ARTÍCULO ORIGINAL

Genotoxic assessment of aqueous extract of *Rhizophora mangle* L. (mangle rojo) by spermatozoa head assay

Evaluación genotóxica del extracto acuoso de *Rhizophora mangle* L. (mangle rojo) mediante el ensayo de la cabeza del espermatozoide

Deyanira Carnesoltas Lázaro[I]; Yanisey Izquierdo López[II]; Ana Iris Frías Vázquez[III]; Anibal Domínguez Odio[IV]; Jorge Ernesto González[V]; Luz María Sánchez[VI]; Neivys García Delgado[VII]

[I]Licenciada en Ciencias Farmacéuticas. Máster en Farmacología. Investigadora Auxiliar. Profesora Auxiliar. Departamento de Farmacología, Instituto Nacional de Oncología y Radiobiología. Ciudad de La Habana, Cuba.
[II]Licenciada en Bioquímica. Aspirante a Investigadora. Laboratorio de Inmunología, Instituto Nacional de Oncología y Radiobiología. Ciudad de La Habana, Cuba.
[III]Licenciada en Biología. Máster en Farmacología. Profesora Auxiliar. Departamento de Biología Animal y Humana, Facultad de Biología, Universidad de La Habana. Ciudad de La Habana, Cuba.
[IV]Doctor en Medicina Veterinaria. Investigador Agregado. Asistente. Centro de Toxicología Médica. Santiago de Cuba, Cuba.
[V]Licenciado en Ciencias Farmacéuticas. Máster en Toxicología. Investigador Agregado. Centro para la Protección e Higiene de las Radiaciones, Pedro Pí. La Habana, Cuba.
[VI]Doctor en Medicina Veterinaria. Investigador Auxiliar. Departamento de Farmacología, Centro Nacional de Sanidad Agropecuaria. San José, La Habana, Cuba.
[VII]Licenciada en Microbiología. Reserva científica. Departamento de Biología Animal y Humana, Facultad de Biología, Universidad de La Habana. Ciudad de La Habana, Cuba.

ABSTRACT

INTRODUCTION: the aqueous extract bark of *Rhizophora mangle* L. (red mangrove) has traditionally been used in Cuban folk medicine due to its wide array of curative properties: astringent, haemostatic, febrifuge and antifungal. Previous chemical characterization studies of the liophylized aqueous extract bark, revealed that tannins are the main components, although the presence of other compounds such as epicatechin, catechin, clorogenic, gallic and ellagic acids, as well as galotannins have been also described.
OBJECTIVE: this work was designed to determine if any components of the extracts has the ability to produce genotoxic effects in germ cells of Cenp:NMRI mouse models using the abnormal shape of spermatozoa test.
METHODS: the lyophilized aqueous extract bark was given by oral gavages (500, 1000 and 2000 mg of total solids /kg bw) in three series of the classical protocol of Wyrobeck and Bruce, in intervals of 24 hrs for 5 days.
RESULTS: in series I, the animals were sacrificed on day 4 after starting the administration. In series II the animals were sacrificed on day 21 while in series III the day of sacrificed was the 35th.
CONCLUSIONS: no cytotoxic effect was observed in the 3 series and in all doses proved and only the highest dose of the extract in the series I provoked a slight genotoxic effect when increase the percentage in the number of abnormal spermatozoa.

Key words: *Rhizophora mangle* L., red mangrove, germinal cell test, plant extract, genotoxicity.

RESUMEN

INTRODUCCIÓN: el extracto acuoso de *Rhizophora mangle* L. (mangle rojo), se ha usado tradicionalmente en Cuba por su amplio espectro de propiedades curativas: como astringente, hemostático, febrífugo y antifúngico. Los estudios previos de caracterización química del extracto acuoso liofilizado de la corteza de la planta revelaron que los taninos son los componentes principales, aunque también se ha descrito la presencia de otros compuestos como las epicatequinas, el ácido clorogénico, ácido gálico y ácido elágico, así como galotaninos.
OBJETIVO: el presente estudio fue diseñado para determinar si alguno de los componentes del extracto tiene la capacidad para producir efectos genotóxicos en células germinales de ratones Cenp:NMRI utilizando el ensayo de anormalidades de la cabeza del espermatozoide.
MÉTODOS: el extracto liofilizado fue administrado por vía oral (500, 1 000 y 2 000 mg de material vegetal/kg) en 3 series del protocolo clásico de *Wyrobeck* y *Bruce*, en intervalos de 24 h por 5 d consecutivos.
RESULTADOS: en la serie I los animales fueron sacrificados el dia 4 después de iniciada la administración. En la serie II los animales fueron sacrificados el día 21, mientras que en la serie III fueron sacrificados el día 35.
CONCLUSIONES: no se observó efecto citotóxico en las 3 series y en todas las dosis probadas, y solamente la dosis mayor del extracto en la serie I provocó un ligero efecto genotóxico al incrementar el porcentaje de espermatozoides anormales.

Palabras clave: *Rhizophora mangle* L., mangle rojo, ensayo en célula germinal, extracto de planta, genotoxicidad.

INTRODUCTION

The introduction in the clinical practice of newly discovered drugs from natural sources should be submitted to pharmacological as well as toxicological studies prior to clinical trials. Therefore, in order to estimate the risk associated to the use of natural products, the study of genotoxicity, embriotoxicity and/or teratogenicity should be conducted, together with conventional toxicological evaluation. Although natural products have generally been regarded as a safe choice for the treatment of different pathologies, some of them have been demonstrated to display mutagenic effects. In this direction, it has described the induction of mutagenic effects in *Salmonella typhimuriun* strains, by the aqueous or methanolic extracts obtained from *Brosimum gaudichaudii*.[1] Using the same assay, has similarly demonstrated the occurrence of mutagenicity after exposure to a stem bark extract from *Schinus terebinthifoliusi*.[2] Also, it have been showed that the hydroalcoholic extract of *Punica granatum* (Punicaceae) whole fruits, used in Cuban traditional medicine as an effective drug for the treatment of respiratory diseases, is genotoxic when tested both *in vitro* and *in vivo* assays that detect DNA damage at different expression levels.[3]

In general, genotoxicity tests are carried out in order to identify if specific compounds have the ability to interact with nucleic acids at low concentrations and thus able to modify certain hereditary characteristics. These interactions may cause a direct or indirect toxicity in the genetic materials of sexual cells, probably via liver metabolism, which could in turn trigger a chain of events leading to carcinogenicity or genetic alterations in successive generations.[4]

Red mangrove, *Rhizophora mangle* L., is a widely distributed tree in some low, muddy and swamp areas of the Caribbean region. This specie has traditionally been used in Cuban folk medicine due to its wide array of curative properties: astringent, haemostatic, febrifuge and antifungal.[5] Particularly, the aqueous extract obtained from this plant has been demonstrated to exhibit antibacterial, wound healing, antioxidant and gastric anti-ulcer activity and mouth mucosa healing properties.[6-10] Another study has shown a remarkable COX-2 and sPLA2 inhibitory *in vitro* activity in the aqueous extract and polyphenols of this plant.[11] The chemical characterization studies of the extract, tannins have revealed that are the main components, although the presence of other compounds such as epicatechin, catechin, clorogenic, gallic and elagic acids, as well as galotannins have been also described. Moreover, this extract also appears to contain bound carbohydrates such as xilose, ramnose, fucose, arabinose, mannose and galactose as well as saturated and unsaturated long-chain fatty acids, essential oils and fitosterols.[12]

Taking in account all the above considerations, the aim of current paper was to carry out a genotoxicity evaluation of the aqueous extract obtained from *R. mangle* in male mice germ cells assay, using morphological criteria as toxicity endpoint.

METHODS

Animals

Experiments were performed using 8-12 weeks, Cenp:NMRI out bred, male mice, provided by *Centro para la Producción de Animales de Laboratorio* (CENPALAB), Havana, Cuba. Animals were kept at room temperature and room relative humidity and exposed to the natural light-dark cycle. They were randomly distributed in

groups of 6 animals per dose in each treatment, and the standard rodent diet and tap water were *ad libitum*. All the animal procedures reported here, were carried out in accordance with the Cuban regulations on the protection of animals.[13] The experimental protocol was also revised by ethical committee and conducted humanely.

Plant extract preparation

R. mangle specimens were authenticated by Department of Botany, National Center of Animal Health (CENSA), Cuba, and a voucher sample 6539, HAJB was deposited at the Herbarium of this Institution. The extract was gently supply by Dr. Luz María Sanchez from Pharmacology Department of National Center of Animal Health (CENSA), and the quality of each batch was carefully monitored by Quality Assurance Department of CENSA.

Experimental design

Three different experimental series (referred as I, II and III) were designed to evaluate the possible genotoxic effect of the aqueous extract of *R. mangle*. In all cases, the extract was dissolved in saline solution (NaCl 0.9%) and given by oral administration using 3 different dose levels (500, 1000 and 2000 mg of total solids/kg b.w.). An equal volume of saline solution was considered as a negative control group in all experimental series. In series I, the extract was administered during 3 days within 24 hours-time interval and the animals were humanely sacrificed on day 4 after starting the administrations. In series II, the extract was also given once per day, but the treatments lasted 5 days and the animals were sacrificed on day 21. In series III, the administrations of the extract were conducted as in series II, but the animals were sacrificed on day 35.

Sperm Morphology Test

The test was performed according to the method described by *Wyrobek* and *Bruce*[14] and *Dobrzyriska* and *Gajewski*.[15] The animals were sacrificed by cervical dislocation on days 4, 21 and 35 after the first injection, according to the different series detailed previously. Both caudal epididymus were removed and placed in a Petri plaque containing 1 mL of saline solution. The sperms were released after mechanical disruption and washing of the epididymus. The sperms concentrations in each sample were determined by spermatic counts on Newbauer chamber (DDR, Germany). In addition, an aliquot of the sperm suspension was stained by 0.1 % eosin. Briefly, a drop was taken and smeared on a clean slide, and 1000 spermatozoa of each mouse were analyzed in a DMLS microscope (Leyca, Germany) with 100x amplification. The following abnormalities were scored: lacking hook, amorphous and banana-shaped head.

Statistical analysis

Results of sperm count and morphology are presented as the mean ± standard deviation (SD). Data of normal distribution and variance homogeneity were studied by Kolmogorov-Smirnov and Bartlet tests, respectively. Treatments were compared using ANOVA and Dunney´s *post* test. $p < 0.05$ was considered significant.

RESULTS

The effect of the oral administration of the aqueous extract of *R. mangle* in terms of germ cells viability is presented in table 1. The spermatic count was statistically similar in control animals and animals treated with the different dose (500; 1 000; 2 000 mg/kg b.w.) of *R. mangle* aqueous extract.

Results of the sperm morphology test, conducted in the three different experimental series are shown in table 2. In experimental series I, the exposure to the highest dose of the plant extract (2 000 mg/kg b.w.) produced an increase of anomalous sperms, with prevalence of hook and banana type cells. However, the effect was not observed after exposure to the lower dose of the plant extract (500 and 1 000 mg/kg). On the other hand, in experimental series II and III, no increment in the frequency of appearance of anomalous head was registered after exposure to the plant extract.

On the other hand, in series II and III we appreciated that none of the doses of the aqueous extract (500, 1 000 and 2 000 mg/kg b.w.) induced variations in the percentage of appearance of anomalous sperms when it is compared with the values in the control group of mice treated with saline solution (NaCl 0,9 %).

DISCUSSION

From ancient times, plants have been used for medicinal purposes. Nowadays, primary health care in 80 % of the world population basically relies on plant and plant-derived products,[16] mainly due to medicinal plants constitute the base of health care systems in many societies and because the recovery of the knowledge and practices associated with these plant resources are part of an important health strategy in discovery of new medicines.[17] However, plants compounds can also have toxic effects in the human body, as they markedly differ from endogenous substances.[18]

When evaluating the effect of the aqueous extract of *R. mangle* over the germinal cells viability it was shown that it does not induce cytotoxicity on the sperm tides that are in differentiation roads to sperms (spermatogenesis phase) or on the sperms that are already formed at 4, 21 or 35 days, corresponding to experimental series I, II and III respectively. Altogether, these results reveal that the plant extract does not affect neither the viability of the primary and secondary spermatocytes (meiosis phase) nor the viability of the spermatogonium (mitosis phase).

The absence of toxicity observed in germinal cells after exposure to the aqueous extract of the bark of *R. mangle* can be explained on the basis of its chemical components. No toxic effects on *in vivo* models have been attributed to condensed and hydrolysable tannins, phytosterols, semi volatile compounds and fatty acids, which are constituents of *R. mangle* aqueous extract.[12] Similarly, it has reported no toxic effects on germs cells viability after exposure to limit dose of the extracts of caisimón of anis (*Piper auritum* H.B.K) and majagua (*Hibiscus elatus* Sw), which also contain tannins in their chemical composition. [19] In addition, no toxic effects have been found for high doses (1000 and 2000 mg/kg) of the aqueous extract of *Mangifera indica* L, which contains an important amount of polyphenolic compounds.[20]

However, a significant increase in abnormal spermatozoa was observed in the group administered with the higher dose of the plant extract within the experimental series I, pointing out to a genotoxic effect on the germ cells,

particularly at the spermatogenesis phase. The finding could be related to certain components of the extract such as hydrolysable tannins (e.g. gallic and ellagic acids) or its metabolites able of exert toxicity particularly during the spermatogenesis process.

Researchers have documented the *in vitro* cytotoxicity and genotoxicity induced by ellagic and gallic acid (15-240 mM concentration range) as measured in culture of B14 cell line.[21] The results of this study showed that tannins could decrease the viability of cells and their cytotoxicity was highest at the concentration of 60 mM. Also the data obtained from the Comet assay also supported the ability of both compounds to contribute to formation of DNA single-strand breaks. Although, both chemical entities could be at least partially responsible for the mutagenic effect of the *R. mangle* extract at high doses, we thinks that in this case we must be cautious and careful because these elements are common in our diets.

On the other hand, the mutagenic effect of the extract could be also related to the lacking of effective cell repair mechanisms at this stage or to the nature of the damage, as in some cases no endogenous mechanism exists for its reparation. In any case, the values of anomalous sperms observed at the higher dose of 2000 mg/kg fall below the values reported in similar studies by,[19] even though they are significant when compared to control group. Consequently, above results generates questions that should be corroborated and discussed in further studies.

Current results show biggest susceptibility in the sperm tides cells, in disagreement with previous approaches in which the late spermatogonial cells and/or early primary spermatocytes appear to be the most susceptible.[14,22] But in support of our finding, researchers have also found the late sperm tides undergoing differentiation process and the sperms already formed as preferable targets of the genotoxicity, exerted by the independent and combined treatment of acryl amide and X-rays on the germinal and somatic cells.[15]

The exposure to *R. mangle* aqueous extract lasting 21 and 35 days did not increase the frequency of appearance of anomalous sperms, according to the data in experimental series II and III. One line of thoughts, guided by results of *Wyrobek* and *Bruce*,[22] could lead us to conclude that *R. mangle* aqueous extract does not induce DNA damage in germ cells on mitosis and meiosis stages. But, a second view should be also considered, as certain genotoxicity of the extract was observed in experimental series I at high doses. Therefore, it can be speculated the occurrence of genotoxicity after exposure to *R. mangle* extract, which could be generally repaired, except during certain conditions, such as those observed in experimental series I. Above considerations could be perfectly permissible as strong genetic cellular control exists in the organism designed to allow the minimal quantity of mutations to be transmitted to progeny cells.

R. mangle (red mangrove) represents an ethno botanically relevant plant in Cuba, traditionally used for different biomedical applications.[5] It's variety of empirical uses with different doses, frequencies of administration and duration of treatments, make necessity to perform a complete set of toxicity studies. Current investigation gives one step towards this general purpose and demonstrates the occurrence of moderate genotoxic effects at doses 4 times higher than maximum therapeutic dose.

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MSc. *Deyanira Carnesoltas*. Departamento de Farmacología, Instituto Nacional de Oncología y Radiobiología. 29 y E, Vedado, CP 10400, Ciudad de La Habana, Cuba. Correo electrónico: deya@infomed.sld.cu; neivys@fbio.uh.cu

Table 1. Sperm counts in NMRI mice treated with *Rhizophora mangle* L.

Treatments		Concentration (10^6/mL)		
		Series I	Series II	Series III
Control		15.6 ± 3.9	13.4 ± 4.3	14.1 ± 2.0
R. mangle	500	13.8 ± 2.0	13.5 ± 1.8	12.1 ± 1.5
	1 000	16.1 ± 4.5	13.8 ± 3.3	11.9 ± 3.2
	2 000	14.5 ± 4.4	12.7 ± 2.8	9.7 ± 4.1

Values represent mean ± standard deviation (SD) of experiments (n= 6/dosis).

Table 2. Results of sperm morphology assay in NMRI mice treated with *Rhizophora mangle* L.

Treatments		Percentage of Abnormal Spermatozoa (% ± SD)		
		Series I	Series II	Series III
Control		2.9 ± 0.8	3.1 ± 0.3	2.9 ± 0.4
R. mangle	500	2.9 ± 0.5	2.4 ± 0.8	3.2 ± 0.7
	1 000	3.3 ± 0.9	3.3 ± 1.0	3.6 ± 0.8
	2 000	4.8 ± 1.03*	2.7 ± 0.6	3.5 ± 0.5

Values represent mean ± standard deviation (SD) of the experiments (n= 6/dosis). ANOVA and Dunney *post* test ($p< 0.05$).