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THE EFFECT OF THE AQUEOUS EXTRACT OF "MASHUA" TROPAEOLUM TUBEROSUM " ON SPERM QUALITY AND ITS IMPLICATION IN PREIMPLANTATION EMBRYONIC DEVELOPMENT. PRE-CLINICAL TEST

EFECTO DEL EXTRACTO ACUOSO DE "MASHUA" TROPAEOLUM TUBEROSUM "EN LA CALIDAD DEL ESPERMA Y SU IMPLICANCIA EN EL DESARROLLO PREIMPLANTACIONAL EMBIONARIO. PRUEBA PRE CLÍNICA

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ABSTRACT

Introduction: The "mashua," Tropaeolum tuberosum, is a plant species native to Peru. Investigations carried out have verified its effects on the fertility of mammals. **Objective:** To evaluate the biological action of the aqueous solution of T. tuberosum in the development of preimplantation embryos of Mus musculus and the reproductive capacity of male Mus musculus. **Methods:** Pre-clinical experimental study of cases and controls. The sample consisted of 32 mice. The case group made up 24 mice grouped in three groups, each one of 8 mice, to which aqueous extract was administered ad libitum, in a concentration of 50 g / Kg of body weight during 8, 16 and 35 days respectively, the control group integrated it 8 mice, who received only tap water. Each group of males was crossed with females of fertile age, the presence of a vaginal plug indicated day 0 of embryonic development. At 72 hours, the uterine horns and oviducts were perfused, and the degree of development, condition and preimplantation embryonic morphology of Mus musculus were evaluated until reaching the blastocyst stage. **Results:** Statistically significant differences were found between the case and control groups. Our results demonstrate that the administration of aqueous T. tuberosum to male mice alters the mouse's reproductive quality, affecting the embryo's ability to develop naturally until the blastocyst stage. **Conclusion:** The aqueous Tropaeolum tuberosum (mashua) extract affects preimplantation embryonic development and reproductive capacity in male Mus musculus.

Key words: Mashua; Tropaeolum; Fetal dominance; Preimplantational development (source: MeSH NLM).

RESUMEN

Introducción: La "mashua", Tropaeolum tuberosum, es una especie vegetal nativa del Perú. Investigaciones realizadas han comprobado sus efectos sobre la fertilidad de mamíferos. **Objetivo:** Evaluar la acción biológica de la solucion acuosa de T. tuberosum en el desarrollo de los embriones preimplantacionales de *Mus musculus* y la capacidad reproductiva de *Mus musculus* macho. **Métodos:** Estudio experimental preclínico de casos y controles. La muestra estuvo conformada por 32 ratones, el grupo casos lo conformó 24 ratones agrupados en tres grupos, cada uno de 8 ratones, a quienes se les administró extracto acuoso ad libitum, en una concentración de 50 g/Kg de peso corporal durante 8, 16 y 35 días respectivamente, el grupo control lo integró 8 ratones, quienes recibieron sólo agua de grifo. Cada grupo de machos se cruzaron con hembras de edad fértil, la presencia de tapón vaginal nos indicó el día 0 de desarrollo embrionario. A las 72 h se perfusionaron los cuernos uterinos y oviductos y se evaluó el grado de desarrollo, condición y morfología embrionaria preimplantacional de *Mus musculus* hasta alcanzar el estadío blastocisto. **Resultados:** Se encontraron diferencias estadísticamente significativas entre los grupos de casos y control. Nuestros resultados demuestran que la administración de solución acuosa de *T. tuberosum* a ratones machos, altera la calidad reproductiva del ratón afectando la capacidad del embrión para desarrollarse normalmente hasta el estadio de blastocisto. **Conclusión:** El extracto acuoso de *Tropaeolum tuberosum* (mashua) afecta el desarrollo embrionario preimplantacional y la capacidad reproductiva en *Mus musculus* macho.

Palabras clave: Mashua; Tropaeolum; Dominancia fetal; Desarrollo preimplantacional (fuente: DeCS BIREME).

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INTRODUCTION

Mashua (Tropaeolum tuberosum), belongs to the group of tubers that grow in the Andean region, and has been used since ancient times as a source of food and medicine by the natives. Some of its biological properties would be related to certain substances such as compounds phenolics, glucosinolates, isothiocyanates and anthocyanins[1,2,3]. Johns et al.[4] suggested an anti-reproductive effect of Mashua in male rats due to a 45% drop in testosterone/dihydrotestosterone in blood samples, without show no effect on the ability to get female rats pregnant. However, in another study, no differences were observed in serum testosterone levels between rats treated with a vehicle or Mashua for 42 days. Still, a reduction in the number of testicular spermatids and daily sperm production was observed from day 12 to day 42 of treatment. Due to this, Mashua's hypothetical effects would be related to the alteration of testicular function by reducing the number, daily production, and affecting the transit time of epididymal sperm[5].

In the study by Leiva-Revilla et al.[6], a decrease in daily sperm production and the number of epididymal and deferent sperm, as well as a decrease in sperm motility, was observed in male rats treated with Mashua; On the other hand, Mashua increased the percentage of abnormal sperm morphology and the rate of epididymal sperm transit. These effects in male rats treated with Mashua were reversible after 24 days of recovery time. In another study, mice treated with a hydroalcoholic extract of Mashua showed a decrease in progressive sperm motility and sperm count in the caudal epididymis. An increase in immobile sperm count after 21 days of treatment[7]. Mashua's anti-reproductive effects would be related to the high content of p-methoxybenzyl-glucosinolate, its transformation into isothiocyanates by the intestinal myrosinase enzyme, and its antiproliferative and proapoptotic properties[8,9,5].

METHODS

Design and study area

Study pre-clinical experimental case and controls, area of experimental biology.

Population and sample

24 male mice aged 6-8 weeks of age, of the Swiss albino strain, were kept in a room with controlled temperature (22-25 ° C) with light and dark cycles of 12 h. Water and mouse food was provided.

Variables

Independent variables: Sex (male, female), treatment of male mice, pregnant females on day 1 and day 4, weight of pregnant females on day 1 and day 4, presence of corpus luteum, number of embryos, embryonic grade, embryo stage.

Dependent variables: reproductive quality of the male mouse, preimplantation embryonic development.

A data collection sheet was made with all the variables of the research study where the findings were recorded.

Procedures

1. Preparation of aqueous extract: Mashua was obtained from commercial sources (Lima, Peru) and processed into a paste at the Institute of Natural Resources, Faculty of Pharmacology, at the Universidad Nacional Mayor de San Marcos.

   The paste was dissolved in distilled water (50 mg of paste per ml) and stored at 4 ° C until use.

2. Treatment: Male mice of fertile age were randomly selected and placed in individual cages for bioassays. Three experimental groups of 8 mice each were established that drank ad libitum for 8, 16 and 35 days an aqueous extract of mashua at a 50 mg/ml concentration. The control group drank tap water.

   A fresh supply of extract was replaced every 24 h, and volumes ingested were recorded every day. Nulliparous females were individually caged with male mice overnight. The next morning, the females with a vaginal plug were considered postcoital females, on day 1 of pregnancy. Only 7 mice from the control group were pregnant, and 7 from treatment day 35, from treatment days 8 and 16, 8 mice were crossed each day.

3. Early differentiation and scaling of preimplantation mouse embryos After treatment, male mice were caged with nulliparous female mice. The presence of a vaginal plug was observed in the females to determine if copulation occurred. The pregnant females were kept for up to 4 days. On day 4 of pregnancy, the animals were sacrificed by cervical dislocation. The oviducts and uterine horns were

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isolated in an embryological plate and perfused with a Phosphate Buffer Saline (PBS) (pH 7), obtaining the embryos for later evaluation.

Three grading categories were established to classify the embryos: 1) Grade I: excellent or good; 2): Grade II: regular; 3) Grade III: poor; 4) Degenerate[10]. The grading system is as follows: 1) Grade I: embryos with a symmetrical mass, blastomeres uniform in size, color and density with at least 85% of the intact cellular material, viable embryonic mass; 2) Grade-II: embryos with a moderate irregularity in their shape, size, color and density of individual cells, at least 50% of the entire embryo; 3) Grade III: embryos with more significant irregularity in shape, size, color and density of individual cells in at least 25% of the entire embryo; 4) Degenerated: dead or severely deteriorated embryos or oocytes, oocytes or non-viable embryos of a cell[11].

Statistical analysis

Statistical software SPSS V25.0 was used. ANOVA analyzed differences in weight. The stages of development and the degrees of embryo quality were analyzed using the chi-square test.

Ethical aspects

The care and handling of the animals were carried out in accordance with the ethical guidelines of our institution.

RESULTS

There was a difference in maternal weight between one group (Trat35D) and the other experimental group treatments on day 1 of pregnancy. This event disappeared on day 4 of pregnancy without showing any statistical difference between the experimental groups. There were no statistical differences between the treatment groups to the number of embryos and corpora lutea (Table 1).

Table 1. Reproductive parameters of female mice mated with male mice treated with an aqueous solution of T. tuberosum.

Treatment of male mice	Control group	T-8d	Case group T-16d	T-35d
Pregnant females	7	8	8	7
Females weight day 1	26.50 ± 0.56	25.11 ± 0.47	24.94 ± 1.00	21.94 ± 0.69*
Females weight day 4	25.96 ± 1.02	26.05 ± 0.44	27.53 ± 1.09	24.23 ± 0.82
Embryos	9.86 ± 1.22	9.00 ± 0.65	8.37 ± 1.49	10.00 ± 0.72
Corpus luteum	11.29 ± 1.06	9.63 ± 0.53	10.63 ± 0.89	10.57 ± 0.53

* Statistical difference is significant - 0.05 ANOVA. Values are expressed as means ± SE.

Control group; Case group (experimental): T-8d: male mice treated with aqueous solution of T. tuberosum for 8 days; T-16d: male mice treated with aqueous solution of T. tuberosum for 16 days; T-35d: male mice treated with aqueous solution of T. tuberosum for 35 days.

Evaluation of early differentiation in preimplantation mouse embryos showed that the control group produced 78.57% blastocysts, 20% compacted morula, and 1.43% embryos in other late development stages. After 8 days of ingestion, male mice produced 80.56% of blastocysts, 4.17% of compacted morula, and 15.28% of embryos in other late stages of development. The 16-day cases produced 71.79% of the blastocysts, 2.56% of the compacted morula, and 23.08% of the embryos in other late stages of development. Finally, the 35-day-old cases produced 80% of the blastocysts, 1.43% of the compacted morula, and 18.57% of the embryos in other late stages of development (Table 2).

Table 2. Embryonic development stage related to sperm quality in mice treated with aqueous solution of T. tuberosum.

Treatment of male mice	Control group	T-8d*	Case group T-16d**	T-35d**
Pregnant females	7	8	8	7
Embryonic stage	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Blastocyst	55 (78.57)	58 (80.56)	56 (71.79)	56 (80.00)
Morula	14 (20.00)	5 (6.94)	2 (2.56)	1 (1.43)
1 to 8 cells	1 (1.43)	3 (4.17)	2 (2.56)	0 (0.009
Indeterminate	0 (0.00)	6 (8.33)	18 (23.08)	13 (18.57)

N: number of embryos, (%): percentage of embryos. Statistically significant differences: * p = 0.0026; ** p <0.0001 (chi-square)

Control group; Case group (experimental): T-8d: male mice treated with aqueous solution of T. tuberosum for 8 days; T-16d: male mice treated with aqueous solution of T. tuberosum for 16 days; T-35d: male mice treated with aqueous solution of T. tuberosum for 35 days.

These results indicate a progressive increase in the number of retarded embryos that did not pass the compacted morula stage relative to the progenitors of male mice treated with aqueous mashua extract. The differences between the control and the experimental groups were statistically significant.

The evaluation of the embryo quality showed in the control group that 97.14% corresponded to Grade 1-Grade 2 (embryos with the best score: better appearance, blastomeres of equal size and with little or no cytoplasm fragmentation), while 0% corresponded to Grade 3, and 2.86% to degenerated embryos (embryos with the worst score: irregular appearance, blastomeres of unequal size and with fragmentation of cytoplasm).

The parents of male mice that drank the aqueous extract of Mashua for 8 days produced 79.12% of embryos corresponding to Grade 1-Grade 2, while 8.33% were Grade 3 and 12.5% were degenerate embryos. The progenitors of male mice that drank the Mashua aqueous extract for 16 days produced 73.07% of embryos corresponding to Grade 1-Grade 2, while 2.56% were Grade 3 and 24.36% were degenerate embryos. The progenitors of male mice that drank the aqueous Mashua extract for 35 days produced 67.24% of embryos corresponding to Grade 1-Grade 2, while 14.29% were Grade 3 and 18.57% were degenerate embryos (Table 3). These results indicate a progressive decrease in the embryo's quality of male mice's progenitors treated with aqueous mashua extract. The differences between the control and the experimental groups were statistically significant.

Table 3. Classification of embryos related to sperm quality in mice treated with aqueous solution of T. tuberosum.

Treatment of male mice	Control group	T-8d*	Case group T-16d**	T-35d**
Pregnant females	7	8	8	7
Embryonic grade	**n (%)**	**n (%)**	**n (%)**	**n (%)**
I	42 (60)	0 (0.00)	17 (21.79)	8 (11.43)
II	26 (37.14)	57 (79.17)	40 (51.28)	39 (55.71)
III	0 (0.00)	6 (8.33)	2 (2.56)	10 (14.29)
Degenerate	2 (2.86)	9 (12.5)	19 (24.37)	13 (18.57)

N: number of embryos, (%): percentage of embryos. Statistically significant differences: * p <0.0001 (chi-square)

Control group; Case group (experimental): T-8d: male mice treated with aqueous solution of T. tuberosum for 8 days; T-16d: male mice treated with aqueous solution of T. tuberosum for 16 days; T-35d: male mice treated with aqueous solution of T. tuberosum for 35 days.

These results indicate a progressive increase in the number of retarded embryos that did not pass the compacted morula stage with the progenitors of male mice treated with aqueous extract of mashua. The differences between the control and the experimental groups were statistically significant. The evaluation of the embryo quality showed in the control group that 97.14% corresponded to Grade 1-Grade 2 (embryos with the best score: better appearance, blastomeres of equal size and with little or no cytoplasm fragmentation), while 0% corresponded to Grade 3, and 2.86% to degenerated embryos (embryos with the worst score: irregular appearance, blastomeres of unequal size and with cytoplasmic fragmentation)

DISCUSSION

Three mechanisms can explain why the quality of sperm affects embryogenesis: 1) weak transcriptional activity in the male pronucleus, 2) abnormal calcium signaling leading to oocyte activation failure and pronuclear developmental abnormalities, and 3) sperm-derived aster abnormalities resulting in both improper pronucleus apposition[12-14] and losses in mitosis[15-17].

In previous research, aqueous Mashua extract was observed to affect head and flagellum morphology in sperm obtained from the epididymis in mice. In general, morphology alterations testify to abnormal genetic material in the sperm[18]. These results are similar to other studies suggesting a possible effect of mashua extract on secondary spermatocytes and round spermatids during spermatogenesis in mice[6,7]. Spermatogenesis is how immature germ cells undergo division, meiosis, and differentiation to give rise to haploid elongated spermatids[19]. When the development of germ cells is complete, mature spermatids enter the epididymis, where they acquire mobility and fertilization capacity of the ovum. In the endocrine and paracrine regulation of spermatogenesis, the production of testosterone by Leydig cells is essential due to its biological effects on the testes and spermatogenesis as a whole[20,21]. In spermiogenesis, the formation of the achromosome and the flagellum, nuclear rearrangement, chromatin condensation, DNA packaging, accumulation of mRNA in the nucleus, and sperm elimination cytoplasm occur[22-24]. Treatment with Mashua affects sperm morphology and produces sperm with an altered ability to produce normal preimplantation embryos. This fact would be related to some type of genetic alteration in the sperm.

Sperm quality is known to depend on chromatin condensation and the nuclear integrity of gametes[24,25]. On the other hand, high-quality

gametes must produce high-quality embryos, and both gamete genomes contribute to the embryonic genome[26,27]. Experimental studies have shown that the development of mammalian embryos depends on the integrity of the sperm DNA and the genetic quality in the sperm cells can cause arrest in early embryonic development[27-29]. If the integrity of the DNA is affected, a slow embryonic development occurs, there is a decreased potential to reach the blastocyst stage producing embryos with altered morphology and low scoring characteristics[30-32]. Poor quality embryo is closely related to sperm DNA quality and this relationship can be assessed in the embryo by taking into account cell number, morphology, size, and blastomere fragmentation[11,33,34]. In embryo fragmentation, the loss of portions of the cytoplasm is related to cytokinesis or imperfect apoptosis[15-17]. These events distort cell division planes or interfere with normal cell-to-cell contact, leading to abnormal compaction, cavitation, and cavitation—blastocyst formation[35]. In humans, maternally inherited mRNA controls the initial zygote divisions. The embryonic genome is inactive until the 4-cell stage. The expression of sperm-derived genes begins, so that paternal influence on the Embryonic development should be obvious until the 8 -cell stage[27,36,37]. The significant reduction in the number of embryos in the morula stage and a non-significant increase in the embryos in the indeterminate stage, as well as a decrease in the quality of the embryos, could be related to both the integrity of the affected DNA in the sperm and with the alteration of the genome activation in early embryos (4 to 8 cells), and the alteration of the centrosomal complex of the sperm cell that could affect mitosis in the embryo[14,27], leading to a delay in the development of the embryo.

Sperm DNA damage can occur due to defective chromatin condensation, alteration of DNA integrity, or oxidative stress resulting from reactive oxygen species (ROS)[5,38,39]. Likely, the sperm of mice exposed to bioactive substances from the aqueous extract of Mashua suffer some alteration during spermatogenesis, which would cause a decrease in their genetic quality that affects both gene expression in the male pronucleus as well as the mitotic apparatus. Mashua isothiocyanates (ITC) are bioactive compounds derived from glucosinolates[40,42]. It has been suggested that the action of ITC is related to the induction of DNA damage, the inhibition of DNA synthesis, the alteration of the chromatin packaging by histone acetylation, the activation of apoptosis, the generation of reactive species of oxygen (ROS), and oxidative stress, and The genetic repression of the androgen receptor (AR)[43-45]. Free radical elements such as ROS can affect the quality of sperm DNA[38,39]. ITC induces ROS production, and oxidative stress is associated with DNA damage. Regardless of ROS's origin, its presence can cause sperm DNA fragmentation, affecting both genomic integrity and embryonic development after fertilization[38,39,19]. ITC can be covalently bound to proteins, inactivating enzymatic activities[46,48]. On the other hand, the p-methoxybenzyl-glucosinolate present in Mashua shows antiproliferative and proapoptotic properties, respectively[8,9]. Both the actions of ITC and p-methoxybenzyl-glucosinolate affect mouse spermatogenesis, sperm production, sperm quality, and embryonic development that alter the male genome's activation centrosomal complex, and mitosis.

Our results show a group of embryos obtained from spermatozoa exposed to Mashua to reach the blastocyst stage that exhibits acceptable embryonic quality. In rats, treatment with benzyl isothiocyanate, a significant component in Tropaeolum tuberosum, showed a non-significant dose-dependent increase in early fetal resorptions before implantation, there were no significant differences in the number of implantation sites, and no there were significant differences in the number of fetal resorptions. This allowed to conclude that the action of this molecule did not change the pregnancy results during the pre and post-implantation periods in rats[49]. These facts could indicate that the developmental capacity of the mouse embryos would not be permanently and irreversibly affected by the Mashua treatment, and the capacity of the embryos to complete their development during the pregnancy period would not be affected.

Poor quality sperm produce poor quality embryos. This is evident in sperm exposed to mashua by increasing the number of retarded embryos that did not pass the compacted morula stage after fertilization. However, this effect would not be permanent and embryos' ability to complete their development until the end of pregnancy is possible.

Our results show that treatment with mashua produces a significant reduction in the number of embryos in the morula stage and a non-significant increase in embryos in the indeterminate stage, as well as a reduction in the quality and capacity of the embryos to develop. Usually until reaching the blastocyst stage.

The limitation of our research work is the sample since it is a preliminary work. A larger sample ensures a representative distribution of the population and is considered representative of the groups of people, objects, processes, etc., studied. Although, of course, the sample size is less relevant in qualitative research.

CONCLUSION

Significant in the number of embryos in the morula stage and a non-significant increase in the embryos in the indeterminate stage, as well as a reduction in the quality and capacity of the embryos to develop normally until reaching the blastocyst stage.

Poor quality sperm produce poor quality embryos. The sperm quality of mice exposed to the action of mashua decreases; Due to this, the number of delayed embryos that did not pass the compacted morula stage after fertilization increases. It is possible that this effect is not permanent and that the embryos maintain the capacity to complete their development until the end of pregnancy.

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