Evaluation of the Insemination doses Additives Using CASA Technology - Maca Peruana

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Abstract
The artificial insemination (AI) represents the most effective biotechnological method used in horse breeding. Therefore, the optimization of already established protocols and their novelization are an important research topic globally with the one goal - to preserve and improve the quality of insemination doses and thus improve the fertility rate. The purpose of the study was to investigate the activity of Maca peruana and determine changes in physiological functions of stallion spermatozoa by adding a solution of Maca dissolved in DMSO and diluted with saline to concentrations 0, 2, 4 and 8% (CON, A, B, C, respectively). The samples were incubated at 37°C throughout the experiment and the measurements were performed in time intervals 0, 60 and 120 minutes. The quality of samples was evaluated by CASA and mitochondrial toxicity test. Motility and progressive motility of spermatozoa of stallions showed stimulative tendency in Maca treated samples especially in experimental group A that exhibited the highest percentages in all measured intervals. The mitochondrial toxicity test showed changes in Maca treated samples, whose survival was several percent higher than the control sample.
In conclusion, the beneficial effect of Maca on spermatozoa has been partially confirmed but mainly when spermatozoa were treated with very low concentrations of additive. The study brings an attention to not widely used plant substance (Maca peruana) and offers numerous opportunities for further research concerned with male reproduction and AI in particular.

Keywords: Lepidium meyenii, fresh cooled semen, Peruvian maca, spermatozoa, stallion

1. Introduction
Spermatogenesis is a complex process highly sensitive to exogenous factors. Many of these factors are associated with the current lifestyle. Male infertility is becoming a global problem in both humans and animals (Tafuri et al., 2021). In horse breeding, artificial insemination provides many benefits such as prevention of problems with the transport of animals, prophylaxis and moreover to improve or maintain the characteristics of the horse breed. Addition of antioxidants and other adjuvants to insemination doses is an essential tool for maintaining the quality of stallion spermatozoa. They can be used in vivo as a dietary supplement or in vitro as a medium supplement (Ciani et al., 2021). The main task of antioxidant additives is to prevent oxidative damage of spermatozoa caused by the preparation and manipulation with ejaculate in vitro. Oxidative damage in spermatozoa can be manifested by decreased motility, viability, DNA damage, or impaired membrane and acrosome integrity.
Oxidative stress has been shown to significantly reduce the success of assisted reproduction (Agarwal et al., 2017).

Numerous studies are currently examining the effects of naturally occurring antioxidants. Plants and their fruits are a source of many natural antioxidants and other substances that have been shown to have beneficial effects on animal cells in vitro, including spermatozoa. Peruvian maca is attracting more and more attention because it has many uses, for example against fatigue, improves spermatogenesis and increases fertility. Recently, Maca has been shown to have high antioxidant activity, especially Maca polysaccharides (Zhang et al., 2017). The continuing interest in this plant is based on the expected effects on male mammalian fertility due to the presence of certain partially specific secondary compounds (Clement et al., 2009).

1.1 Peruvian Maca

The Peruvian Maca (Lepidium meyenii, syn. Lepidium peruvianum) was domesticated probably between 4000 and 1200 BC on the high plateaus of Peru's central Andes. This biennial plant belongs to the family Brassicaceae (Toledo et al., 1998). The plant adapts well to extreme conditions at high altitudes (cold weather, strong UV radiation, low oxygen levels and unstable climate) (Zhang et al., 2016). Hypocotyls are widely used as a nutritional supplement in human medicine to increase fertility and sexual function. Dried Maca hypocotyls are rich in high nutritional elements such as carbohydrates, proteins, lipids, essential amino acids, and free fatty acids. Maca also contains several secondary metabolites, such as macamides, macaridine, alkaloids and glucosinolates. Macamides are specific Maca alcamides that are known for their antioxidant effect (Tafuri et al., 2019). The purpose of this study was to investigate the activity of Maca peruana and the effects on physiological functions of spermatozoa.

2. Data and Methods

2.1 Material

Ejaculate samples were obtained from breeding stallions (n = 6) from breeding stables in western part of Slovakia. The age of stallions was 5-26 years. The collection was realized from stallions of the Holstein horse breed. The horse feed consisted of hay and oats and stallions were housed in stables with straw bedding.

2.2 Ejaculate collection

Ejaculate collection was performed in the morning hours. Preheated artificial vagina (40 – 42°C; Colorado type, Minitube, Tiefenbach, Germany) was used for the collection. After filling with water, the pressure was adjusted by adding the required amount of air. The vagina was lubricated with indifferent sterile vaseline. Ejaculate collection from stallions was performed by phantom jump (Halo et al., 2019).

2.3 Preparation of Maca solution

A stock solution was prepared from maca powder (Bio Maca; GymBeam). In the experiment, 20 mg of maca powder was diluted in 1 ml of DMSO. The DMSO solution was prepared from a commercial solution (Sigma Aldrich, St. Louis, USA) and saline (0.9% NaCl; Braun Melsungen AG, Germany) and adjusted to a concentration of 20%. After extracting the bioactive substances from the maca, we filtered the solution through filter paper. The filtrate thus obtained was prepared in final Maca concentrations of 2, 4 and 8%.
2.4 Preparation of samples
Stallion ejaculate was diluted in ratio 1:2 with three prepared maca DMSO-based solutions diluted in saline: A - 2% solution; B - 4% solution; C - 8% solution. Control samples (CON) were prepared by diluting ejaculates only with saline and DMSO. Spermatozoa motility was assessed at three time intervals: 0, 60 and 120 minutes at temperature 37°C.

2.5 CASA – Computer Assisted Semen Analysis
The basic principle of the CASA microscopy-based system is to obtain a series of sequential images of motile spermatozoa in a static imaging field (Budai et al., 2014). Spermatozoa analyzes were performed by computer sperm analyzer (CASA) with SpermVision software (Minitube, Tiefenbach, Germany) and an Olympus BX 51 microscope (Olympus, Japan). Diluted ejaculate samples (10 μL) were pipetted on the Makler counting chamber (Sefi-Medical Instruments, Germany) pre-heated to 37°C for each analysis. Following parameters were observed - total motility (MOT; %), progressive motility (PRO; %) and other trajectory and velocity parameters (Halo Jr. et al., 2021).

2.6 Determination of cell viability
Spermatozoa viability was determined using the mitochondrial activity test (MTT test) according to Mosman (1983). This method is based on the conversion of the yellow tetrazolium salt (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) to blue formazan particles, which catalyzes the mitochondrial enzyme succinate dehydrogenase of intact mitochondria inside living cells, with intent Formazan staining of samples can be quantified spectrophotometrically using an ELISA microplate reader.

The tetrazole salt (Sigma Aldrich, St. Louis, USA) was dissolved in PBS (Dulbecco's Phosphate Buffer Saline, Sigma Aldrich, St. Louis, USA) at a ratio of 5 mg/mL and 10 μL of this solution was added to wells of 96 chamber microplate containing spermatozoa samples. After two hours of incubation, the tetrazole and succinate dehydrogenase reaction was stopped using isopropanol (Centralchem, Bratislava, Slovakia) (Tvrdá et al., 2015). The optical density at 570 nm versus 620 nm as a reference value was determined with a microplate Elisa reader (Multiskan FC, ThermoFisher Scientific, Vantaa, Finland). The resulting data were expressed as a percentage, while the value of the control sample was standardized to 100%.

2.7 Statistical analysis
Statistical software GraphPad Prism 5 software (GraphPad Software, Inc. La Jolla, California, USA) was used for statistical processing of the results using paired Student's t-test. Significant differences of the control group compared to the experimental groups were determined at the level of statistical significance: * P <0.05; ** P <0.01; *** P <0.001.

3. Results and Discussion
We evaluated the effect of Peruvian maca in three different concentrations for individual parameters of stallion spermatozoa motility in vitro during cultivation at 37°C temperature. The effect of Maca (Lepidium meyenii) was evaluated at time intervals of 0, 60 and 120 minutes.

The Peruvian persimmon has a somewhat positive effect on spermatozoa. The percentage of spermatozoa motility (MOT) was reduced during the first measurement compared to the control sample. However, it increased over the cultivation time where samples A (2%) and B (4%) recorded higher MOT than CON in time intervals 60 and 120 minutes. The obtained
results indicate a positive effect of maca in lower concentrations in all monitored time intervals. However, higher concentrations imply negative effects (Figure 1).

![MOT](image)

**Figure 1:** The effect of Maca on the total spermatozoa motility (%) at 37 °C. A – 2%; B – 4%; C – 8% solution of Maca

The highest decrease in progressive motility (PRO) occurred in sample C (8%) after 60 and 120 minutes (Figure 2). No samples showed significant differences compared to the control sample. For sample A (2%), we observed a slight increase in progressive motility compared to the control at all time intervals.

![PRO](image)

**Figure 2:** The effect of Maca on the progressive spermatozoa motility (%) at 37 °C. A – 2%; B – 4%; C – 8% solution of Maca

We tested the mitochondrial activity of spermatozoa using the MTT assay after experimental treatment with Peruvian maca. MTT detects succinate dehydrogenase activity and thus we determine mitochondrial activity. The test was performed after 120 minutes of cultivation, the results are compared in Figure 3. Increased spermatozoa viability was noticeable in each
experimental sample enriched with Maca. From the results of this analysis, we can state that mitochondrial activity was the most increased in the sample C (8%) compared to the control.

![MTT Graph](image)

**Figure 3: The effect of Maca on the viability (%) of stallion spermatozoa after 120 minutes of incubation at 37 °C. A – 2%; B – 4%; C – 8% solution of Maca**

A previous *in vivo* study on humans showed that Peruvian maca improves ejaculate volume, spermatozoa count (concentration), motile spermatozoa count and spermatozoa motility, although it does not affect the levels of relevant serum hormones, including serum luteinizing hormone, follicle stimulating hormone (FSH), for testosterone or estradiol. This suggests that the improved quality of ejaculate observed because of Maca administration may be due to an increase in bioavailable testosterone or testosterone receptors and an improved Sertoli cell response to FSH (Aoki et al., 2018).

Aoki et al. (2018) examined whether the addition of Maca to the culture medium improved the *in vitro* fertilization (IVF) success rate. As expected, in fertilization, the rate of fertilization in maca-enriched medium was significantly increased compared to control medium. The results showed that human tubal fluid (HTF) medium containing Maca extract at a concentration of 4% was the most suitable for IVF in mice.

The study conducted on bulls reports that ejaculate volume and spermatozoa density were evidently increased with time in the control group and Maca-treated bulls. The same results were proven for spermatozoa density and total spermatozoa count per ejaculate. The percentage of motile spermatozoa increased over time in bulls with the addition of Maca. This study demonstrated positive effects of supplementing ground dried Maca hypocotyl in prepubertal bulls. Supplementation of Maca hypocotyl improved mating patterns, spermatozoa count, and quality. Further research is necessary before definitively recommending of this approach. This could include using higher doses of maca, applying the most effective form of maca, black hypocotyls, and extending the experiment by using more bulls with lowered spermatozoa quality (Clement et al., 2010).

The use of Peruvian maca also induces an increase in testicular size and stimulation of spermatogenesis in rats and mice. Maca reduced spermatogenic damage in mice and prostate size in rats and restored stress-induced homeostasis in mice (Valentová et al., 2006).

Basic research is required to fully understand the mechanism of action of Maca on spermatozoa parameters. Potential bioactive substances in Maca include macaridine, macamides, macaene,
gluosinolates, alkaloids and Maca nutrients. However, these data are insufficient to determine whether the maca is clinically effective (Lee et al., 2016).

4. Conclusion
From the results of the study, we can state that lower concentrations of Peruvian maca have positive effects on motility (MOT) and progressive motility (PRO) of stallion spermatozoa compared to higher concentrations where a negative impact was observed. Interestingly the highest concentration was effective in the case of viability/mitochondrial activity (MTT) of spermatozoa. However, none of the monitored parameters showed significant differences compared to the control. Despite this fact, the effects of this plant on spermatozoa bring interesting results. As it is a not widely used plant, its effects in vitro are still worth further research. In the future, more specific analyses need to be performed to confirm or refuse its positive effects on the spermatozoa quality.

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