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EFFECT OF TROPAEOLUM TUBEROSUM "MASHUA" (TROPAEOLACEAE) ON GENE EXPRESSION RELATED TO SPERMATOGENESIS IN MOUSE

EFECTO DE TROPAEOLUM TUBEROSUM O "MASHUA" (TROPAEOLACEAE) SOBRE LA EXPRESIÓN GÉNICA RELACIONADA A ESPERMATOGÉNESIS EN RATÓN

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ABSTRACT

Introduction: Tropaeolum tuberosum, known as mashua is an Andean tuber that has both economic and nutritional value for low-income populations. It is believed that it affects male fertility because Andean men associate it with impotence and decreased fertilizing capacity. Studies done on rats fed mashua showed that there was a 45% decrease in the testosterone/dihydrotestosterone ratio. The effect of this plant on reproduction is related to its content of isothiocyanates, compounds that bind covalently to proteins, which may be directly or indirectly involved in the spermatogenic process. The purpose of this research was to evaluate the effect of the aqueous extract of mashua on spermatogenesis and reproductive physiology of mice. **Methods:** The in vivo morphofunctional parameters of mouse sperm (spermatogram) were evaluated and the expression of: Cytochrome P450 17α-hydroxylase/17,20-lyase, protein that regulates acute steroidogenesis, cyclin and protamine, related to spermatogenesis, was quantified. **Results:** At 7, 14 and 21 days of dosing, the sperm count is affected, as well as their progressive motility (PM); on the other hand, a delay in their maturation was observed. Regarding gene expression, no significant differences were found between the expression of the two genes studied (cytochrome P450 17α-hydroxylase/17,20-lyase, cyclin). **Conclusion:** The effect of mashua does not occur at the level of the expression of the genes involved in spermatogenesis, but at the level of its functions as a protein.

Keywords: Genic expression; Mashua; Mouse; Spermatogenesis; Tropaeolum tuberosum; Medicinal Plants. (Source: MESH-NLM)

RESUMEN

Introducción: Tropaeolum tuberosum, conocido como mashua, es un tubérculo andino que tiene un valor tanto económico como nutritivo para las poblaciones de pocos recursos. Se cree que afecta la fertilidad masculina, porque los hombres andinos lo relacionan con impotencia y disminución de la capacidad fecundante. Estudios hechos en ratas que se alimentaron con mashua demostraron que hubo un 45% de decrecimiento de la tasa testosterona/dihidrotestosterona. El efecto de esta planta en la reproducción está relacionada a su contenido de isotiocianatos, compuestos que se unen covalentemente a las proteínas, las cuales pueden estar directa o indirectamente involucradas en el proceso espermatogénico. El propósito de esta investigación fue evaluar el efecto del extracto acuoso de la mashua sobre la espermatogénesis y la fisiología reproductiva de ratones. **Métodos:** Se evaluaron los parámetros morfofuncionales in vivo de espermios de ratones (espermatograma) y se cuantificó la expresión de: Cytochrome P450 17α-hydroxylase/17,20-lyase, proteína reguladora de esteroidogenesis aguda, ciclina y protamina, relacionados a la espermatogénesis. **Resultados:** : A los 7, 14 y 21 días de dosificación, se vio afectado el conteo de espermatozoides, así como su motilidad progresiva (MP); por otra parte, se observó un retardo en la maduración de los mismos. En cuanto a la expresión génica, no se encontró diferencias significativas entre la expresión de los dos genes estudiados (cytochrome P450 17α-hydroxylase/17,20-lyase, ciclina). **Conclusión:** El efecto de la mashua no se da a nivel de la expresión de los genes involucrados en la espermatogénesis, sino a nivel de sus funciones como proteína.

Palabras clave: Espermatogénesis; Expresión Génica; Mashua; Ratón; Tropaeolum tuberosum; Plantas medicinales. (Fuente: DeCS- BIREME)

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INTRODUCTION

"Mashua" (Tropaoelum tuberosum) is a tuber of ancient and native use with an economic, nutritional and medicinal value, used by the Andean highlanders [1]. Components of "mashua" with properties such as antibiotics, nematicides, diuretics, and insecticides have been isolated, in addition to effects found on testosterone in males and estrogens in females. Many medicinal uses of "mashua" are related to the presence of isothiocyanates and glucosinolates [2]. In recent years, isothiocyanates (organosulfur compounds) have been shown to induce apoptosis in various cancer cell lines in mice [3] and delay cell cycle progression[4].

These characteristics have been related to covalent binding to cellular proteins [5] and inhibition of enzymes involved in detoxifying carcinogens, among other mechanisms that have not yet been fully described [6]. Furthermore, studies conducted on rats fed "mashua" diets showed a 45% decrease in testosterone/dihydrotestosterone levels[7].

There are no previous reports on the effects of "mashua" on gene expression related to spermatogenesis. Therefore, in the present study, the gene expression of some important proteins in the process of spermatogenesis was evaluated; these were: Cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17), which is crucial for the biosynthesis of cortisol and sex steroids [8], and thus taken as a marker for testosterone biosynthesis; the protein regulating acute steroidogenesis is a gene regulated by androgen receptors [9]; Cyclin, a protein essential for cell division; and Protamine in the process of spermatogenesis. The objective of this study was to evaluate the effect of the aqueous extract of T. tuberosum on the basic morphofunctional parameters of sperm (motility, morphology, and concentration) and to relate them to the effect on the expression of some genes involved in spermatogenesis.

METHODS

Study Design and Study Area

Experimental preclinical study of cases and controls in the field of experimental biology.

Population and Sample

The sample consisted of 20 male mice (Mus musculus) of the Swiss Rockefeller albino strain, 6-7 weeks old, obtained from the animal facility of the National Institute of Health in Lima, Peru. The experimental group (cases) consisted of 10 mice, to whom an aqueous extract was administered orally via nasogastric tube No.18 (Fisher Scientific, Pittsburgh, PA, USA) at a concentration of 1000 mg/kg of body weight for 21 days. The specimens were kept in the animal facility under conditions of 22-24 °C ambient temperature, with 14 hours of light and 10 hours of darkness, with free access to a pellet diet (Purina, Peru) and water ad libitum.

Plants

"Mashua" tubers were acquired from local markets (Huancayo, Peru). In the laboratory, the plants were certified by the Department of Botany of the Universidad Nacional Mayor de San Marcos (UNMSM).

Chemicals

Ethyl alcohol 70°, distilled water, PBS solution (Phosphate buffer saline - Sigma), DNA-freeTM Kit (Ambion), SYBR Green I, Trizol.

Extract Obtaining

The tuber was weighed, liquefied, and immediately macerated with 70° alcohol for 2 days, and then the liquid phase was separated from the solid part. The liquid part was taken to the oven to evaporate the alcohol, obtaining a paste that was considered a standard compound.

Experimental Design

The mice were divided into 4 treatment groups. One group served as the Negative Control (CN) and was treated with distilled water. The treatment groups (T7, T14, and T21) were administered the aqueous extract (20%) of T. tuberosum orally for 7, 14, and 21 days, respectively. The total weight (g) of the mice was recorded at the start of the treatment and on the evaluation day. After each treatment, the mice were euthanized by cervical dislocation, and samples of testicles and epididymis were obtained. One testicle and one epididymis were placed in Trizol inside a cryovial (100mg/ml) and stored at -196° C in liquid nitrogen until RNA extraction.

Spermatozoa were obtained from the second epididymis to measure sperm parameters (sperm concentration and motility) according to WHO (2010) guidelines. RNA extraction was performed using Trizol, following the protocol for RNA isolation from Invitrogen Cat. No. 15596-18. Once the RNA was obtained, contaminating DNA was removed using the DNA-freeTM Kit from Ambion, following the protocol described in Cat. No. AM 1906. From the DNA-free RNA, cDNA synthesis was performed using RT-PCR, as described in Cat. No. 18080-051. Quantification was done by real-time RT-PCR using the LightCycler® 2.0 system from Roche Applied Science, where the PCR product can be detected and measured by the fluorescent signal of SYBR Green I.

The principle is that SYBR Green I binds to the minor groove of the DNA double helix and intercalates in the DNA helix [10]. In solution, the dye exhibits very low fluorescence, but fluorescence (at 530 nm wavelength) is enhanced upon binding to DNA. Thus, during PCR, the increase in SYBR Green I fluorescence is directly proportional to the amount of double-stranded DNA generated, and this emission is detected by the optical filter of the LightCycler® 2.0 system.

Quantification was relative to the housekeeping gene, beta-actin, which is theoretically expressed constantly in the testicular tissue of mice and served as a reference point for measuring the expression of the target genes. This relative measurement is given by the ratio of the expression of the target genes to the expression of the housekeeping gene in the treated groups at different time periods with the aqueous extract of "mashua" compared to the control group.

Statistical Analysis

Homoscedasticity (equal variances) of the data was tested using Levene's test. To check if the data obtained for the different variables being evaluated were normally distributed, the Shapiro-Wilk test was performed. When the variable being evaluated showed a normal distribution, the means were compared using t-Student test; otherwise, the Mann-Whitney U test was applied for non-parametric tests. The results were expressed as mean ± SE (standard error). The statistical program SPSS Ver 17.00 was used, considering the significance level of $p \leq 0.05$.

Ethical Aspects

The care and handling of the animals were carried out in accordance with the ethical guidelines of our institution and the National Research Council for the care and use of laboratory animals[11].

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RESULTS

Body Weight and Reproductive Organs

No significant differences were observed in the increase in body weight and the weight of the testicles, epididymis, and prostate ($p>0.05$) among the analyzed groups. The results are summarized in Table 1.

Table 1. Differences in initial and final body weights and reproductive organ weights (g).

Parameters	Control group	Mashua 7 days	Mashua 14 days	Mashua 21 days
DifWs	8.27 ± 1.500	6.39 ± 1.180	8.7 ± 1.630	9.02 ± 1.350
Dif Wtestis	0.100 ±.100	0.091 ± 0.116	0.107 ± 0.120	0.0987 ± 0.130
DifWepid	0.037 ± 0.067	0.033 ± 0.064	0.0382 ± 0.056	0.035 ± 0.071
Wprost	0.0078 ± 0.043	0.0072 ± 0.036	0.0073 ± 0.048	0.0071 ± 0.042

Control Group 0 % TT and Treatment Groups 20% TT. Media ± EE; analyzed by t-Student.
DifWs: body weight difference, DifWTestis: testis weight difference, DifWepid: epididymis weight difference, Wprost: prostate weight

Table 2. Sperm Motility and Concentration.

Seminal Parameters	Control Groups	Mashua 7 days	Mashua 14 days	Mashua 21 days
Mot A	38. 095 ± 2.057	34.976 ± 1.840	30.528 ± 1.936	25. 017 ± 3.156 *
Mot B	14.856 ± 2.132	15.978 ± 2.080	20.573 ± 2.418	19.702 ± 2.879
Mot C	9.473 ± 1.673	9.57 ± 1.880	7.661 ± 1.875	5.167 ± 1.561
Mot D	37.576 ± 2.298	39.475 ± 2.577	41.238 ± 2.577	39.001 ± 3.868
Concent Sps	25.06 ± 2.260	19.11 ± 2.120	18.555 ± 1.539	13.455 ± 2.622 *

Control Group 0 % TT and Treatment Groups 20% TT. Media ± EE; analyzed by t-Student.
Mot A: Motility type A, Mot B: Motility type B, Mot C: Motility type C, Mot D: Motility type D, Concent Sps: Sperm count.
*(p <0.05) ANOVA

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The results of gene expression quantification of two out of four genes (Cytochrome P450 17α-hydroxylase/17,20-lyase, Cyclin) showed no significant difference compared to the control group in relative quantification to Beta Actin expression (data not shown).

DISCUSSION

The present study aims to investigate the effects of a single dose of Tropaeolum tuberosum "mashua" on sperm quality and gene expression during mouse spermatogenesis, considering the antiandrogenic background of "mashua" [2,12]. Based on the results shown, there are no systemic toxic effects on body weight and reproductive organs.

The effect of the aqueous extract of "mashua" reduces sperm concentration in all three treatment groups compared to the control group, with statistical significance observed in the 21-day treatment group. In terms of motility, a decrease in progressive motility was also observed, with a higher incidence in the 21-day treated group, where a greater number of immature spermatozoa (presence of cytoplasmic droplets) was observed, possibly explaining the decrease in progressive motility.

These effects do not seem to be related to changes in the expression of genes involved in spermatogenesis, as no differences in gene expression were observed compared to the control group. This would indicate that the effect of "mashua" on reproductive physiology may rather have a post-transcriptional effect, where it may be affecting the proteins that play an important role in this process, possibly due to the action of organophosphate compounds like isothiocyanates present in "mashua," which have the ability to covalently bind to proteins and deactivate enzymatic activities [3,13-15].

However, further study is required in this regard, perhaps by expanding the range of genes to be studied. If some proteins are affected, there might be transcription factor-related proteins that could be influencing the expression of other genes directly or indirectly related to the spermatogenesis process. Thus, this could possibly explain the effect observed on sperm maturation in this study, possibly related to estrogen receptors at the epididymal level.
progressive motility.

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