups M60, M120 and M180 were orally given 60 mg, 120 mg and 180 mg, respectively, of maca nano-emulsion. Maca was administered orally twice a week for 10 wk. Rabbit does were divided into four equal groups and prepared for insemination with chilled and frozen semen from the best concentration of the treatment (M60=980±69.65×10^6/mL spermatozoa) and the control. Semen was collected and evaluated once weekly for 10 wk, and testosterone hormone was also analysed once at fortnightly intervals for the same period. Results revealed that maca significantly increased (P<0.05) ejaculate volume (0.41 *vs*. 0.56 mL for M0 *vs*. M60), sperm progressive motility (61.00 *vs*. 75.19% for M0 *vs*. M60), sperm count, the total number of sperm per ejaculate and testosterone levels (0.45 *vs*. 1.5 ng/mL for M0 *vs*. M60). It also improved sperm morphology compared to the control. M60 was the most effective concentration, so semen was collected from M60 rabbit bucks, chilled and frozen and used in the fertilising ability test. Finally, administering the nano-emulsion of black maca orally (60 mg/twice weekly/buck/10 wk) has been suggested for increasing rabbit buck semen quality, testosterone hormone levels and fertilising ability of chilled and cryopreserved rabbit buck spermatozoa. The pregnancy and kindling rates were 66.66 *vs*. 100% for M0 *vs*. M60 of chilled semen, as well as 33.33 *vs*. 83.33% for frozen/thawed sperm.

Key Words: maca, nano-emulsion, rabbit bucks, semen analysis, testosterone hormone, fertilising ability.

INTRODUCTION

Rabbits are an important animal species for satisfying the nutritional requirements of the world's growing population due to the high conversion rate of feed. Fortunately, Egypt has become one of the top ten producers of rabbit meat, contributing 56 metric tonnes or 3.8% of the world's total production (FAOSTAT, 2022).

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Due to the specificity of rabbit sperm membrane, fast cooling rates from room temperature to 5°C are likely possible in this species (Mocé *et al.*, 2003; Mocé and Vicente, 2009). Nevertheless, differences between rabbit breeds in the resistance of sperm to the cryopreservation process have been reported (Mocé *et al.*, 2003). When artificial insemination (AI) is used in rabbit farms, semen quality can affect the fertility and prolificacy of rabbit does (Alvariño, 2000). In market rabbit production, fresh semen is appropriate for routine AI if kept for a short period (Morrell, 1995; Castellini, 1996) To extend storage time, frozen semen is required, but the freezing process is associated with a reduction in motility, viability and fertilising ability or prolificacy after AI (Kashiwazaki *et al.*, 2006). Enormous efforts have been made to reduce these detrimental effects and increase the efficiency and reliability of sperm cryopreservation in the rabbit. Several studies have been conducted to address the components of sperm freezing extenders, although the results were not always satisfying. Some additional supplements can improve the efficiency in sperm cryopreservation (Beharry and Heinrich, 2018). Numerous studies on improving rabbit sperm cryopreservation have been undertaken from a variety of perspectives, including freezing procedure, type, concentration and combination of cryoprotectants. Despite this, a standard protocol for rabbit sperm cryopreservation has not been well established due to the various and irreproducible results from each study. It is known that freezability of rabbit sperm varies among individuals (Mocé, *et al.*, 2005) or breeds (Kulíková *et al.*, 2017).

Maca is a Peruvian herb belonging to the Brassicaceae family and grows only between 4000 and 4500 m altitude in the highlands of Peru (Zheng *et al.*, 2000; Cicero *et al.*, 2001). Maca is recognised as a "Peruvian national treasure", known as "Peruvian ginseng" for its nutraceutical and medicinal properties (Chen *et al.*, 2021). In rats, black maca had greater benefits in sperm counts and epididymal sperm motility than red or yellow maca (Gonzales *et al.*, 2006). Maca also improved the spermatogenesis, fertility and sexual behaviour of rats (Wang *et al.*, 2007). Rabbit diets containing more than 15% crude protein are indicated to guarantee optimal sperm production (Nizza *et al.*, 2000). A large proportion of spermatozoa lipids are polyunsaturated fatty acids (PUFA) of the n-3/n-6 series, which change membrane fluidity (Meizel and Turner, 1983). Therefore, diets must contain sufficient amounts of these fatty acids. Maca is thus considered a hidden treasure among all herbals. The dried hypocotyls of maca contain roughly 13-16% protein, 59% carbohydrates, 2.2% fats and 8.5% fibre (Pawlosky *et al.*, 2003). Maca also contains free fatty acids, the most prevalent of which are linoleic, palmitic and oleic. Unsaturated fatty acids account for 52.7%, while saturated fatty acids comprise 40.1% (Dini *et al.*, 1994; Gonzales, 2012). In addition, maca contains many secondary metabolites (Valerio and Gonzales, 2005). The secondary metabolites are macaridine, macaene, glucosinolates, isothiocyanates, polysaccharides, polyphenols, macamides and maca alkaloids, which are unique to this plant (Gan *et al.*, 2017) and have not yet been discovered in other plant species (Zheng *et al.*, 2000). This lipid portion of maca (macamides) is thought to have an important role in improving sexual behaviour (Cicero *et al.*, 2002). In pre-pubertal breeding bulls fed a mixture of all three (black, yellow and red) dried maca phenotypes at 233 mg/kg body weight, an increase in sperm motility was noted with an increase in the ejaculation volume. The effect was visible in animals with an initially poorer sperm quality and only in those that had been fed for a longer period of time of 10 weeks (Clément *et al.*, 2010). However, no increase in ejaculation volume or sperm concentration was seen after an 8-wk long supplementation with milled maca dried hypocotyls in rams at the same dose (Lavana *et al.*, 2013).

Nanoparticles have physicochemical characteristics superior to those of mass materials owing to their large surface-to-volume ratio, higher reactivity, stability, bioactivity, bioavailability, controlled particle size, controlled release of drugs, site-specific targeting and controlled arrival of medications (Zadeh and Kor, 2013). Nanotechnology plays an important role in medication delivery because of the capacity of nanoparticles to enter cells, tissues and organs compared to macro particles, which have poor bio-accessibility and high toxicity (Underwood and Van Eps, 2012). However, nanoparticles or nano-emulsions are ten times more potent than the plant itself (Onoue *et al.*, 2010; Lin *et al.*, 2011; Yu and Huang, 2012).

The limited survival of sperm after cryopreservation is a significant disadvantage of using frozen semen in artificial insemination programmes for livestock animals, including rabbits. In rabbits, sperm cryopreservation has been applied for experimental purposes (Mocé and Vicente, 2009). Chilling, freezing and thawing rabbit spermatozoa significantly impacted semen quality due to the changes in osmotic balance and temperatures caused by cooling, freezing and rewarming, leading to ice crystal formation and, in turn, decreased sperm mortality (Holt, 2000).

Therefore, the present study aimed to determine the potential beneficial effects of administering the nano-emulsion of black maca (*Lepidium meyenii*) to mature New Zealand (NZW) rabbit bucks orally to improve fresh, chilled and frozen rabbit semen and its promising effects on fertility and testosterone hormone levels.

MATERIAL AND METHODS

Ethical approval

The Animal Care and Ethical Use Committee of the Faculty of Veterinary Medicine at Cairo University reviewed and approved the experimental protocol (Vet CU 25122023859).

Preparation of maca nano-emulsion

The material used in this study is maca dried powder was purchased from Sun Food Company for pharmaceutical raw materials (Saudi Arabia).

Maca nano-emulsion preparation method

Maca product (50.0 mg/mL) was prepared by mixing 25 mL Tween-20 and 25 mL Tween-80 with a Probe homogeniser (LK Lab, Korea) at 2500 rpm for 20 min, then slowly adding 50 mL of deionised water to the mixed oil phase. Maca nano-emulsion was made according to the method described by El-Oksh *et al.*, (2022).

Characterisation method

Transmission electron microscopy (TEM): Characterisation of maca nano-emulsion determined the physical and chemical properties, to evaluate its biological activity. A transmission electron microscope (TEM) was used to determine the shape and surface topography of the maca nano-emulsion (Jeol model, JEM-2100 high-resolution, Japan).

Dynamic light scattering (DLS).

Instrument: Malvern Zetasizer Nano ZS.

Sample preparation

Disperse the nanoparticles in a suitable solvent (e.g., deionised water or ethanol) at an appropriate concentration. Filter the nanoparticle suspension through a 0.45 µm or 0.22 µm filter to remove any large aggregates or impurities.

Measurement conditions

Under 25°C, 173°C scattering angle of backscatter, refractive index specifies the refractive index of the dispersant and nanoparticle material, 120 s or as-needed equilibration time, 3-5 runs per measurement and an appropriate analysis model.

Semen characteristics

Animals, housing and feeding

Forty mature healthy NZW white rabbit bucks were used in the present study. Their body weight was 2700 to 3000 g and they were 8 to 10 mo old.

Throughout the study, the rabbits were individually housed in flat-deck cages and provided with a 16 h light/8 h dark photoperiod. All bucks were fed a commercial pelleted basic diet (17.5% crude protein, 2.3% ether extract, 16.8% crude fibre, 2600 kcal digestible energy/kg) as recommended by the National Research Council (Council, 1994), and had free access to water. This work was done from December 2023 to May 2024.

Experimental design

Forty mature NZW white rabbit bucks were allocated arbitrarily into four equal groups. The control group received a basic diet only (M0). The treatment groups, M60, M120 and M180, were given orally, twice weekly for 10 wk, 60 mg, 120 mg and 180 mg, respectively, of maca nano-emulsion, equivalent to 10% of that previously used (Ragab *et al.*, 2022) in rabbits.

Semen collection

Several weeks before the experiment began, all rabbit bucks were trained daily to mount an anoestrus doe as a teaser. Once a week, ejaculate was collected from each buck early in the morning for ten weeks using a lubricated and prewarmed (42-45°C) artificial vagina (All Vet. Supply Inc., France).

Evaluation of the fresh semen

Immediately after semen collection, the ejaculates were transferred to the laboratory and placed in a warm water bath at 37°C. Semen samples were evaluated for ejaculate volume after removing the gel mass using a graduated collection tube. Mass motility was estimated subjectively (0-5 score; Moule, 1965) using a light microscope with low power. Progressive sperm motility was subjectively assessed by visual estimation by dropping 10 µL semen onto a clean glass slide prewarmed at 37°C, diluted with warm sodium citrate 2.9%, and covered with a cover slip immediately after collection (Rosato and Iaffaldano, 2011). Progressive sperm motility was determined in three microscopic fields per semen sample using a light microscope with high power. The percentage of progressive sperm motility was subjectively assessed from 0 to 100% (El-Desoky *et al.*, 2017). The live and abnormal sperm percentages were determined by staining duplicate smears of freshly collected ejaculate with Eosin-Nigrosine stain to determine live (unstained) and morphologically abnormal sperm percentage (Campbell *et al.*, 1956). A total of 200 sperm cells were examined randomly under an oil immersion lens (×1000 magnification). The functional integrity of the plasma membrane of the sperm was assessed using the hypo-osmotic swelling (HOST) test (El-Desoky *et al.*, 2017). Briefly, in a 1:10 ratio, 30 µL of semen was mixed with 300 µL of a hypoosmotic solution (0.90 g fructose plus 0.49 g sodium citrate per 100 mL of distilled water; 100 mOsm/kg) and incubated for one hour at 37°C. The sperm percentage that responded to HOST with swollen and curled tails (functionally integrated plasma membrane of spermatozoa) was recorded in 200 sperm cells under a light microscope (×40 magnification). The sperm cell concentration was determined using a direct cell count on an advanced Neubauer haemocytometer (GmbH Co.), and the total number of sperm per ejaculate was calculated by multiplying the ejaculate volume by the concentration.

Semen processing

Semen was collected from the M0 and M60 groups, processed and inseminated based on its characteristics. The gel plug was removed immediately following semen collection, and the ejaculates were kept in a 37°C incubator.

To eliminate animal variations, ejaculates from each group were pooled. Pooled semen samples with a final volume for each group were tested for basic semen characteristics such as motility, live sperm percentage and sperm cell count. Pooled semen samples with >70% motility, >75% alive sperm, and >250×10^6 sperm cells/mL were processed for chilling and freezing (Mocé *et al.*, 2003).

Chilled and frozen-thawed semen

Pooled ejaculates were diluted using Tris-citrate glucose (TCG; composed of tris 3.028 g/100 mL, citric acid 1.69 g/100 mL, and glucose 0.931 g/100 mL) extender (Roca *et al.*, 2000; Mocé and Vicente, 2009). Each extender contained 20% egg yolk, 25 mg of gentamicin and 50 000 IU of penicillin per 100 mL. The dilution rate was calculated assuming that each insemination dose (1 mL chilled semen) contained at least 35×10^6 motile sperm cells. The diluted semen samples were gradually cooled to +5°C over 90 min. For cryopreservation, the same diluent used in chilling was prepared and supplemented with 8% dimethyl sulfoxide (Iaffaldano *et al.*, 2012), 2% sucrose as the non-permeable cryoprotectant (Cortell and Viudes-De-Castro, 2008; Di Iorio, 2015). After 90 min of chilling at +5°C, the equilibration time was increased to two hours to improve sperm viability and motility after freezing. Plastic straws (0.5 mL) were filled with diluted semen samples (at least 75×10^6 motile sperm cells), sealed with a sealing powder

and placed at a level of 5 cm above the liquid nitrogen vapour in a foam box for 10 min at –125/–130°C before being plunged into the liquid nitrogen tank (Cristanelli *et al.*, 1985). Thawing of frozen straws was performed in a water bath at 50°C for 10 s (Mocé *et al.*, 2003; Di Iorio, 2015).

Fertilising ability

Choosing the most effective treatment, AI was carried out with control semen (M0) and the best-treated buck group (M60). Eighty female rabbits (aged 9-10 mo and weighing between 2800 and 3200 g) were divided into four equal groups of twenty each (females with one parity, non-lactating)

The does were inseminated with M0 chilled semen, M60 chilled semen, M0 frozen/thawed semen and M60 frozen/thawed semen. To synchronise their oestrus, all females underwent the following bio-stimulation protocol: flushing by increasing feed amount per doe from 180 g/d to *ad libitum* (3 d before insemination), cage change (3 d before insemination) and photoperiod increase from 16 to 24 h (2 d before insemination) of light (Theau-Clément, 2000).

Females chosen for insemination were assumed to be sexually receptive (with the red tone of vulvar lips). They received an intramuscular injection of 0.3 mL Receptal (GnRH analogue or 1.05 µg Buserelin acetate; Hoechst Marion Roussel, S.A., Madrid, Spain) before insemination. Rabbit does were inseminated with 0.5 mL of frozen-thawed semen (containing at least 75×10^6 motile sperm cells) immediately after GnRH injection (Iaffaldano *et al.*, 2012*;* Di Iorio *et al.*, 2018). The frozen semen was used in insemination after 2 wk from the freezing process. Following 24 h of storage at +5°C, rabbit does were inseminated with 1 mL of chilled semen (containing at least 35×10^6 motile sperm cells). Pregnancy was detected by ultrasonography (Idris *et al.*, 2016) nine days after insemination and foetuses were detected by trans-abdominal palpation 12-15 d post-insemination to determine the fertility rate (Mocé *et al.*, 2003). Litter size was determined for each doe directly after kindling. Pregnancy rate (number of pregnant does/number of inseminations), kindling rate (number of does giving birth/number of inseminations) and the number of kits born alive (total liveborn/kindling) were determined to assess the reproductive performance (Di Iorio *et al.*, 2018) of the rabbit does.

Blood sampling

Blood samples were collected from the Vena auricularis into vacutainer tubes (Jeffrey and Jenkins, 2008). Blood was collected at fortnightly intervals early in the morning from each buck, until the end of the experiment, and was centrifuged at 600 ×*g* for 20 min. The serum was collected and kept at –20°C until testosterone assay. Testosterone was quantified by enzyme-linked immunosorbent assay (ELISA) using commercial ELISA kits (Diasino, China). The sensitivity of the assay was 0.05 ng/mL, and intra- and inter-assay precessions were 6.25 and 7.14%, respectively.

Statistical analyses

The results were presented as mean±standard error of mean. The data were analysed using statistical software SPSS version 20.0. A simple one-way analysis of variance (ANOVA) was used to examine the effect of week within treatments and the effect of treatment within weeks. Duncan's Multiple Range test was used to distinguish between significant means at $P<0.05$. Univariate two-way ANOVA model (4 treatments ×10 wk). A student t-test was used to compare the fertility parameters of the control and the selected maca treatment. The significance level was set at $P<0.05$.

RESULTS

Transmission electron microscope (TEM)

TEM images of the created maca nano-emulsion (NPs) revealed that the resulting particles were spherical to subspherical structures in the form of clusters with a maximum thickness of about 30-50 nm (Figure 1C). Furthermore, it was demonstrated that maca nano-emulsion did not aggregate in specific areas.

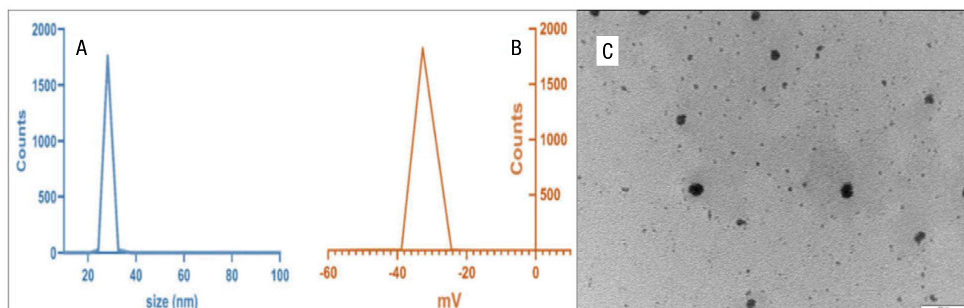

Figure 1: The Dynamic light scattering Zeta size (A), Zeta potential (B) patterns and Transmission electron microscope of maca nano-emulsion (C).

Zeta size and Zeta potential

The Zetasizer results showed that the average particle size of synthesised maca NPs was 37.5 nm, with a narrow size distribution [polydispersity index (PDI) is 0.04], as shown in Figure 1A. The surface charge of synthesised nanoparticles is -31.21 mVas (Figure 1B). The higher colloidal stability in water indicated the high bioactivity of the prepared maca NPs.

Fresh semen characteristics

During the experiment, treated maca groups had significantly higher mean values ($P<0.05$) for ejaculate volume, mass and progressive sperm motilities, sperm cell concentration, total number of sperm per ejaculate, normal sperm morphology and plasma membrane integrity percentages compared to the control group (Table 1). The M60 treatment group had peak values for mass and progressive sperm motilities, sperm cell concentration, total number of sperm per ejaculate, live sperm percentage and plasma membrane integrity percentage (Table 1).

Table 1: Fresh semen characteristics of mature NZW rabbit bucks treated orally with M0, M60, M120 and M180 mg of nano-emulsion twice a week for ten weeks (mean±standard error of mean).

	Maca-treated groups (mg)				
Semen parameters	M0	M60	M120	M180	*P*-value
Volume (mL)	0.41 ±0.02 ^{A}	0.56 ±0.02 ^{B}	0.70 ±0.02 ^{C}	0.71 ±0.04 ^{C}	0.0001
Mass motility (Score 0-5)	1.17 ±0.06 ^{A}	3.25 ±0.19 ^{C}	2.02 ±0.15 ^{B}	1.88 ±0.10 ^{B}	0.0001
Progressive motility (%)	61.00 ±0.93 ^{A}	75.19 ±1.70 ^{B}	72.79 ±1.33 ^{B}	72.81 ±1.64 ^{B}	0.0001
Sperm cell concentration ($\times10^6$/mL)	275.00 ±15.69 ^{A}	980.08 ±69.65 ^{C}	665.00 ±40.13 ^{B}	564.58 ±42.51 ^{B}	0.0001
Total number of sperm /ejaculate ($\times10^6$)	121.10 ±10.12 ^{A}	586.35 ±60.05 ^{C}	448.48 ±26.12 ^{B}	429.89 ±47.92 ^{B}	0.0001
Live sperm (%)	83.11 ±1.15 ^{A}	87.02 ±1.13 ^{B}	85.47 ±1.48 ^{AB}	85.65 ±0.91 ^{AB}	0.182
Abnormal sperm (%)	35.57 ±1.58 ^{C}	20.63 ±1.29 ^{A}	18.64 ±1.17 ^{A}	28.75 ±1.49 ^{B}	0.0001
(HOST) Plasma membrane integrity (%)	78.66 ±0.80 ^{AB}	82.58 ±1.34 ^{B}	80.21 ±1.26 ^{B}	74.84 ±1.61 ^{A}	0.004

Means with different superscripts within rows (A, B, C), are significantly different, at least at $P<0.05$, (n=100 ejaculates/group). HOST=hypo-osmotic swelling test.

The maca treatments (*P*<0.0001), weeks of the experiment (*P*<0.001) and treatments×weeks (*P*<0.0001) affected ejaculate volume, sperm mass motility, sperm progressive motility, sperm cell concentration, total number of sperm/ejaculates, abnormal sperm and sperm plasma membrane integrity. Treatments×weeks did not affect sperm cell concentration, but tended to influence (*P*>0.05) total sperm/ejaculate. At the end of the experiment (Week 10), M60 had the highest mass motility (*P*<0.0001), sperm progressive motility (*P*<0.0001), sperm concentration (*P*<0.0001), total sperm/ejaculate (*P*=0.006) and the lowest (*P*<0.0001) abnormal sperm percentage.

The *ejaculate volume* at W0 was nearly similar in the M0, M60, M120 and M180 maca-treated groups (Figure 2A). *Semen volume* (mL) increased significantly (*P*<0.0001) in M60, M120 and M180 compared to the control group (0.50±0.06; 0.83±0.04; 0.63±0.04 *vs.* 0.35±0.07, respectively) two weeks after treatment (Figure 2A). The highest (*P*<0.05) ejaculate volume was recorded at W10 in the treated groups compared to the control (Figure 2A).

The *mass motility* (score 0-5) of the sperm (Figure 2B) started to increase significantly (*P*=0.003) at week 4 in the rabbit bucks supplemented with M60 nanoparticles (2.67±0.33), M120 (1.67±0.33) and M180 (1.67±0.17) compared to M0 (1.00±0.00), and continued to increase to reach their maximum values at week 10 (Figure 2B).

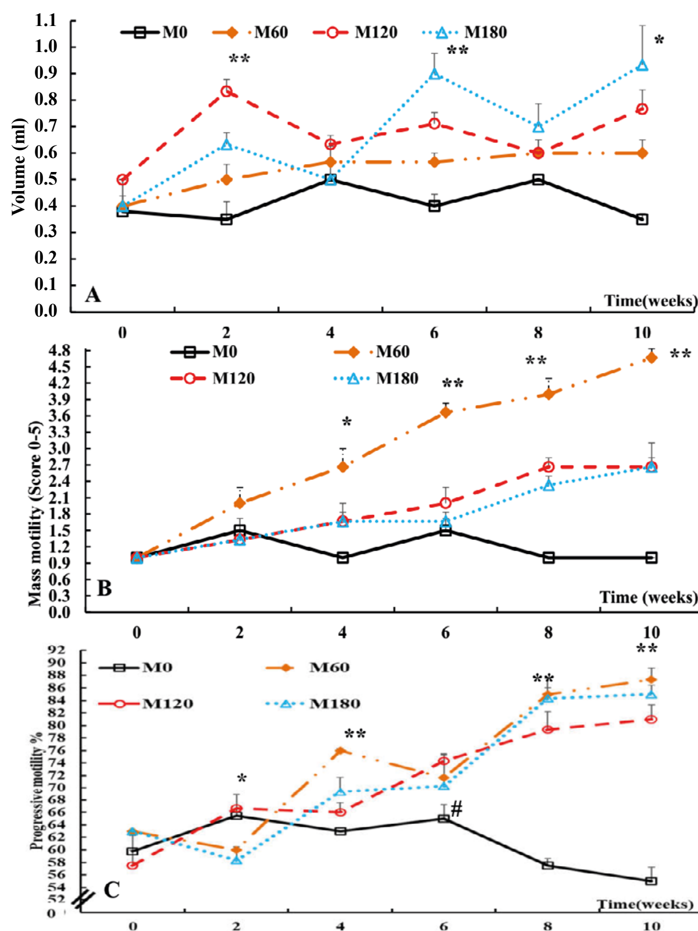

Figure 2: Mean ejaculate volume (A), semen mass motility (B) and sperm progressive motility percentage (C) of control and treated male rabbits, with error bars. **P*<0.01; ***P*<0.0001; #*P*>0.05.

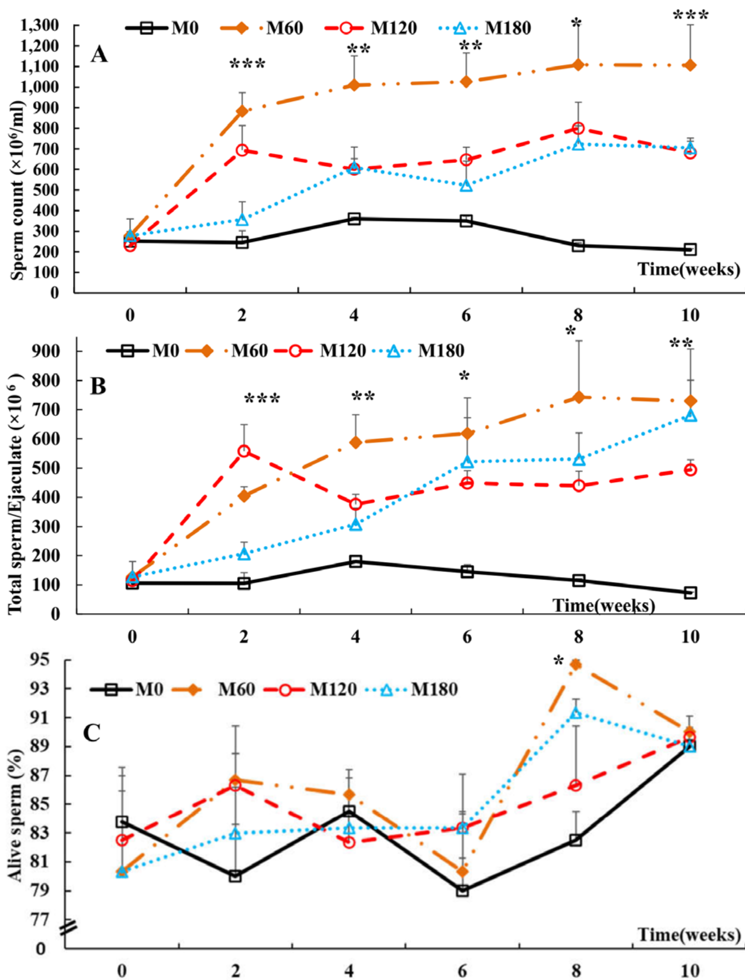

Figure 3: Mean sperm cell concentration×10^6/mL (A), total sperm number/ejaculate x10^6 (B) and live sperm % (C) in control and treated male rabbits, with error bars. *P<0.01; **P<0.001; ***P<0.0001.

The *progressive motility* percentage of the sperm (Figure 2C) increased significantly (P<0.0001) at week 8 (85.00±2.50; 84.33±1.74; 79.33±2.83) and week 10 (87.33±1.86; 85.00±1.44; 81.00±2.29) in M60, M180 and M120, respectively in the treated groups compared to M0 (57.50±1.12 and 55.00±2.24), respectively.

The *sperm cell concentration* (Figure 3A) began to increase (P<0.001) two weeks after starting the supplementation with M60, M120 and M180 compared to M0 with the following values: 882.67, 693.33 and 356.67×10^6/mL *vs.* 245.00×10^6/mL, respectively (Figure 2A). The significant (P<0.001) increase in the sperm cell concentration continued throughout the experiment until week 10, with peak values for the M60 treated group.

The *total number of sperm/ejaculate* (Figure 3B) started to increase (P<0.0001) two weeks after treatment in the rabbit bucks supplemented with M60, M120 and M180 compared to the control (Figure 3B). In terms of ejaculate volume and sperm cell concentration, the total number of sperm per ejaculate displayed a similar pattern throughout the experimental period (Figure 3B).

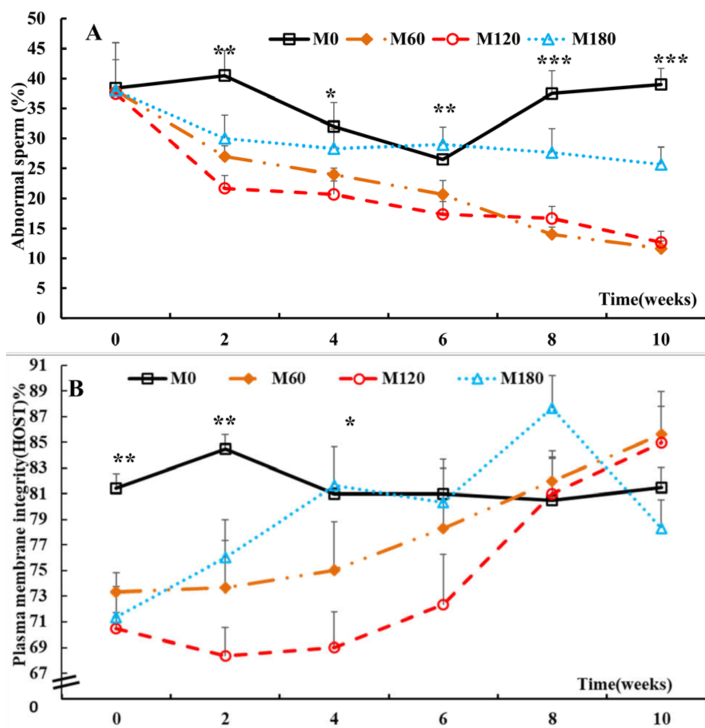

Figure 4: Mean abnormal sperm % (A) and sperm plasma membrane integrity (HOST) percentage (B) in control and treated male rabbits, with error bars. *P<0.05; **P<0.01; ***P<0.0001.

The *live sperm* percentage did not vary significantly among treated and control groups (Table 1) but reached high (P<0.01) values at week 8 in all the experimental groups. Both M60 (P<0.0001) and M180 (P=0.003) groups showed the peak values of live sperm percentage at week 8 and week 10 (Figure 3C).

The *abnormal sperm* percentage (Figure 4A) started to decrease (P=0.003) from week 2 after treatment in the rabbit bucks supplemented with M60, M120 and M180 compared to the M0. The significant improvement in the normal sperm morphology percentages continued throughout the experimental period until week 10. The lowest value (P<0.001) of abnormal sperm percentage was recorded in favour of M60 group (11.67±1.20) at W 10 compared to M180 (25.67±2.92), M120 (12.67±1.89) and M0 (39.00±2.68).

The *sperm plasma membrane integrity* (HOST) did not change significantly in the M0 group throughout the experimental period Figure 4B). In contrast, M60 and M120 treated groups showed a consistent significant (P<0.05) improvement, with a peak value (85.67%) noted for the M60 treated group in the tenth week (Figure 4B).

The changes in the blood serum *testosterone concentrations* of male rabbits treated with M0, M60, M120 and M180 mg maca nano-emulsion throughout the experiment revealed that serum testosterone concentrations increased significantly (P<0.0001) in all maca-treated groups compared to the control, reaching peak levels at weeks 8 and 10 (Table 2).

With regard to chilled and frozen-thawed semen, M60 significantly (P<0.05) increased sperm progressive motility and alive sperm percentages in chilled (+5°C) and frozen-thawed semen compared to the M0 groups (Table 3). Nonetheless, there were no significant differences in sperm cell abnormalities between the M60 and M0 groups.

Table 2: Testosterone concentrations (ng/mL) in the serum of male rabbit bucks treated with M0, M60, M120 and M180 mg nano-emulsion for 10 wk (mean±standard error of mean).

	Maca-treated groups (mg)				
Weeks	M0	M60	M120	M180	*P*-value
0	0.49 ±0.08^{cd}	0.51 ±0.13^{a}	0.48 ±0.15^{a}	0.40 ±0.15^{ab}	0.953
2	0.17 ±0.00^{Aa}	2.00 ±0.57^{Bb}	1.70 ±0.45^{Bab}	2.20 ±0.23^{Bbc}	0.111
4	0.51 ±0.05^{Acd}	1.83 ±0.37^{Bb}	0.44 ±0.05^{Aa}	0.52 ±0.08^{Aab}	0.0001
6	0.67 ±0.07^{ABd}	1.27 ±0.03^{Ba}	0.90 ±0.25^{Bab}	0.30 ±0.08^{Aa}	0.005
8	0.29 ±0.02^{Aab}	1.31 ±0.36^{ABab}	1.92 ±0.49^{Bb}	2.14 ±0.59^{Babc}	0.051
10	0.44 ±0.04^{Abc}	2.50 ±0.34^{Bb}	2.08 ±0.34^{Bb}	3.60 ±0.78^{Cc}	0.0001
P-value	0.0001	0.001	0.005	0.0001	
Overall	0.45 ±0.03^{A}	1.50 ±0.18^{B}	1.37 ±0.17^{B}	1.71 ±0.23^{B}	0.0001

Means with different superscripts within rows (A, B, C) within columns (a,b,c,d) are significantly different, at least at $P<0.05$. (n=50 serum samples/group).

Effect of maca treatment on the fertilising ability of chilled (+5°C) and frozen-thawed rabbit bucks' semen

The pregnancy and kindling rates were similar in each group, whether treated or not (Table 4). This was attributed to the fact that the number of pregnant females equalled the number of females giving birth. Furthermore, females inseminated with M60 chilled ($P<0.0001$) and frozen semen ($P=0.002$) had larger litter sizes than the control group. The mortality rate of kits was low in those inseminated with M60 chilled ($P<0.0001$) or frozen-thawed semen (Table 4).

DISCUSSION

To the best of our knowledge, this study is the first to discuss the effects of oral supplementation with black maca nano-emulsion on the semen quality of NZW rabbit bucks and its beneficial effects on testosterone levels. Furthermore, the fertilising ability of chilled (+5°C) and cryopreserved rabbit buck spermatozoa was determined using pregnancy rate, kindling rate, total born (litter size), liveborn and mortality percentage.

Maca is an Andean bulbous crop that native inhabitants have used for centuries to improve overall vitality and treat infertility in humans and domestic animals (Canales *et al.*, 2000). Even today, maca products have many traditional therapeutic applications and are well-known for their purported fertility-boosting properties in both men and women.

Throughout the experimental period, the present results showed rising trends in semen characteristics after two weeks of maca nano-emulsion administration.

The ejaculate volume increased in the treated groups compared to the control starting two weeks after oral supplementation and reached its peak values in the tenth week. A similar increase in the ejaculate volume was reported in infertile and healthy men (Gonzales *et al.*, 2001a; Melnikovova *et al.*, 2015) and dogs (Gattuso *et al.*, 2024). On the other hand, Del Prete *et al.* (2018) indicated that the ejaculate volume did not change in stallions supplemented with 20 g of yellow maca powder daily for a total of 60 d. Semen volume resulted from the contributions of seminal vesicles, prostate and epididymis; all of these glands are androgen-dependent. In the current study, the

Table 3: The effects of M0 and M60 mg nano-emulsion supplementation on the quality of chilled and frozen-thawed semen of rabbit bucks (mean±standard error of mean).

	Maca-treated groups (mg)					
	M0			M60		
Semen characteristics (%)	Chilled	Frozen	*P*-value	Chilled	Frozen	*P*-value
Progressive motility	48.80±0.72	27.39±0.65	0.0001	70.80±0.78	45.00±0.68	0.0001
Alive sperm	60.51±0.66	39.98±0.72	0.0001	74.90±0.35	58.44±0.43	0.0001
Sperm abnormalities	23.22±0.40	24.43±0.63	0.142	22.30±0.33	23.41±0.92	0.121

(n= 10 replicates).

Table 4: Fertilising ability of chilled and frozen-thawed semen collected from rabbit bucks treated with M0 and M60 nano-emulsion (mean±standard error of mean)

	M0		M60	
Parameters	Chilled semen +5°C	Frozen-thawed semen	Chilled semen +5°C	Frozen-thawed semen
Pregnancy rate (%)	66.66 [b]	33.33 [a]	100 [d]	83.33 [c]
Kindling rate (%)	66.66 [b]	33.33 [a]	100 [d]	83.33 [c]
Total born/doe	4.00 ±0.00 [a]	3.00 ±0.00 [a]	7.00 ±0.49 [b]	7.00±0.54 [b]
Liveborn %	50.00 (2/4) [b]	33.33 (1/3) [a]	100 (7/7) [d]	71.42 (5/7) [c]
Mortality%	50.00 ±8.33 [c]	66.67 ±0.00 [d]	0.00 ±0.00 [a]	28.57 ±4.8 [b]

Means with different superscripts within rows (a,b,c,d) within rows are significantly different, at least at $P<0.05$. (n=20 does/group).

level of testosterone, a representative hormone of androgen, was elevated in the treated groups, especially in the last two weeks of the experiment. This result correlated with the study in rats that attributed the testosterone elevation to the enhanced ability of testosterone production by Leydig cells (Ohta *et al.*, 2016), as the cytoplasmic area of Leydig cells was increased by feeding the maca extract powder. This suggests that maca extract has a stimulatory effect on Leydig cell growth directly or indirectly, or may be due to its androgen-like actions (Gonzales *et al.*, 2001b). Moreover, Ohta *et al.* (2016) added that feeding hydroalcoholic extract powder of *Lepidium meyenii* (maca) increases serum testosterone concentration and enhances the steroidogenic ability of Leydig cells in male rats.

Mass and progressive sperm motilities reported herein indicated a tremendously noticeable increase, especially in the M60 nano-emulsion-treated group. The present finding is consistent with reports in infertile and healthy men showing improvement in semen motility after treatment with maca (Gonzales *et al.*, 2001a). Sperm motility was likewise androgen-dependent; mass motility and the percentage of motile cells significantly increased rabbit doe production (+1 live births/AI) when the semen showed at least a commencement of wave movement, or when the percentage of motile cells was more than 84% (Brun *et al.*, 2002). One study demonstrated that maca also restored lead acetate-induced impairment to spermatogenesis in male rats (Rubio *et al.*, 2006). The concomitant increase in the strength of mass motility of the sperm depends on and coincides with the significant increase in sperm cell concentration and progressive sperm motility reported in the current study. Similarly, several studies in rats indicated that maca and its extracts improved acrosome reaction, sperm motility and sperm count by preserving the structural and functional integrity of testosterone-producing Leydig cells (Valdivia Cuya *et al.*, 2016; Onaolapo *et al.*, 2018). Clément *et al.* (2010) indicated that maca promoted sperm production, motile sperm count and the number of sperm with normal morphology in bulls.

In the present study, sperm cell concentration and total number of sperm/ejaculates increased significantly, especially in the M60 maca-treated group. This could be attributed to the thought that maca increases the mitotic phases of spermatogenesis (Gonzales *et al.*, 2003; Gasco *et al.*, 2007). Being a function of ejaculate volume and sperm cell concentration, the total number of sperm per ejaculate reported herein displayed a similar pattern. These findings differ from those described in infertile men in which there were no differences in sperm concentration after treatment with maca (Quelca Tancara *et al.*, 2010; Poveda *et al.*, 2013).

The present results revealed an obvious reduction in the abnormality percent of the sperm in the maca-treated groups, especially the M60 treated group. A similar effect has been shown in rats, as maca prevented lead acetate-induced spermatogenesis damage, implying that it enhances sperm production (Rubio *et al.*, 2006). Maca supplementation has been shown to improve spermatogenesis after malathion poisoning (Bustos-Obregón *et al.*, 2005) and attenuate spermatogenic disorders caused by high altitude conditions in rats (Gonzales *et al.*, 2004), which aligns with the present findings.

There were no significant differences in the overall mean values of live sperm percentage among the four groups throughout the experimental period, although the sperm membrane integrity of the treated groups, particularly M60 and M120, had increased significantly compared to the control. In agreement with our results, oral administration

of maca extracts at 400 or 600 mg/head weekly enhanced sperm membrane integrity in male rabbits affected by environmental heat stress (Ragab *et al.*, 2023).

Results of the current study recorded that the M60 groups (chilled and frozen/thawed semen) had significantly higher pregnancy, kindling, liveborn and litter size rates than the control group (Table 4). In addition, litter size at weaning is an important economic trait that determines the number of kits for future breeding programmes and lifetime production (El-Sheikh *et al.*, 2019). Results of the present study showed that the oral administration of black maca nano-emulsion at level M60 /head twice weekly significantly (P<0.05) increased litter size after AI, either with chilled or frozen semen, compared to the control group. This could be attributed to maca's beneficial effects on sperm quality, particularly progressive sperm motility and viability after stress exposure during freezing and thawing, as well as its antioxidant activity. Furthermore, maca-supplemented rabbit bucks have 20% more post-thawing sperm motility than the control group. The fundamental mechanisms that endorse the effect of *Lepidium meyenii* (maca) on rabbit semen remain imprecise. Further research is necessary to validate and quantify the absorption of black maca nano-emulsion in rabbits, to clarify the mechanisms of *Lepidium meyenii* special effects on semen production and to determine the possible influence of black maca nano-emulsion on the systemic antioxidant capacity in rabbits. Moreover, the effect of black maca nano-emulsion on the steroidogenic activity of Leydig cells is needed to indicate the reason for increasing testosterone levels.

CONCLUSIONS

Results of the current study reported that, for the first time, oral supplementation with 60 mg nano-emulsion of black maca extract/rabbit buck twice weekly for ten weeks could improve semen characteristics, serum testosterone levels and rabbit buck fertility. This is indicated by the highly significant pregnancy, kindling and liveborn rates of rabbit progenies from dams inseminated with chilled and frozen semen of rabbit bucks treated groups.

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