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Tropaeolum tuberosum (Mashua) reduces testicular function: effect of different treatment times

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Summary

Tropaeolum tuberosum Ruiz & Pavon, along with other several species, is an edible-tuber crop that grows in the Andean region. Folk medicine describes the use of mashua to reduce reproductive function in men. The present study aimed to evaluate the effect of mashua (1 g kg^{-1}) on sperm production in rats during 7, 12, 21 and 42 days of treatment. The following parameters were assessed: reproductive organ weights, spermatid count and daily sperm production (DSP), sperm count in epididymis and sperm transit and serum testosterone levels. Freeze-dried extract of mashua had 3.7 g 100 g^{-1} of benzyl glucosinolate. Mashua-treated rats showed a reduction in testicular spermatid number and DSP from day 12 to day 42; meanwhile, the effect of mashua was noted in epididymal sperm count after 12 and 42 days of treatment. In addition, epididymal sperm transit time was delayed at day 7 and it was accelerated on days 12 and 21 of treatment. No differences in serum testosterone levels were found between rats treated with vehicle and mashua after 42 days of treatment. Finally, mashua reduces testicular function after one spermatogenic cycle by reducing spermatid and sperm number, DSP and epididymal sperm transit time.

Introduction

Tropaeolum tuberosum Ruiz & Pavon, along with other several species, is an edible-tuber crop that grows in the Andean region (Grau *et al.*, 2003). *Tropaeolum tuberosum* belongs to the Tropeolaceae family and it is known by its Spanish and commercial name: mashua (Grau *et al.*, 2003). In the Andes, mashua is traditionally used for its nutritional and medicinal properties including beneficial effects on liver and kidney (Hodge, 1946; Oblitas, 1969) and to cure prostate and urinary disorders (Salcedo, 1986; Brack, 1999). In addition, folk medicine describes the use of mashua to reduce reproductive function in men (Hodge, 1951; Leon, 1967; Oblitas, 1969; Brack, 1999).

To our knowledge, little is known about the effect of mashua on male reproductive parameters. In fact, the only scientific evidence reported showed that mashua has antireproductive effects on male rats by reducing serum levels of testosterone and dihydrotestosterone; however, no effect was observed in the capability to impregnate female rats. In this study, mashua was mixed in the food, for such reason the dose administered was not controlled (Johns *et al.*, 1982).

The present study aimed to evaluate the effect of the oral administration of an aqueous extract of mashua on sperm production in male rats during different times of administration. For this purpose, rats treated with vehicle and mashua (1 g kg^{-1}) were orally administered for 7, 12, 21 and 42 days and the following parameters were assessed: reproductive organ weights, spermatid count and daily sperm production (DSP), sperm count in epididymis, sperm transit and serum testosterone levels at day 42 of treatment.

Material and methods

Animals

Forty-eight male rats from the Holtzman strain (three-months old) were obtained from the animal house of the Universidad Peruana Cayetano Heredia (Lima, Peru). Rats were housed at six per cage and were maintained under controlled conditions at 22 °C with a 12 : 12 h light/dark cycle in the animal house at the Universidad Peruana Cayetano Heredia. Rats were provided with food and water *ad libitum*.

Experimental protocol

Rats were divided randomly into two groups (24 rats each) according to treatments: Control (vehicle) and mashua. Each group was subdivided into four additional subgroups (six rats per group) according to the time of treatment: 7, 12, 21 and 42 days. Animals were treated according to the standards of the National Institute of Health for the care and use of laboratory animals (National Research Council, 1996). All experiments were approved by the Institutional Review Board at the Universidad Peruana Cayetano Heredia.

Preparation of the aqueous extract *Tropaeolum tuberosum* (mashua)

Tubers of *Tropaeolum tuberosum* were obtained from Cerro de Pasco, Peru at 4340 m altitude. For the present study, the freezed-dried extract was prepared as follows: First, 500 g of tubers were cut into pieces, placed in a container with 1500 ml of water and boiled for 60 min. Next, the preparation was cooled, filtered and freeze-dried. One gram of tubers of mashua produced 0.10 g of freeze-dried aqueous extract. The freeze-dried mashua extracts were further diluted to obtain a dose equivalent to 1 g raw material per kg BW in 1 ml of vehicle. These solutions were placed in small vials and kept in a refrigerator at 4 °C until use.

Quantification of benzyl glucosinolate in mashua tubers

Previous studies demonstrated that the major secondary metabolite presented in mashua is p-methoxybenzylglucosinolate (Johns *et al.*, 1982). It has been shown that glucosinolates have anti-proliferative and proapoptotic properties (Gonzales *et al.*, 2005; Rubio *et al.*, 2006). For these reasons, the glucosinolate content was measured by high-performance liquid chromatography (HPLC) in freeze-dried mashua extract. For this purpose, 1 g of freeze-dried mashua extract was diluted in 45 ml of 70% ethanol (JT Baker, Phillipsburg, NJ, USA) and stirred for 30 min at 40 °C. Then, the solution was centrifuged at 1000 ***g*** for 10 min and decanted. This procedure was repeated and the combined extractions were diluted to a final volume of 100 ml with 70% ethanol. A standard of benzyl glucosinolate and glucotropaeolin (30 μg ml^{-1}), was also diluted with 70% ethanol. All samples were filtered through a membrane filter (0.45 μm) and analysed using an automatic Hewlett Packard HPLC series 1100 (Hewlett Packard, Waldbronn, Germany) with an RP-C (18) column at 235 nm wavelength. Sample injection volume was 100 μl. The mobile phase consisted of a mixture of 1006 mg tetraoctylammonium bromide (Fluka, Buchs, Switzerland) in 600 ml of methanol (Fisher Scientific, Fair Lawn, NJ, USA) and 1137 mg of disodium hydrogen phosphate anhydrous (Mallinckrodt-Baker, Edo de Mexico, Mexico) in 400 ml of water, adjusted to pH 7.0 with phosphoric acid (Mallinckrodt-Baker, Edo de Mexico, Mexico) (flow rate 1.0 ml min^{-1}). The running programme consisted of a constant flow of mobile phase and a column temperature of 30 °C. The quantification was carried out by comparing the peak area of the samples with the mean peak area of the standard.

Body and organ weights

Rats were weighed at the beginning and 24 h after last treatment (initial and final body weights, respectively). After the animals were killed (24 h after last treatment), selected organs (testes, epididymis, seminal vesicles and ventral prostate) were carefully dissected out, cleaned of adhering connective tissue and accurately weighed.

Daily sperm production and sperm transit

Testes were homogenised in 10 ml of 0.9% saline–0.05% (v/v) Triton X-100 solution for 1 min by a homogeniser (Takahashi & Oishi, 2003). After ten times dilution, the number of homogenisation resistant elongated spermatid nuclei per testis was determined with a haemocytometer. Counts of the four chambers of the haemocytometer were averaged. Daily sperm production (DSP) and its efficiency (DSP per g testis) were determined by division of the elongated spermatid count per testis and spermatids per g 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 epididymal sperm number by the daily sperm production (Dalsenter *et al.*, 2003).

Epididymal sperm count

Homogenisation-resistant epididymal sperm from non-perfused rats were counted as described previously

(Gonzales *et al.*, 2006). Caput and corpus epididymis were cut and homogenised separately to the cauda epididymis. Homogenisation was performed in 5 ml saline (NaCl 0.9%). Homogenates were kept refrigerated at 4 °C for 24 h to allow spermatozoa to be released from the walls. Then, 5 ml of eosin (2%) was added and vortexed. One millilitre of this mixture was diluted with 2 ml eosin (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 $\times 10^6$ per caput per corpus or cauda). Data are referred as sperm per caput per corpus or cauda epididymidis.

Determination of serum testosterone levels

Serum testosterone levels were determined using RIA with 125I-testosterone as the radioactive marker. The assay was performed using a commercial kit (Diagnostic Products Co., Los Angeles, CA, USA). All samples were run in the same assay period. The within-assay variation was 5.5% and sensitivity was 4.0 ng dl^{-1}.

Statistical analyses

Data were analysed using the statistical package STATA (v. 8.0) for PC (Stata Corporation, College Station, TX). Data are presented as mean ± standard error of the mean (SEM). Barlett test was performed to determine the homogeneity of variances. When 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. A value of $P < 0.05$ was considered to be statistically significant. Mann–Whitney U nonparametric test was used when variances were not homogeneous.

Results

Body and organ weights

Rats treated with vehicle (control group) and mashua (1 g kg^{-1}) showed increased body weight at the end of the experiments. At day 21, control group showed a greater increase in body weight when compared with the mashua group ($P < 0.05$). Regarding reproductive organ weights, only rats treated with mashua during 12 days reported a reduction in epididymal weight ($P < 0.05$) (Table 1).

Spermatid count and daily sperm production (DSP)

Figure 1 shows the effect of oral administration of mashua on spermatid (Fig. 1a) and DSP (Fig. 1b). Rats treated with mashua showed a reduction in spermatid count and DSP from day 12 to day 42 when compared with control groups ($P < 0.05$).

Epididymal sperm count

Sperm counts in epididymis are shown in Fig. 2. Regarding the epididymal sperm count, rats treated with mashua showed lower values than control group at days 12 and 42 ($P < 0.05$). No differences were observed in epididymal sperm count at day 7 and 21 between mashua and control groups.

Sperm transit

Figure 3 shows the effect of mashua on sperm transit in rats. At day 7, a reduction was observed in rats treated with mashua when compared with the control group ($P < 0.05$). However, an increase in sperm transit was observed at days 12 and 21 in the mashua group compared

Table 1 Body and organ weights (in grams) in rats treated with *Tropaeolum tuberosum* (Mashua) for 7, 12, 21 and 42 days

	Increase in body weight	Left testis	Left epididymis	Seminal vesicle	Prostate
7 days					
Control	12.25 ± 6.72	1.76 ± 0.05	0.62 ± 0.02	1.28 ± 0.11	0.33 ± 0.02
Mashua	19.00 ± 2.07	1.63 ± 0.15	0.56 ± 0.04	1.24 ± 0.11	0.27 ± 0.03
12 days					
Control	21.33 ± 7.65	1.75 ± 0.07	0.62 ± 0.02	1.11 ± 0.14	0.26 ± 0.02
Mashua	10.60 ± 2.36	1.64 ± 0.06	0.51 ± 0.02*	1.18 ± 0.06	0.29 ± 0.03
21 days					
Control	69.50 ± 3.15	1.96 ± 0.22	0.55 ± 0.02	1.19 ± 0.03	0.38 ± 0.01
Mashua	56.00 ± 4.50	1.69 ± 0.07	0.52 ± 0.02	0.98 ± 0.08	0.37 ± 0.02
42 days					
Control	54.20 ± 16.19	1.52 ± 0.15	0.50 ± 0.05	0.99 ± 0.12	0.34 ± 0.06
Mashua	86.75 ± 11.81	1.59 ± 0.04	0.50 ± 0.02	1.11 ± 0.13	0.38 ± 0.03

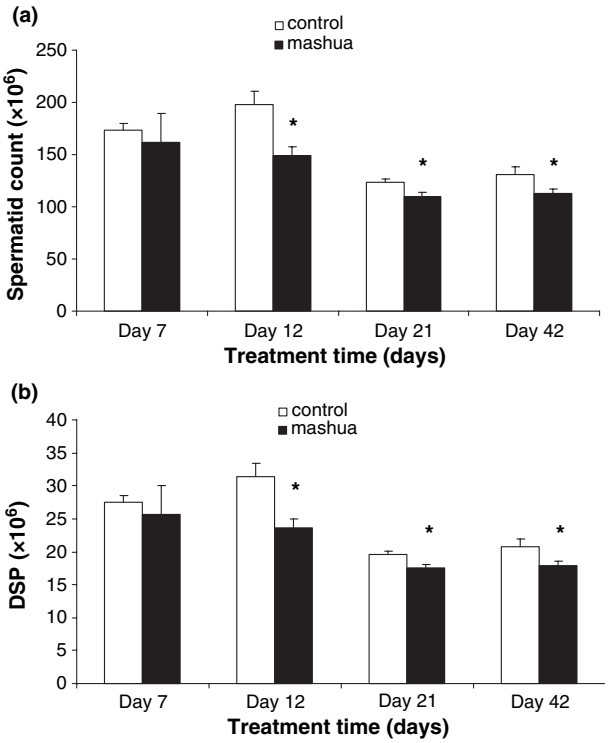

Fig. 1 Effect of mashua (1 g kg^{-1}) on spermatid count (a) and daily sperm production (DSP, b) in adult rats. Data are expressed as mean ± SEM.

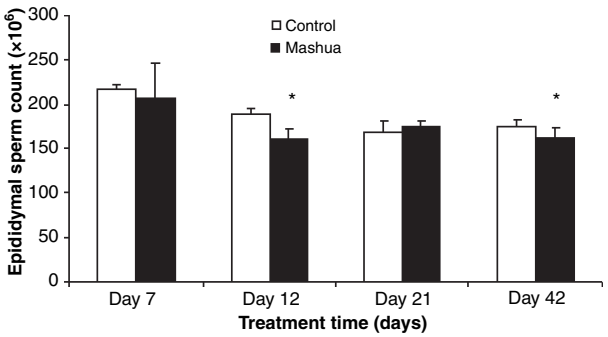

Fig. 2 Effect of mashua (1 g kg^{-1}) on epididymal sperm count in adult rats. Data are expressed as mean ± SEM.

with the control group ($P < 0.05$). The increase observed after 42 days of treatment was not statistically significant.

Serum testosterone levels

Mann–Whitney *U* nonparametric test showed no significant differences in testosterone levels between control and mashua-treated groups after 42 days of treatment [49.02 (22.25–214.64) ng dl^{-1} versus 116.05 (14.87–630.40) ng dl^{-1}, median (interquartile range); P : NS].

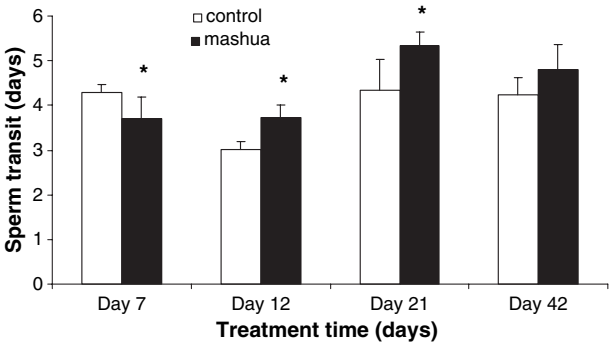

Fig. 3 Effect of mashua (1 g kg^{-1}) on sperm transit in adult rats. Data are expressed as mean ± SEM.

Benzyl glucosinolate content

High-performance liquid chromatography analysis of benzyl glucosinolates content in freeze-dried mashua extract revealed that this extract had 3.7 g 100 g^{-1} of benzylglucosinolates. After correction per 100 g of mashua tubers, the quantity of benzyl glucosinolates was 0.37 g 100 g^{-1} mashua tubers.

Discussion

The aim of the present study was to evaluate the effect of mashua on testicular function at different times of administration. As mentioned above, there is only one previous report about the anti-reproductive effect of mashua where rats were fed a diet containing the tuber (Johns *et al.*, 1982). In this previous study, experimental and control rats did not show any differences in impregnating females, although rats receiving mashua had a diminished testosterone/dihydrotestosterone ratio. However, the doses administered were not controlled because the tubers were mixed with the food and this may be a limitation for the final results presented (Johns *et al.*, 1982).

The duration of the seminiferous cycle in rats is 12.5 days (Aslam *et al.*, 1999). Previous studies demonstrated that the restoration of advanced spermatids (steps 17–19) in rats occurred 42 days after termination of treatment with GnRH antagonist (Hikim & Swerdloff, 1994). In the present study, mashua effect on testicular function was observed after 12 days of treatment where experimental rats showed a reduction in spermatid number and DSP when compared with control rats. For these reasons, it is suggested that the effect of mashua may be related to a reduction in late spermatid maturation in testis. Moreover, mashua treatment diminished epididymal sperm number after 12 and 42 days of treatment, supporting the fact that mashua may alter spermatid maturation and/or

release from testicular seminiferous tubules. The effect of mashua on epididymal sperm transit showed differences according to the time of treatment. For instance, sperm transit rate was reduced in rats treated with mashua for 7 days; meanwhile, rats treated for 12 and 21 days showed an increased sperm transit rate through the epididymis. In fact, previous studies demonstrated that epididymal sperm transit may play a role in the process of sperm maturation, and an alteration to the transit can negatively affect this process resulting in a reduction of epididymal sperm number (Fernandez *et al.*, 2008). Animals treated with mashua showed an acceleration of epididymal sperm transit after 12 and 21 days of treatment and consequently a decrease in epididymal sperm number. The latter makes possible to suggest that an effect of mashua in epididymis should not be discarded.

Testis, epididymis and particularly seminal vesicle and prostate are androgen dependent organs (Almenara *et al.*, 2001; Gonzales *et al.*, 2001; Nishino *et al.*, 2004); so it is possible to suggest that the effect of mashua on reproductive organ weights should not be related to any alteration in androgen levels. In fact, it is known that adrenergic, cholinergic, and nonadrenergic noncholinergic systems are related to vasoactivity within the testis and sperm transport in the epididymis (Kempinas *et al.*, 1998a,b) and it has been demonstrated that the administration of diethylstilbestrol and guanethidine sulphate accelerates and delays epididymal sperm transit time, respectively (Fernandez *et al.*, 2008). Additional research is needed to determine the mechanism related to the anti-reproductive effect of mashua tubers.

The compounds in mashua related to its anti-reproductive effect have not been elucidated yet. However, it was suggested that the effect of mashua on reproductive function may be due to its content of p-methoxybenzyl-glucosinolate as its major secondary metabolite (Johns *et al.*, 1982). Previously, it has been demonstrated that glucosinolates after ingestion by humans or rats are metabolised to isothiocyanates by gut mirosinase enzyme (Shapiro *et al.*, 1998; Rouzaud *et al.*, 2003). As mentioned above, these compounds have antiproliferative and pro-apoptotic properties (Gonzales *et al.*, 2005; Rubio *et al.*, 2006). HPLC results showed that freeze-dried extract of mashua had a high content of benzyl glucosinolate (3.7 g 100 g^{-1}). The content of benzyl glucosinolates in mashua tubers is higher than in other plants, i.e. *Lepidium meyenii* (Maca) (Gonzales *et al.*, 2008). *Lepidium meyenii* has beneficial effects on male reproductive system, so it is suggested that a higher content of benzyl glucosinolates in mashua could be related to its effect on male reproductive system. Then, it is probable that glucosinolates in mashua tubers may be related to their anti-reproductive effects. Other compounds present in mashua with anti-reproductive effects have not been elucidated yet. More studies are needed to clarify this issue.

To sum up, the present study demonstrated that mashua reduced testicular function. The effect of mashua could be related to its capacity to reduce the number of intratesticular spermatids and to accelerate epididymal sperm transit.

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