ORIGINAL ARTICLE

Effect of Black maca (*Lepidium meyenii*) on one spermatogenic cycle in rats

G. F. Gonzales, J. Nieto, J. Rubio & M. Gasco

Instituto de Investigaciones de la Altura, and Department of Biological and Physiological Sciences (Faculty of Sciences and Philosophy), Universidad Peruana Cayetano Heredia, Lima, Peru

Keywords
Black maca—intratesticular testosterone—*Lepidium meyenii*—spermatogenesis—spermatogenic cycle

Correspondence
Gustavo F. Gonzales, Instituto de Investigaciones de la Altura or Laboratory of Investigation and Development (LID), Faculty of Sciences and Philosophy, Universidad Peruana Cayetano Heredia, PO Box 1843, Lima, Peru.
Tel.: 00 511 482 1195;
Fax: 00 511 482 1195;
E-mail: iiad@upch.edu.pe

Accepted: July 4, 2006

Summary

Lepidium meyenii (Maca) grows exclusively between 4000 and 4500 m above sea level in the Peruvian central Andes. The hypocotyls of this plant are traditionally used in the Andean region for their supposed fertility-enhancing properties. The hypocotyls have different colours. Of these, Black maca has better effects on spermatogenesis. The present study aimed to test the hypothesis that Black maca has early effects during a spermatogenic cycle (12 days) of male rats. For this, testicular spermatid, epididymal sperm and vas deferens sperm counts were measured after 1, 3, 5, 7 and 12 days of treatment with Black maca. Aqueous extract of Black maca was given orally by daily gavage at a dose of 2 g kg^{-1}. In a spermatogenic cycle, compared with day 1, daily sperm production (DSP) was lower at day 7 (control), whereas with Black maca, the difference was observed at day 12. Epididymal sperm count was higher in rats treated with Black maca at days 1, 3 and 7, but similar to controls at days 5 and 12; similarly sperm counts in vas deferens was higher in rats treated with Black maca in days 3, 5 and 7, but similar to controls at days 1 and 12. From this, it is suggested that first action of Black maca was at epididymal level increasing sperm count after 1 day of treatment, whereas an increase in sperm count was observed in vas deferens at day 3 of treatment. Finally, an increase in DSP was observed after 7 days of treatment with Black maca. Testicular testosterone was not affected after 7 days treatment with Black maca. In conclusion, Black maca affects sperm count as early as 1 day after beginning of treatment.

Introduction

Maca (*Lepidium meyenii*) is a Peruvian plant that belongs to the Brassicaceae family, and grows exclusively between 4000 and 4500-m above sea level (a.s.l) in the central Peruvian Andes. This plant, of which the hypocotyls, traditionally are used, is known for its property to enhance fertility. Oral administration of an aqueous extract from the hypocotyls of Yellow *L. meyenii* (Yellow maca) for 7 days at a dose of about 2 g kg^{-1} of body weight (BW) to male rats increased length of spermiation stage (stage VIII; Gonzales *et al.*, 2004; Chung *et al.*, 2005), whereas, a treatment length of 14 days increased the lengths of stages in which first mitosis occurs (stages IX–XI) (Gonzales *et al.*, 2001). Furthermore, maca also enhanced sperm count and sperm motility in normal men without affecting serum testosterone, luteinizing hormone (LH) or follicle stimulating hormone (FSH) levels (Gonzales *et al.*, 2001a).

Maca is observed in several varieties characterized by different colours of the hypocotyls (Valerio & Gonzales, 2005) and different biological properties have been described for Red maca, Yellow maca and Black maca (Gonzales *et al.*, 2005). Long-term treatment for 42 days, a time enough for recovery of spermatogenesis, (Hikim & Swerdloff, 1994) showed that Black maca has a better effect than Yellow maca whereas Red maca was ineffective with regards to increasing sperm production (Gonzales *et al.*, 2006).

The duration of the seminiferous cycle in rat is 12.5 days (Aslam *et al.*, 1999). It would be interesting to

know early effects of Black maca on a spermatogenic cycle. For such a reason, the present study was designed to determine if treatment with Black maca for 1, 3, 5, 7 and 12 days affects daily sperm production (DSP), epididymal and vas deferens sperm counts.

Materials and methods

Animals

Sixty, four-month old, male rats from the Holtzman strain obtained at the animal house of the National Institute of Health (Lima, Peru) were used. Rats were divided randomly into two groups of treatment: Control (vehicle treated group) and Black maca treated group. Rats were housed at six per cage. Rats were maintained at environmental temperature (22 °C) with a 12 : 12 h light/dark cycle in the animal house at the Universidad Peruana Cayetano Heredia. Rats were provided with Purina laboratory chow and tap water *ad libitum*.

Preparation of aqueous extract of *Lepidium meyenii* Maca

The dried hypocotyls of *L. meyenii* were obtained from Carhuamayo, Junin at 4000 m a.s.l. The identity of the plant was authenticated by Irma Fernandez, a Botanist of the Department of Pharmaceutical Sciences, Universidad Peruana Cayetano Heredia.

For the present study, 500 g of the dried hypocotyls were pulverized and then placed in a container with 1500 ml of water and boiled for 60 min. The preparation was left standing to cool and was then filtered. The filtrate, containing an amount equivalent to 333 mg of the dry hypocotyls (starting material) in 1 ml was placed in small vials and kept in 4 °C refrigerator for no more than 12 days.

Experimental protocol

Maca was administered for 1, 3, 5, 7 and 12 days. This allowed the study of one spermatogenic cycle in the rat. A No. 18 intubation needle (Fisher Scientific, Pittsburgh, PA, USA) for nasogastric feeding was used to orally administer around 2 ml water (with or without maca). The amount of maca administered daily to each rat was 2 g kg^{-1}. This amount is comparable with human consumption in the highlands of Peru (Valerio & Gonzales, 2005). The Institutional Review Board of the Scientific Research Office from the Universidad Peruana Cayetano Heredia approved the study. One day after the end of the treatments, rats were sacrificed by decapitation and a blood sample was obtained from the cervical trunk. Blood samples were centrifuged and the sera separated, placed in vials and kept frozen until assayed for serum hormones. In the evaluation of sperm counts, histological analysis and hormone level measurements, the investigators were blinded to treatment group.

Daily sperm production

Testes were homogenized in 10 ml of 0.9% saline-0.05% (v/v) Triton X-100 solution for 1 min by a homogenizer (Takahashi & Oishi, 2003). After a dilution 1/10, the number of homogenization-resistant elongated spermatids nuclei per testis was determined with a haemocytometer. Counts for four haemocytometer chambers were averaged. DSP and its efficiency (DSP per gram testis) were determined by division of the elongated spermatid count per testis and spermatids per gram testis by 6.3 days of spermatogenesis time during steps 17–19 spermatids for Holtzman rats (Kubota *et al.*, 2003; Takahashi & Oishi, 2003). The epididymal sperm transit rate was calculated by dividing the cauda epididymal sperm number by the DSP (Dalsenter *et al.*, 2003).

Epididymal sperm count

Homogenization-resistant epididymal sperms from 60 nonperfused rats were counted as described previously (Gonzales *et al.*, 2004) with some modifications. Modifications included measurements in caput/corpus and cauda epididymides. Caput and corpus epididymis were cut and homogenized separately to the cauda epididymis. Homogenization was performed in 5 ml saline (NaCl 0.9%). Homogenates were kept refrigerated at 4 °C for 24 h to allow sperm to be released from the walls. Then, 5 ml of eosine (2%) were added and vortexed. One millilitre of this mixture was diluted with 2 ml eosine (2%) and a sample was placed in a Neubauer chamber and head sperms were counted in 25 squares. Sperm counts in the 25 squares were multiplied by 0.06 (sperm $\times 10^6$ ml^{-1}) and then by 5 ml (sperm $\times 10^6$/caput/corpus or cauda). Data are referred as sperm per caput/corpus or cauda epididymis.

Vas deferens sperm count

Vas deferens was dissected in two parts, one corresponding to the proximal end and the second to the distal end. Each part was homogenized with 1 ml saline. An aliquot was diluted with two parts of eosine (2%). Homogenization-resistant sperm heads were counted in the 25 squares of the Neubauer chamber. Four chambers were measured in each sample and they were averaged. Results from each part (proximal or distal end) were multiplied by 0.03 and

defined as sperm $\times 10^6$/part of vas deferens. Data were expressed as total amount of sperms in vas deferens (sperm count in proximal end + sperm count in distal end).

Histological study

Right testis and epididymis were excised and dissected free of fat. Then, testes and epididymis were immersion-fixed in Bouin's fixative and embedded in paraffin. The tissue blocks were sectioned into 5 μm thickness and stained with haematoxylin and eosine (H&E) and then observed under a light microscope.

For testes, tubular area (μm^2) and lumen area (μm^2) were measured by sampling 10 random sections for each rat. In epididymis, 10 random sections with 10 measurements of epithelial height (μm) for each section per rat were measured. Epididymal tubular areas were also measured (μm^2). All assessments were performed using an axiostar plus microscope (Carl Zeiss, Thornwood, NY, USA). The images were captured by a Moticam2000 (Richmond, BC, Canada) coupled to a personal computer. MOTIC IMAGE PLUS 2.0 software (Motic Instruments Inc., Xiamen, China) was used for measurements of testicular seminiferous tubule area and duct luminal area and for measurements of epididymal epithelial height and calculated by statistic ANOVA test.

Hormone assays

Serum testosterone concentrations were measured by radioimmunoassay using commercial kits (Diagnostic Products Co, Los Angeles, CA, USA) in rats treated for 7 days with vehicle, or Black maca. The hormone labelled with iodine-125 was used as radioactive marker. Samples were run in the same assay to avoid inter-assay variation. The intra-assay variation was 5.5% for testosterone. The minimum testosterone detection level was 0.04 ng ml^{-1}.

Intratesticular testosterone (ITT) levels were determined in six rats treated with vehicle (control) and six rats treated with Black maca administered for 7 days as described previously (Kubota *et al.*, 2003). Briefly, left testis was homogenized in 10 ml of phosphate buffered saline (PBS, pH 7.4) and the homogenate (1 ml) was then extracted with diethyl ether and the ether fraction was dried. The dried lipophilic substances were re-suspended in 1 ml of the buffer included in the kit and processed for measurement following the manufacturer's description. Data are referred as nanogram per gram testis.

Statistical analysis

Data were analysed using the statistical package STATA (version 8.0) for personal computer (Stata Corporation, 702 University Drive East, College Station, TX, USA). Data are presented as mean ± SEM. Homogeneity of variances was assessed by the Bartlett test. If variances were homogeneous, differences between groups were assessed by analysis of variance (ANOVA). If *F*-value in the ANOVA test was significant, the differences between pair of means were assessed by the Scheffé test.

When variables were not homogeneous, the Kruskal–Wallis test was used to assess differences between groups. If the result was statistically significant, the differences between pair of medians were assessed by the Mann–Whitney *U*-test. A value of $P < 0.05$ was considered to be statistically significant.

Results

A decrease in DSP from day 1 to 7 was observed in the control group ($P < 0.05$; Fig. 1). After 12 days of treatment with vehicle (control), an increase in DSP occurred as compared with the previous value at day 7 ($P < 0.05$). Treatment with Black maca modified the pattern observed in the control group. DSP increased from day 1 to 7 of treatment ($P < 0.01$). However, a reduction in DSP was observed at day 12 of treatment with Black maca, such that the difference with the control group disappeared (Fig. 1). From these results, DSP was higher in the group treated with Black maca for 7 days ($P < 0.01$) with respect to the control group at the same time. After 12 days of treatment, DSP was similar in the maca treated group and controls (Fig. 1). ITT levels (168.8 ± 20.3 and 172.2 ± 36.7 ng g^{-1} for maca and control groups respectively; *P*, not significant) or serum testosterone levels (264.7 ± 34.1 and 229.5 ± 47.2 ng dl^{-1} for maca and control groups respectively; *P*, not significant) were not affected after 7 days of treatment with Black maca.

Epididymal sperm count was higher in rats treated with Black maca at days 1, 3 and 7 but similar to controls at days 5 and 12 (Fig. 1); similarly sperm counts in vas deferens was higher in rats treated with Black maca at days 3, 5 and 7 but similar to controls at days 1 and 12 (Fig. 1).

Analysis of the seminiferous tubular area and seminiferous luminal area showed that the areas of seminiferous tubule were not affected by treatment with Black maca for 7 or 12 days (Fig. 2a). However, seminiferous luminal area was reduced in the group treated for 7 days with Black maca and similar to the control in the group treated for 12 days with Black maca (Fig. 2b). When the per cent of tubular lumen related to tubular area is assessed, this is reduced in the Black maca group treated for 7 days ($P < 0.01$) but in the groups treated for 12 days, no differences were observed between maca treated and control groups (Fig. 3).

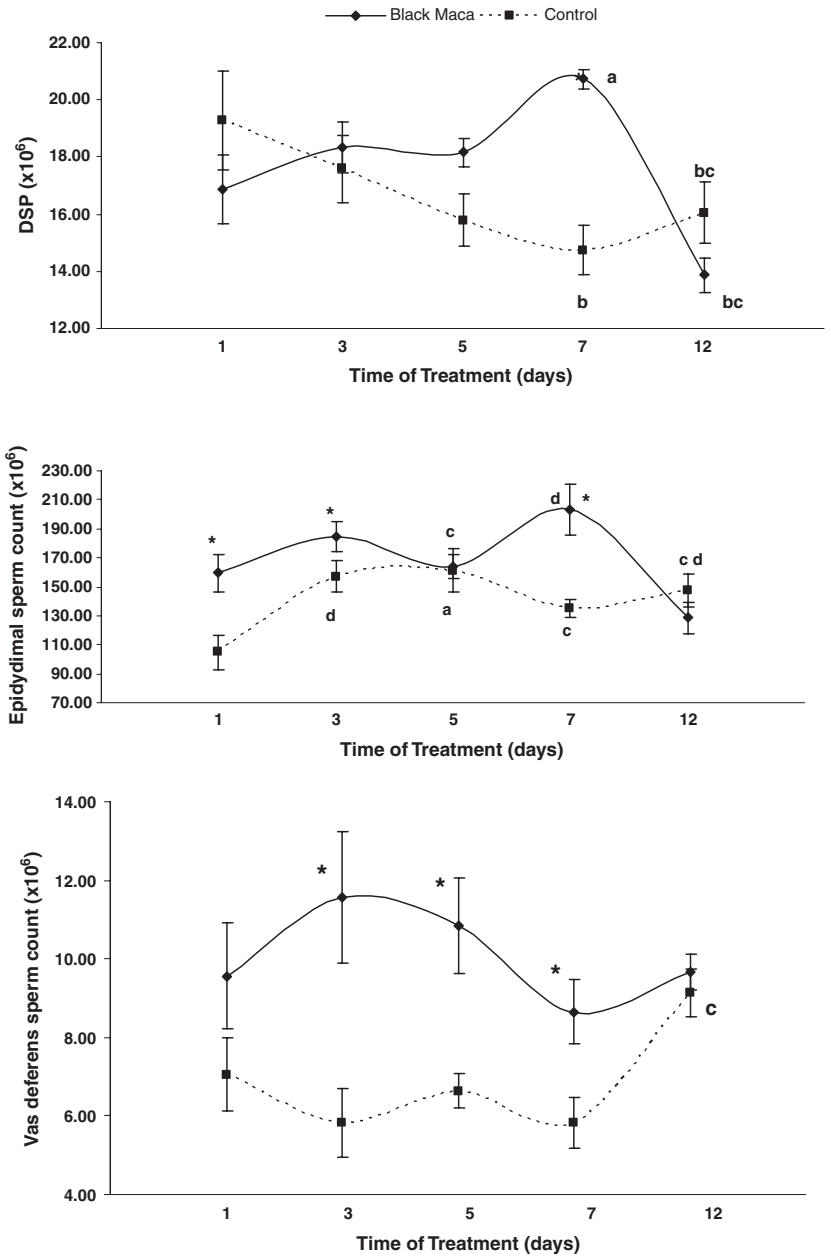

Fig. 1 Effect of Black maca on daily sperm production (DSP), epididymal sperm count and sperm count in the vas deferens of adult rats. Data are mean ± SEM of six animals. Black maca or vehicle were administered for 1, 3, 5, 7 and 12 days at dose of 2 g kg^{-1}. *$P < 0.01$; **$P < 0.05$ with respect to control values. a, $P < 0.01$; b, $P < 0.05$ with respect to values at day 1 of treatment. c, $P < 0.01$; d, $P < 0.05$ with respect to the previous day.

Epithelial height in epididymis was not affected after treatment with Black maca for 7 or 12 days. Epithelial heights were 63.24 ± 1.17 and 62.32 ± 0.60 μm (mean ± SEM) in control and Black maca groups after 7 days of treatment respectively (P, not significant). Moreover, after 12 days of treatment with vehicle (control) or Black maca, epithelial heights were 61.80 ± 0.69 and 61.20 ± 0.89 μm respectively (P, not significant). However, as sperm number in the epididymal lumen increased, the area of the epididymal tubules in the group treated for 7 days with Black maca (581.54 ± 38.86 × 10^3 μm^2) increased in comparison with control group (409.42 ± 29.19 μm^2; $P < 0.01$).

In the groups treated during 12 days with Black maca (374.85 ± 22.42 × 10^3 μm^2) or vehicle (412.8 ± 24.97 × 10^3 μm^2), the areas of the epididymal tubules were not different (P, not significant).

Discussion

Maca is naturally present in different varieties which are characterized by external colour (Valerio & Gonzales, 2005). Previous study showed differences in the biological responses of three varieties of maca: Red, Yellow and Black. Black maca appeared to have more beneficial

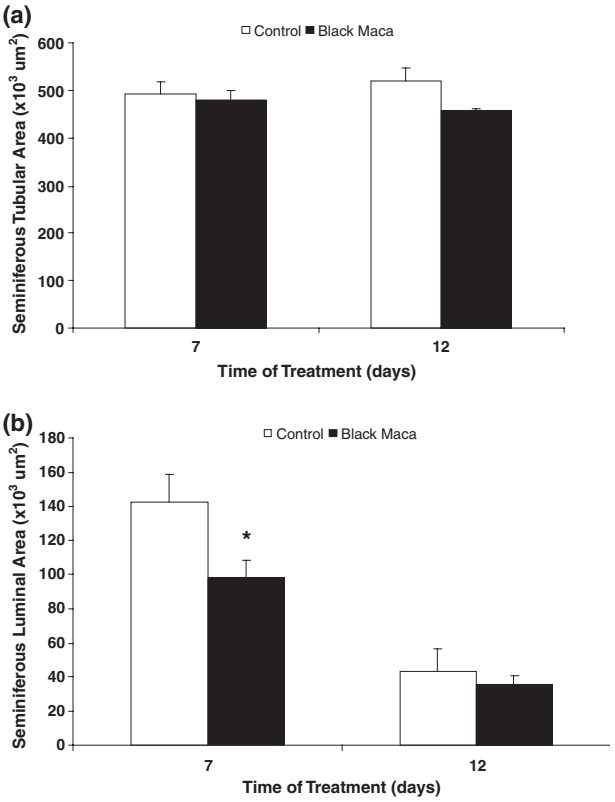

Fig. 2 Seminiferous tubular area (a) and seminiferous luminal area (b) of rats treated with Black maca administered during 7 and 12 days. Data are mean ± SEM. *$P < 0.05$ with respect to control group. Differences between groups were assessed with Mann–Whitney *U*-test.

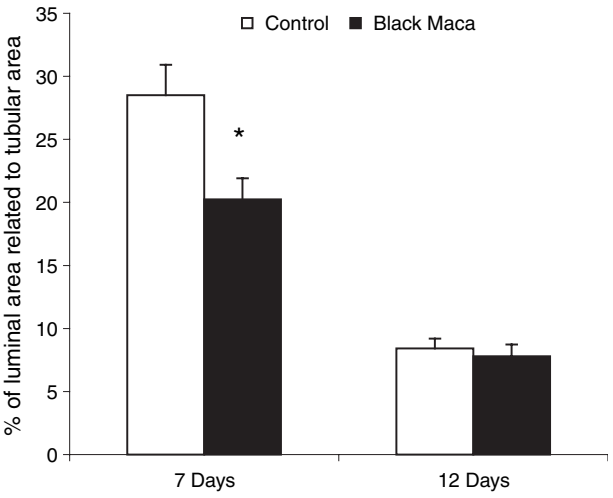

Fig. 3 Percentage of luminal area related to tubular area in seminiferous tubules of rats treated with vehicle (control) or Black maca for 7 or 12 days. *$P < 0.01$.

effects on sperm counts and epididymal sperm motility after 42 days of treatment (Gonzales *et al.*, 2006). The present study showed that DSP, epididymal and vas deferens sperm counts are affected by Black maca during a spermatogenic cycle of the male rat.

It is not possible for Maca to affect spermatogonia and change sperm count within 12 days. Then, one could speculate that spermiation was enhanced, however quantitative studies on spermiation in Sprague Dawley rats show that at least 97% of spermatids are spermiated in normal adult rats (Saito *et al.*, 2000), meaning that enhancement of an already successful process would be difficult. In this case it is possible that a modulation could be occurring between DSP and sperm count in the epididymis and/or vas deferens.

Animals used in the present study were virgins. In this model, an accumulation of sperm as an effect of longer time of sexual abstinence was not observed. This suggests that a mechanism is operating at epididymal and/or vas deferens levels that avoids an excessive accumulation of sperm due to sexual abstinence. One question is how epididymal sperm count is maintained. Several studies during the 1970s failed to substantiate the hypothesis that, in the normal animal, significant numbers of spermatozoa disappear during epididymal passage (Amann, 1970; Jones, 2004). The balance of evidence does not support the view that, in the normal animal, the epididymis routinely absorbs significant numbers of defective or dead spermatozoa (Jones, 2004). The epididymis, a natural sperm reservoir, has maturational and storage functions and it protects spermatozoa from oxidative injury by encouraging scavengers of reactive oxygen species (ROS) (Zini & Schlegel, 1997). This later effect is further supported by the finding that supplementation with the antioxidant ascorbic acid, increased epididymal sperm concentration in normal male rats (Sonmez *et al.*, 2005). This raises the possibility, that sperm number may be regulated at epididymal and/or vas deferens level through a balance between oxidant and antioxidant status.

It is possible that spermatozoa undergo degradation and dissolution by DNases, proteinases and hydroxylases within the epididymal lumen (Jones, 2004) when oxidant status prevails (Rao & Sharma, 2001). For instance, lead acetate induces oxidative stress and it has been demonstrated in male rats that it reduces DSP and epididymal sperm counts, whereas maca reversed these effects (Rubio *et al.*, 2006).

It was interesting to observe that in the control animals, a reduction in DSP was observed after 7 days of treatment, whereas Black maca delayed this reduction up to day 12. Similarly, epididymal sperm count was not maintained constant in the control group at different times of treatment (1, 3, 5, 7 and 12 days). A wave in the

control group with increasing values from day 1 to 5 was observed and thereafter, a reduction was observed at day 7 and again an increase in epididymal sperm count occurred at day 12. Treatment with Black maca resulted in higher values at days 1, 3 and 7 but similar values with control were observed at days 5 and 12. The modulation was also observed in the vas deferens. These changes were also corroborated with the histological study. At day 7, less tubules per field as a consequence of replenishment of sperms in the lumen of epididymis was observed. An effect on testosterone levels was discarded as intratesticular and serum testosterone levels were not affected by treatment with Black maca. Moreover, recently it has been reported that maca is not acting through the androgen receptor (Bogani *et al.*, 2006).

All of these results suggest that Black maca is acting firstly on epididymal sperm count as values increased after 1 day of treatment, then is followed by an increase of sperms in vas deferens observed after 3 days of treatment, whereas an increase on DSP is observed only after 7 days of treatment as a consequence of the delayed reduction of DSP. This suggests any kind of regulation between DSP, epididymal sperm count and vas deferens sperm count.

Histological study in the epididymis showed that epithelial height was not affected by the treatment with Black maca for 7 or 12 days. The only difference was at the level of number of tubules per field. In fact, after 7 days of treatment with Black maca, less tubules were seen when compared with controls. This was not observed after 12 days of treatment. This was due to the fact that after 7 days of treatment more sperm were within the epididymis, whereas after 12 days of treatment, epididymal sperm count was similar in the control and Black maca treated group.

The active secondary metabolites present in the plants responsible for the maca actions are still unknown. Some novel compounds have been recently identified as two new imidazole alkaloids (lepidine A and B; Cui *et al.*, 2003). Muhammad *et al.* (2002) described a benzylated derivative of 1, 2-dihydro-N-hydroxypyridine, named macaridine, together with the benzylated alkamides (macamides), N-benzyl-5-oxo-6E, 8E-octadecadienamide and N-benzylhexadecanamide, as well as the acyclic keto acid, 5-oxo-6E, 8E-octadecadienoic acid. However, fertility-enhancing properties of these compounds have not been assessed. As the results from the present study have been obtained using aqueous extract of maca, none of these alkaloids may be responsible of the activity on sperm number.

In conclusion, data presented here show that Black maca improved sperm counts in epididymis as early as 1 day after treatment and that this effect seems to be a regulatory mechanism of the distribution of sperm produced in the testis rather than a real higher production of sperm. This is the reason why epididymal sperm count resulted similar in the group treated during 12 days with maca and the control. If this change in the dynamics of sperm movement during a spermatogenic cycle is the key to induce higher sperm production after 42 days of treatment needs to be investigated.

Acknowledgements

The Vicerrectorate of Investigation of the Universidad Peruana Cayetano Heredia and PROCOM 2005 from the Peruvian National Council of Sciences, Technology and Innovation (CONCYTEC) supported this study.

References

Amann RP (1970) The male rabbit. IV. Quantitative testicular histology and comparisons between daily sperm production as determined histologically and daily sperm output. *Fertil Steril* 21:662–672.

Aslam H, Rosiepen G, Krishnamurthy H, Arslan M, Clemen G, Nieschlag E, Weinbauer GF (1999) The cycle duration of the seminiferous epithelium remains unaltered during GnRH antagonist-induced testicular involution in rats and monkeys. *J Endocrinol* 161:281–288.

Bogani P, Simonini F, Iriti M, Rossoni M, Faoro F, Poletti A, Visioli F (2006) *Lepidium meyenii* (Maca) does not exert direct androgenic activities. *J Ethnopharmacol* 104:415–417.

Chung F, Rubio J, Gonzales C, Gasco M, Gonzales GF (2005) Dose-response effects of *Lepidium meyenii* (Maca) aqueous extract on testicular function and weight of different organs in adult rats. *J Ethnopharmacol* 98:143–147.

Cui B, Zheng BL, He K, Zheng QY (2003) Imidazole alkaloids from *Lepidium meyenii*. *J Nat Prod* 66:1101–1103.

Dalsenter PR, de Araujo SL, de Assis HC, Andrade AJM, Dallegrave E (2003) Pre and postnatal exposure to endosulfan in Wistar rats. *Hum Exp Toxicol* 22:171–175.

Gonzales GF, Ruiz A, Gonzales C, Villegas L, Cordova A (2001) Effect of *Lepidium meyenii* (Maca) roots on spermatogenesis of male rats. *Asian J Androl* 3:231–233.

Gonzales GF, Córdova A, Gonzales C, Chung A, Vega K, Villena A (2001a) Improved sperm count after administration of *Lepidium meyenii* (Maca) in adult men. *Asian J Androl* 3:301–304.

Gonzales GF, Gasco M, Córdova A, Chung A, Rubio J, Villegas L (2004) Effect of *Lepidium meyenii* (Maca) on spermatogenesis in male rats acutely exposed to high altitude (4340 m). *J Endocrinol* 180:87–95.

Gonzales GF, Miranda S, Nieto J, Fernandez G, Yucra S, Rubio J, Yi P, Gasco M (2005) Red Maca (*Lepidium meyenii*) reduced prostate size in rats. *Reprod Biol Endocrinol* 3:5.

Gonzales C, Rubio J, Gasco M, Nieto J, Yucra S, Gonzales GF (2006) Effect of short-term and long-term treatments with three ecotypes of *Lepidium meyenii* (Maca) on spermatogenesis in rats. *J Ethnopharmacol* 103:448–454.

Hikim AP, Swerdloff RS (1994) Time course of recovery of spermatogenesis and Leydig cell function after cessation of gonadotropin-releasing hormone antagonist treatment in the adult rat. *Endocrinology* 134:1627–1634.

Jones R (2004) Sperm survival versus degradation in the mammalian epididymis: a hypothesis. *Biol Reprod* 71:1405–1411.

Kubota K, Ohsako S, Kurosawa S, Takeda K, Qing W, Sakawe M, Kawakami T, Ishimura R, Tohyama C (2003) Effects of Vinclozolin administration on sperm production and testosterone biosynthetic pathway in adult male rat. *J Reprod Dev* 49:403–412.

Muhammad I, Zhao J, Dunbar DC, Khan IA (2002) Constituents of *Lepidium meyenii* ''Maca''. *Phytochemistry* 59:105–110.

Rao MV, Sharma PS (2001) Protective effect of vitamin E against mercuric chloride reproductive toxicity in male mice. *Reprod Toxicol* 15:705–712.

Rubio J, Riqueros MI, Gasco M, Yucra S, Miranda S, Gonzales GF (2006) *Lepidium meyenii* (Maca) reversed the lead acetate induced-damage on reproductive function in male rats. *Food Chem Toxicol* 44:1114–1122.

Saito K, O'Donnell L, McLachlan RI, Robertson DM (2000) Spermiation failure is a major contributor to early spermatogenic suppression caused by hormone withdrawal in adult rats. *Endocrinology* 141:2779–2785.

Sonmez M, Turk G, Yuce A (2005) The effect of ascorbic acid supplementation on sperm quality, lipid peroxidation and testosterone levels of male Wistar rats. *Theriogenology* 63:2063–2072.

Takahashi O, Oishi S (2003) Testicular toxicity of dietarily or parenterally administered bisphenol A in rats and mice. *Food Chem Toxicol* 41:1035–1044.

Valerio LG, Jr, Gonzales GF (2005) Toxicological aspects of the South American herbs cat's claw (*Uncaria tomentosa*) and Maca (*Lepidium meyenii*): a critical synopsis. *Toxicol Rev* 24:11–35.

Zini A, Schlegel PN (1997) Identification and characterization of antioxidant enzyme mRNAs in the rat epididymis. *Int J Androl* 20:86–91.