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EFFECT OF CONSUMPTION OF THE FRUIT EXTRACT OF "CAMU CAMU"ON THE INTEGRITY OF SPERM DNA OF MICE PRE-TREATED WITH A SINGLE DOSE OF CYCLOPHOSPHAMIDE

EFECTO DEL CONSUMO DEL EXTRACTO DEL FRUTO DE "CAMU CAMU" EN LA INTEGRIDAD DEL ADN ESPERMÁTICO DE RATONES PRETRATADOS CON DOSIS UNICA DE CICLOFOSFAMIDA

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ORIGINAL PAPER

ABSTRACT

Introduction: Myrciaria dubia known as "camu camu" is a fruit that grows in the Amazon and its main characteristic is its high content of vitamin C. Ascorbic acid has a protective role in spermatogenesis as it is a compound that has excellent reducing action. The purpose of this research was to evaluate in vivo the cytoprotective capacity of the aqueous extract of the fruit of Myrciaria dubia (Kunth) McVaugh "camu-camu" against the mutagenic damage produced by the antineoplastic drug cyclophosphamide (CP) on the male germ line. **Methods:** Mice (n= 60) were divided into five treatment groups: T1= negative control (without treatment); T2 ingested the aqueous extract (10mgkg-1), T3 ingested the aqueous extract (50mgkg-1), T4 ingested the aqueous extract (100mgkg-1); T5 is the positive control. All of them were injected with a single dose of CP (50 mgkg-1) intraperitoneally. Treatment with camu-camu continued for 45 days, then the mice were euthanized to determine sperm quality and the frequency of DNA damage using the Index protocol. Sperm DNA fragmentation – Halomax protocol. **Results:** The effect of camu-camu extract was observed in all trials ($p< 0.05$) compared to the negative control. Group T4, which was administered the highest concentration of the aqueous extract of the fruit, evidenced the cytoprotective effect of camu-camu ($p< 0.05$). **Conclusion:** The damaging effect on DNA due to the oxidative action of CP could be inhibited by the aqueous extract of the "camu camu" fruit.

Keywords: Camu-camu; Myrciaria dubia; Cyclophosphamide; DNA fragmentation; Mouse; Semen. (Source: MeSH – NLM)

RESUMEN

Introducción: Myrciaria dubia conocido como "camu camu" es una fruta que crece en la Amazonía y tiene como principal característica su alto contenido de vitamina C o ácido ascórbico, el cual tiene el rol de protección en la espermatogénesis por ser un compuesto con excelente acción reductora. El proposito de esta investigacion fue evaluar la capacidad citoprotectora in vivo del extracto acuoso del fruto de Myrciaria dubia (Kunth) McVaugh "camu-camu" frente al daño mutagénico producido por el antineoplásico ciclofosfamida (CP) sobre la línea germinal masculina. **Métodos:** Se utilizaron ratones (n= 60) divididos en cinco grupos tratamiento: T1= control negativo (sin tratamientos); T2 ingirió el extracto acuoso (10mgkg-1), T3 ingirió el extracto acuoso (50mgkg-1), T4 ingirió el extracto acuoso (100mgkg-1); T5 es el control positivo (se le administró solamente CP). A todos se inyectaron una dosis única de CP (50 mgkg-1) vía intraperitoneal., El tratamiento con camu-camu continúo por 45 días, luego los ratones fueron eutanizados para determinar la calidad espermática y la frecuencia del daño al ADN mediante el protocolo de índice de fragmentación de ADN espermático – protocolo Halomax. **Resultados:** Se observó en todos los ensayos el efecto del extracto de camu-camu ($p< 0,05$) respecto al control. El grupo T4, el cual se administró la mayor concentración del extracto acuoso del fruto (100 mgkg-1), evidenció el mayor efecto citoprotector del camu-camu ($p< 0,05$). **Conclusión:** El efecto dañino al ADN por la acción oxidativa del CP podría estar siendo inhibido o modulado por el extracto acuoso del fruto de "camu camu".

Palabras clave: Camu-camu; Myrciaria dubia; Ciclofosfamida; Fragmentación del ADN; Ratón; Semen. (Fuente: DeCS-BIREME)

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ORIGINAL PAPER

INTRODUCTION

Infertility is defined by the World Health Organization as a disease of the reproductive system characterized by the inability to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse [1]. This condition affects nearly 20% of couples of reproductive age, with the male factor contributing to 50% of the cases. The first analysis performed on a man visiting an assisted reproduction center to predict his fertile potential is the semen analysis, which generally includes macroscopic and microscopic examination of the seminal fluid. Additionally, new complementary tests are now considered, evaluating other aspects of sperm such as the integrity of their genetic material[2].

The importance of analyzing sperm DNA lies in the fact that various studies have shown that the integrity of sperm DNA affects clinical outcomes in assisted reproduction treatments. Despite the information provided by the semen analysis to evaluate sperm quality, approximately 10% to 15% of men diagnosed with infertility present semen parameters within normal ranges but may have defects in sperm DNA. Sperm DNA strand breaks are attributed to several causes, including excessive production of free radicals in the ejaculate, as well as exposure to environmental, occupational factors, and toxic habits [3]. High sperm DNA damage has been correlated with infertility, defective embryonic development, implantation failure, and an increase in recurrent miscarriages[4].

Cyclophosphamide (CP) [N, N-bis(2-chloroethyl) tetrahydro-2H-1,3,2-oxazaphosphorin-2-amino 2-oxide] is an alkylating agent commonly used as an antineoplastic and immunosuppressive drug. CP's cytotoxicity is mediated by DNA alkylation at the N7 position of guanine and the formation of DNA-DNA and DNA-protein cross-links, as well as single-strand DNA breaks [5-7]. Cyclophosphamide induces infertility by interrupting meiosis before the pachytene stage, causing genotoxic damage to the germline and impairing Leydig cells [8-10]. Eukaryotic cells, to maintain genetic stability, halt their cell cycle, which allows for the activation of DNA repair mechanisms[11].

However, when the damage is severe, cell death pathways such as apoptosis are activated[12]. Apoptosis describes a unique morphological pattern of cell death characterized by chromatin condensation, membrane blebbing, and DNA fragmentation; this mechanism plays an important role in the homeostasis of multicellular organisms. Abnormal apoptosis function has been associated with several human diseases, including neurodegenerative disorders and cancers.

"Camu camu" (Myrciaria dubia) is a fruit that grows in the Amazon and is notable for its high vitamin C or ascorbic acid content [13]. Ascorbic acid has been reported to have a protective role in spermatogenesis due to its excellent reducing action, making it a good antioxidant[14-16]. Reports have shown that the aqueous extract of Myrciaria dubia H. B. K. Mc Vaugh "camu camu" has an antimutagenic effect against damage caused by fluoride salts, demonstrated through the in vivo micronucleus assay in mouse bone marrow. When administered beforehand, it also has a cytoprotective effect on the same cell line[17]. Another study conducted in our laboratory confirmed the protective effect of camu camu on three cell lines previously treated in vivo with potassium bromate[18].

Based on the reported scientific evidence, the objective of this research is to determine whether "camu camu" can reverse the negative effect of CP on the male germline in mice, focusing this evaluation on the DNA fragmentation index of sperm. The achieved results could be extrapolated for human use, thereby restoring fertility in patients who have required the use of this drug in their cancer treatment. The Halomax test is a technique used to identify and evaluate sperm with damaged DNA. It identifies sperm with damaged genetic material (DNA) and differentiates them from those without damage. This test establishes the proportion of sperm with fragmented DNA in the total analyzed sample. It is estimated that, using normal reproduction methods, a percentage of sperm with fragmented DNA above 30% reduces or, in some cases, eliminates the possibility of achieving a full-term pregnancy.

The purpose of this research was to evaluate in vivo the cytoprotective capacity of the aqueous extract of Myrciaria dubia (Kunth) McVaugh "camu camu" fruit against the mutagenic damage caused by the antineoplastic cyclophosphamide (CP) on the male germline in mice.

METHODS

Design and study area

Preclinical experimental study in the field of experimental biology.

Population and sample

The sample consisted of 60 male albino BALB/c mice (Mus musculus) aged 6 to 8 weeks, obtained from the animal facility of the Instituto Nacional de Salud in Lima, Peru. The treatment groups were administered the aqueous extract via nasogastric tube No. 18 (Fisher Scientific, Pittsburgh, PA, USA). The mice were maintained under standard animal facility conditions: 14-hour light/10-hour dark photoperiod, temperature of 25°-27°C, relative humidity of 90%, with free access to a pellet diet (Bedoce, Peru) and water ad libitum. After an acclimatization period in the faculty's animal facility, the mice were randomly distributed into cages in five treatment groups (n=12). On the study's start day, they were administered CP (50 mgKg-1) once intraperitoneally, except for the negative control group.

Study variables

The present study evaluated reproductive organ weights, semen analyses, motility analysis, vitality analysis, sperm morphology analysis, precise sperm count, plasma membrane integrity, and sperm DNA fragmentation index evaluation.

PROCEDURES

Plants

"Camu camu" fruits, Myrciaria dubia (Kunth) McVaugh, were collected in the city of Pucallpa, Peru; transported by air to Lima and immediately transferred to the Laboratory of Reproduction and Developmental Biology of the Universidad Nacional Mayor de San Marcos (UNMSM). The plants were certified by the Botany Department of UNMSM. In the laboratory, the fruit was weighed and blended; the pulp of the "camu camu" fruits was extracted and dried at 60°C for 24 hours in a dry air convection oven.

Subsequently, a 10% (w/v) aqueous extract was prepared for 24 hours at 60°C. After 24 hours, the extract was decanted, filtered, quantified, and stored at -20°C; another 2% (w/v) final aqueous extract was prepared from this extract. The "camu camu" fruit was lyophilized and stored for later use. The distribution of the lyophilized "camu camu" was resuspended in distilled water as a vehicle in three different doses (10 mgKg-1, 50 mgKg-1, and 100 mgKg-1). "Camu camu" was administered daily via Fisher nasogastric tube No. 18 for 45 days.

Experimental design

The mice were separated into cages with the following distribution: a negative control group NC (n=12) was administered saline solution intraperitoneally for the same period; a group T2 (n=12) was administered camu camu extract (10 mgkg-1 BW) for 45 days; a group T3 (n=12) was administered camu camu extract (50 mgkg-1 BW) for 45 days; a group T4 (n=12) was administered camu camu extract (100 mgkg-1 BW) for 45 days; a group T5 (positive control) was administered cyclophosphamide intraperitoneally (50 mgKg-1 BW) once. Throughout the treatments, body weights were obtained daily, and at the end, all specimens from each group underwent two evaluations: sperm analysis and sperm DNA fragmentation index evaluation. After treatments, the mice were euthanized and dissected to separate reproductive organs, isolate them from fat bodies, and place them in saline solution at 37°C to perform the respective sperm analyses according to WHO-approved parameters [19], including motility analysis, vitality analysis, sperm morphology analysis, precise sperm count, plasma membrane integrity, and sperm DNA fragmentation index evaluation.

Obtaining Reproductive Organs

With the aid of a stereoscope, the following organs from the male reproductive system (right and left sides) were separated: testis, head and body of the epididymis, tail of the epididymis, and vas deferens. They were then weighed and maintained in saline solution at 37°C during the sperm analysis protocol application. The epididymal tail was sectioned in 0.5 mL of phosphate-buffered saline (PBS) at 37°C for sperm DNA fragmentation analysis following the Halomax Kit protocol (HALOTECH DNA SL). Sperm with fragmented DNA are considered those with a large halo and chromatin dispersion spots, while sperm without fragmented DNA have a small and compact chromatin dispersion halo.

Statistical analysis

The results were properly tabulated and entered into Excel 2007 software to be processed using SPSS version 17.0 for Windows. Results will be shown as mean ± standard deviation (SD) and contrasted using ANOVA with Levene's test (to assess homogeneity of variances), Kolmogorov-Smirnov test (normal distribution of weights and sperm concentration), and Tukey and Bonferroni tests for parametric data (morphology, vitality, and sperm integrity) with significance levels of $p<0.05$ and $p<0.01$.

Ethical Aspects

The care and handling of the animals were conducted in accordance with the ethical guidelines of the Universidad Nacional Mayor de San Marcos and the National Research Council for the care and use of laboratory animals [20].

RESULTS

No significant differences were observed in the increase of body weight, weight of the testes, epididymis, and prostate ($p>0.05$) (Table I); as well as in sperm morphology (not included in the tables), among the groups analyzed during the experiment. Similarly, the results of vitality, motility, membrane integrity, and sperm count are detailed in Table II.

The cytological differences between fragmented and non-fragmented sperm are shown in Figures 1 and 2. In Figure 1, representing the positive control group (cyclophosphamide only, 50 mgKg-1 BW), large halos are observed, indicating sperm DNA damage in a value higher than those in Figure 2, where sperm from group 4 (cyclophosphamide only, 50 mgkg-1 BW) + camu camu extract (100 mgkg-1 BW) show few sperm with large halos. It is important to note that the presence of the flagellum differentiates sperm from other possible cells involved.

Table 1. Weight (g) of reproductive organs in male mice. Data are expressed as mean ± SD.

GROUP	TESTICULAR WEIGHT	EPIDIDYMAL WEIGHT	PROSTATE WEIGHT
Negative Control	0,1277 ± 0,0025	0,0413 ± 0,0012	0,0513 ± 0,0022
Treatment 1	0,1066 ± 0,0054	0,0401 ± 0,0012	0,0510 ± 0,0016
Treatment 2	0,1121 ± 0,0046	0,0219 ± 0,0239	0,0500 ± 0,0008
Treatment 3	0,1123 ± 0,0033	0,0377 ± 0,0021	0,0459 ± 0,0017
Positive control	0,0899 ± 0,0691	0,0338 ± 0,0028	0,0428 ± 0,0029

$p<0.05$ Treatments vs control

Table 2. Effect of Myrciaria dubia H. B. K. McVaugh "camu camu" on motility (PM, NPM, IM), vitality, membrane integrity, and sperm count in mice treated with cyclophosphamide (50 mgKg-1 BW). Data in percentage. 45-day treatment.

Treatment	Motility			Vitality (viable)	Membrane Integrity (viable)	Sperm count (million/ml)
	PM	NPM	IM			
NC	54.399±14.311	11.623±8.096	33.978±10.749	60.944±20.221	60.342±13.745	$1.079x10^{6}$±51.563
PC	49.564±11.361	12.558±9.965	37.878±10.624	65.722±18.777	56.978±14.844	$1.310x10^{6}$±25.797
10mgkg^{-1}	42.121±20.103	12.476±9.508	45.403±18.788	46.401±31.631	51.555±20.636	$1.059x10^{6}$±25.658
50mgkg^{-1}	29.391±12.306**	14.559±7.600	56.050±14.123**	40.714±13.082	49.289±18.396	$0.795x10^{6}$±30.568**
100mgkg^{-1}	46.297±7.147	18.446±7.692	35.257±5.886	56.787±14.220	58.867±9.421	$1.646x10^{6}$±29.698

NC=negative control; PC=positive control. PM (rapid and slow progressive motility) Sperm moving actively in a straight line or large circles regardless of speed. NPM (Non-Progressive Motility) Sperm showing movement without locomotion.. IM (Immotility) Complete absence of motility.
Values are expressed as Mean ± SD
**Significant for $p<0.05$ compared to positive control.

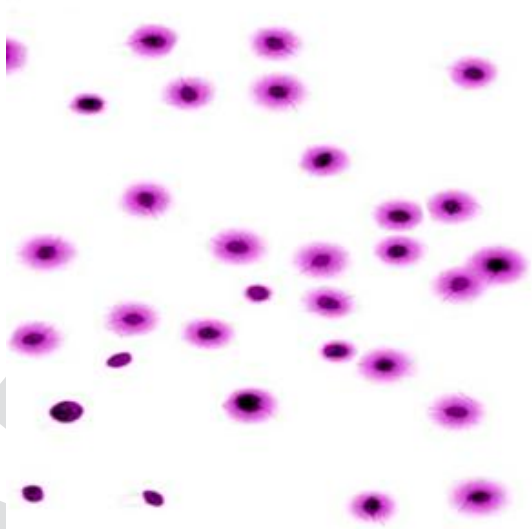

Figure 1. Results of the Halomax Test on mouse sperm treated with cyclophosphamide (50 mgKg-1 BW). The presence of large halos indicates DNA damage, a sign of fragmentation, 400x magnification.

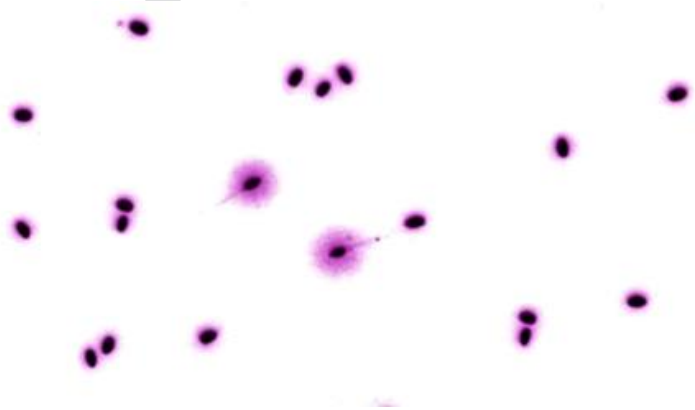

Figure 2. Results of the Halomax Test on mouse sperm treated with cyclophosphamide (50 mgKg-1 BW) and aqueous extract of camu camu (100 mgKg-1 BW). The presence of few fragmented sperm suggests the protective effect of the fruit. 400x magnification.

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DISCUSSION

Reproduction and fertility are the foundation of species continuity. However, when referring to our own species, this premise goes beyond simply fulfilling our biological purpose. While infertility as a disease does not cause death, it often leads to situations that can be deemed as lacking psychological and social well-being [1]. From this perspective, any effort to generate knowledge that helps individuals achieve conception should be considered a priority from both biological and clinical viewpoints.

The protective effect of antioxidant substances against genotoxicity can occur in three ways: by decreasing the assimilation of pro-oxidant genotoxicants, preventing their formation within the diet itself; as a reducing agent at the sites of pro-oxidant action, and by inducing detoxifying enzymes capable of reducing active oxygen intermediates [3-16]. Analyzing the protective effect of the aqueous extract of Myrciaria dubia fruit through the sperm DNA integrity test showed no significant differences between NC and the treatment groups. This result suggests a protective effect of the aqueous extract of camu camu fruit against CP oxidative damage. Additionally, it was determined that oral supplementation with vitamin C in humans reduces DNA damage induced by hydrogen peroxide (H_2O_2).

In vivo studies in human cells and in vivo studies in rodents have demonstrated that high intracellular concentrations of ascorbic acid reduce mutations caused by oxidative stress from $KBrO_3$[20]. It is likely that the high content of ascorbic acid (vitamin C) in camu camu fruit is responsible for the protective effect observed in the results, as a similar number of grade 0 cells were found between T1 and T4. It is known that CP induces permanent alterations due to different types of damage, which can be detected in a micronucleus test by blocking cytokinesis[22,23]. These findings indicate that CP induces DNA damage through various mechanisms besides oxidative stress.

CONCLUSIONS

It is concluded that the oral administration of aqueous extract of camu camu can counteract, modulate, and neutralize the effects of CP, as evidenced by the reduced rate of sperm with nuclear DNA damage in treated samples.

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