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Male Rat Infertility Induction/Spermatozoa and Epididymal Plasma Abnormalities After Oral Administration of *Kalanchoe gastonis bonnieri* Natural Juice

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Natural aqueous crude extracts (NACE) of several Crassulaceae family plants have been applied as a vaginal contraceptive by the populace. The aim of this work was to evaluate the inhibition of fertility in male Wistar rats and some physiological and biochemical changes in spermatozoa and epididymal plasma induced by NACE from *Kalanchoe gastonis bonnieri* (K. g. b.) (Crassulaceae). The NACE was obtained by mechanic pressure on grinding fresh plant leaves. Sublethal doses (150–300 mg/kg body weight) of NACE were orally administered to adult and fertile male rats daily for 30 days in a search for a contraceptive effect, and physiological and biochemical modifications on sperm cells and cauda epididymal plasma. The toxicity studies revealed that the lethal dose (LD_{50}) calculated was 11 g/kg body weight. Sublethal doses induced 50%–100% fertility inhibition, with 100% recovery of fertility 30 days after stopping the treatment. The sperm motility, viability and spermatic density were also significantly decreased ($p < 0.001$). The outstanding biochemical change observed in the cauda epididymal plasma was a decrease of carnitine concentration. The NACE of *K. gastonis* contains one substance active on fertility by affecting spermatozoa motility, viability and sperm density with a significantly decreased carnitine and sialic acid ($p < 0.001$) in the caudal epididymal plasma.

Keywords: Kalanchoe gastonis bonnieri; fertility inhibition; motility; viability; biochemical changes.

INTRODUCTION

The worldwide population increase means that human fertility regulation deserves urgent consideration. Even though research on male reproduction has been extensive in the last years, little effort has been made to develop male contraception. Nevertheless, several vulnerable sites that could be used in male contraception have been proposed. Due to the strict sequence and nature of the events that determine male gamete maturity, interference in any step would have serious consequences on the eventual sperm fertilizing capability. A method of interfering in the male reproductive process, without affecting libido, spermatogenesis or the genomic integrity of the sperm cell would be attractive (Hafez, 1979). Recent publications have demonstrated that it is possible to produce a reversible and specific alteration in the epididymal sperm number, viability and motility without inducing side effects by using plant preparations with high glycoside concentrations (Xu and Quian, 1986; Quian, 1987).

Mexican Traditional Medicine reports the use of some natural aqueous crude extracts (NACE) of Crassulaceae plants as vaginal contraceptives. We have demonstrated that some of these plants *in vitro* produce immobilization–agglutination effects, and ultrastructural changes in human spermatozoa (Huacuja *et al*., 1995) as well as a contraceptive effect in male rats (Huacuja *et al*., 1997).

The purpose of this study was to assess the effects on rat cauda epididymis of the NACE obtained from Crassulaceae *Kalanchoe gastonis bonnieri* plant (Raym-Hamet & E.P. Perrier), in relation to contraceptive activity and to correlate it with some physiological and biochemical changes in spermatozoa and cauda epididymal plasma.

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MATERIALS AND METHODS

Preparation of the natural aqueous crude extract (NACE). The plant was harvested from the gardens of Centro de Investigación Biomédica de Occidente,

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Instituto Mexicano del Seguro Social, Guadalajara, Jalisco Mexico. The plant was botanically authenticated by A. Aguilar at Herbarium of the Instituto Mexicano del Seguro Social, and given a voucher registration number (IMSSM-11502). A natural aqueous crude extract was obtained from the emerging plantules growing at the edges of adult leaves of the plant by mechanical pressure in a mortar. The natural juice NACE, was clarified by centrifugation, and stored at –30 °C until assays were performed.

Animals. Adult fertile Wistar female and male rats (250–300 g body weight) were used for this study. They were housed in groups of five per polypropylene cage with free access to standard laboratory food and tap water, and maintained on a 12 h/12 h light-dark cycle, with an ambient temperature of 25° ± 1 °C (Hernández-Pérez *et al.*, 1995).

Toxicity test. The LD_{50} dose of *Kalanchoe gastonis bonnieri* extract was investigated twice in three groups of six animals by the orogastric administration of a single and variable doses equivalent to 4, 8 and 13 g of the total solids of NACE/kg body weight. Symptomatic behaviour as a consequence of the treatment was evaluated each 6 h for 3 days.

Infertility induction and reversibility. Fertility inhibition studies were performed twice in two groups of 15 adult male rats. Group 1 was given oral doses (150 mg/kg body weight) daily for 30 days, and the control group received water only. The NACE dose was calculated weekly from the mean weights of the animals. Fertility inhibition was assessed by pairing 10 male treated rats, each with three 60-day-old virgin females. Successful mating was determined by the presence of spermatozoa in morning vaginal smears. Any male rat that impregnated at least one of the females, was considered fertile. Those resulting fertile rats were considered as subgroup 1, and were treated again with 300 mg/kg body weight also for 30 days after the last dose of the first treatment with 150 mg/kg body weight.

Group 2 was orally administered 300 mg of total solids of NACE/kg body weight daily for 30 days, and the same design coutinued to determine fertility inhibition in group 1. The reversibility of the effect was investigated in all fertility inhibition assays 30 days after stopping treatment. Mated fertile females were caged individually, and observed during the ensuing pregnancy. The actual number of pregnancies that resulted in each group was recorded as the percentage of pregnant females.

Evaluation of the physiological and biochemical changes induced by the extract. Physiological and biochemical changes in the epididymal cauda spermatozoa and cauda epididymal plasma, respectively, were evaluated in infertile rats resulting as a consequence of treatment, and in controls that received water as a vehicle. 24 h after the last dose of NACE, the animals were weighed and killed by ether inhalation. The spermatozoa in the micropuncture samples taken from each epididymal cauda region were diluted in Ham F-10 medium (pH = 7.4), and assayed for spermatozoa motility and viability. Epididymal plasma and spermatozoa were collected from cutting the epididymides cauda in halves, carefully scraping and aspirating spermatozoa and epididymal plasma with an insulin syringe without a needle. Each aspirate was diluted with 1 mL of Ham F-10 medium (pH 7.4) to gently resuspend spermatozoa, and then centrifuged at 12000 × **g** for 30 min in an Eppendorf centrifuge 5415 C: The supernatant volumes were measured, and the excess of 1.0 mL was assigned to the total volume of both cauda epididymides plasma. The epididymal plasma was stored at –40 °C until specific spectrophotometric determination of proteins, sialic acid, carnitine and carbohydrates were performed (Huacuja *et al.*, 1997).

Statistical analysis. Results are given as the mean ±SD. Differences between the groups were evaluated statistically by using Student's paired *t*-test (Excel program). A probability level of 0.05 or less was accepted to be significant.

RESULTS

Toxicity studies

Toxicity studies revealed that 13 g/kg body weight induced 80% lethality and the lethal dose (LD_{50}) was estimated to be 11.0 g/kg body weight (Fig. 1). In all cases the animals developed a general bad state with the following symptoms: abdominal spasms, bristling fur, lethargy, prostration, inactivity and loss of weight. Three rats (50%) died within the first 24 h, and the remaining three rats (50%) survived, with a total recovery 8 days after the treatment.

Infertility and reversibility induction

Two sublethal doses were used to achieve the fertility inhibition: 150 mg/kg body weight produced 50% fertility inhibition (group 1) (Table 1) and 100% inhibitory effect was observed in the five remaining fertile rats (subgroup 1) (Table 1) treated again with 300 mg/kg body weight, 30 days after the first treatment period with 150 mg/kg body weight (Table 1). A significant reduction (50%) in fertility occurred when the NACE was administered at a dose of 300 mg/kg body weight

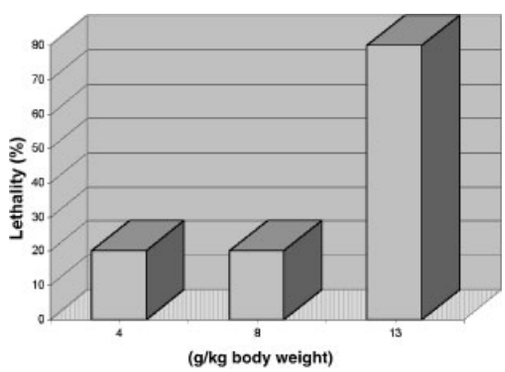

Figure 1. Toxicity profile in male rats with single and variable doses of *Kalanchoe gastonis bonnieri* natural aqueous crude extract. The computed LD_{50} was around 11.0 g/kg body weight.

Table 1. Fertility inhibition of male rats after oral administration of NACE from *Kalanchoe gastonis bonnieri*

Group	Pregnant rats	Fertility inhibition (%)	Fertility recovery after 30 treatment days (%)
(n = 10) Control	10/10	0	–
(n = 10) Group 1 (150 mg/kg)	5/10	50	100
(n = 5) Subgroup 1[a] (300 mg/kg)	0/5	100	100
(n = 10) Group 2[b] (300 mg/kg)	5/10	50	100

[a] This subgroup is formed by resulting fertile rats treated with 150 mg/kg body weight, which were treated again for 30 days with 300 mg/kg body weight 30 days after the first treatment.
[b] This group was treated with 300 mg/kg body weight without previous treatment with 150 mg/kg body weight.

Table 2. Modifications of the physiological function in spermatozoa and cauda epididymal plasma induced with NACE from *Kalanchoe gastonis bonnieri*

Group	Sperm density ($\times 10^6$)	Viability (%)	Motility (%)	Epididymal liquid (μL)
Control	209 ± 27	77 ± 4	70 ± 2	65
Group 1 (150 mg/kg)	150 ± 10[a]	33 ± 2.08[a]	17 ± 15[a]	75
Group 2 (300 mg/kg)	90 ± 26[a]	55 ± 10[a]	16 ± 15[a]	64

The numbers indicate the mean values ± standard deviations of 10 determinations.
[a] ($p < 0.001$) compared with the control groups.

Table 3. Biochemical composition of cauda epididymal plasma following treatments with NACE from *Kalanchoe gastonis bonnieri*

Group	Protein	Total carbohydrate	Sialic acid	Carnitine
Control	4.85 ± 0.80	0.76 ± 0.15	2.14 ± 0.88	0.35 ± 0.1
Group 1 (150 mg/kg)	2.83 ± 0.02[b]	0.87 ± 0.02	0.96 ± 0.02[b]	0.16 ± 0.08[a]
Group 2 (300 mg/kg)	6.07 ± 3.96	1.04 ± 0.57	1.45 ± 0.71	0.11 ± 0.07[b]

Values are expressed in g/100 mL of epididymal plasma of 10 determinations and are indicated as mean ± standard deviation
[a] ($p < 0.05$), [b] ($p < 0.001$) compared with the control groups.

(group 2). The rats in this group did not receive a previous treatment with 150 mg/kg body weight as in subgroup 1 (Table 1). The fertility in the male rats was recovered 100% 30 days after the treatment period.

Evaluation of the physiological and biochemical effects induced by the extract

A significant reduction ($p < 0.001$) in the cauda epididymal spermatozoa reserves, viability and motility were observed compared with the controls; there were no significant changes in the cauda epididymal plasma volume (Table 2).

At the end of the treatment periods, the biochemical composition of the cauda epididymal plasma displayed a significant decreased in carnitine in both individual animal groups ($p < 0.05$)[a], ($p < 0.001$)[b] respectively (Table 3); proteins and sialic acid were also significantly decreased ($p < 0.001$) only in the first group treated with 150 mg/kg body weight.

DISCUSSION

Several studies have shown the contraceptive effects of the oral administration of plant extracts in humans and rats (Quian *et al.*, 1986), mice (Xu and Quian, 1986; Quian, 1987; Hernández-Pérez *et al.*, 1995; Quian *et al.*, 1986) and man (Quian *et al.*, 1986) without any side effects. Isolation, purification and characterization of the active substance that produces a reversible contraceptive effect in male humans and rats has been performed (Matling *et al.*, 1993; Lue *et al.*, 1998). A protocol for screening male fertility regulation by some of the most representative plants of our locality, belonging to the Crassulaceae family has been developed. Some of these plants form part of the Mexican Traditional Medicine the extracts or infusions of which have been used as contraceptives at the vaginal level. *In vitro* immobilizing and agglutinating effects of NACE were produced instantaneously in human and other mammalian spermatozoa species (Huacuja *et al.*, 1995). This activity seems to be a general characteristic of the Crassulaceae family of plant extracts (Huacuja *et al.*, 1985) with possible inhibition of fertility (Huacuja *et al.*, 1997). Male fertility inhibition was assessed after oral administration of sublethal selected doses of *Kalanchoe gastonis bonnieri* NACE.

It is suggested that the total sperm immobilization produced instantaneously *in vitro* by the extract may be due at least in part to a direct effect of NACE's active substance on the dyneine protein–ATP complex axoneme system (Oko and Clerment, 1990) which is drastically affected (Huacuja *et al.*, 1995). Our results on toxicity studies were similar to others performed in *Kalanchoe* species, also belonging to the Crassulaceae

family plants: *K. daigremontana, K. fedtschenki* and *K. tubiflora.* When chicken were given the extracts (10 g/kg body weight), some toxicity effects were observed (William's and Smith, 1984).

The contraceptive activity of NACE was accompanied by a statistically significant decrease in sperm density, motility and viability (Table 2). The inhibition of fertility by 50% and 100% seemed to depend on the oral dose of NACE administered, and on the time of the treatments. The male fertility potency resulting after the treatments, could indicate that for a 100% successful contraceptive effect, it is apparently necessary that rats be subjected to prior treatment with low doses (subgroup 1) of the extract in order to sensitize them, followed by stopping and then treating them on a basis 30/30 days with 300 mg/kg body weight. These physiological effects could in some way also be the result of a considerable decrease in carnitine, sialic acid and protein concentrations in the cauda epididymal plasma (Table 3). These biochemical changes possibly occurred in the cauda epidymal plasma of subgroup 1 also (data not presented). For several tissues, carnitine has been shown to have an important cellular function, for example when transferring long-chain fatty acids across the inner mitochondrial membrane for β-oxidation (Bremer, 1977). Acyl-CoA derivatives are not permeable to the inner mitochondrial membrane. Cytosolic acyl-CoA derivatives enter the mitochondrion by a shuttle system in which carnitine acts as an acyl carrier. In this acyl-CoA transport mechanism carnitine acyl transferase I is located on the outer surface of the inner mitochondrial membrane. This enzyme catalyses acyl group transfers from acyl-CoA to the hydroxyl group of carnitine. Acyl carnitine may serve as a readily accessible energy pool for use in both activation of respiration and motility in mammalian spermatozoa. (Milkowski *et al*., 1976). Carnitine is one of the most important metabolites associated with epididymal sperm maturation, motility and fertilizing capacity (80%) (Casillas *et al*., 1984).

A decrease in carnitine concentration is relevant given that the interrelation between the sperm cells and the environment created by the composition of the epididymal secretions and by the permeability of the epididymal epithelium, are of extreme importance.

The feasibility of using the natural juice of some *Kalachoe* species from the Crassulaceae family plants for male fertility regulation has been demonstrated. This approach was used after our preliminary results concerning the isolation and purification of the active substance (peak 1) which was obtained successfully by column chromatography, as done for *Kalanchoe blossfeldiana* natural juice (Huacuja *et al*., 1995). Similarly peak 1 from *Kalanchoe gastonis bonnieri* which is also rich in sialic acid, uronic acid and hexosamines, possessed powerful immobilizing and agglutinating activities on epididymal cauda spermatozoa (Fig. 2). This paper reports a preliminary study in which male rats were made temporarily infertile by using sublethal doses of NACE, obtained from the emerging small plantules growing at the edges of adult leaves *of Kalanchoe gastonis bonnieri.* Studies are in progress to confirm the male contraceptive effect induced by the substance isolated (peak 1) by gel filtration chromatography procedure obtained from *Kalanchoe gastonis bonnieri* natural juice.

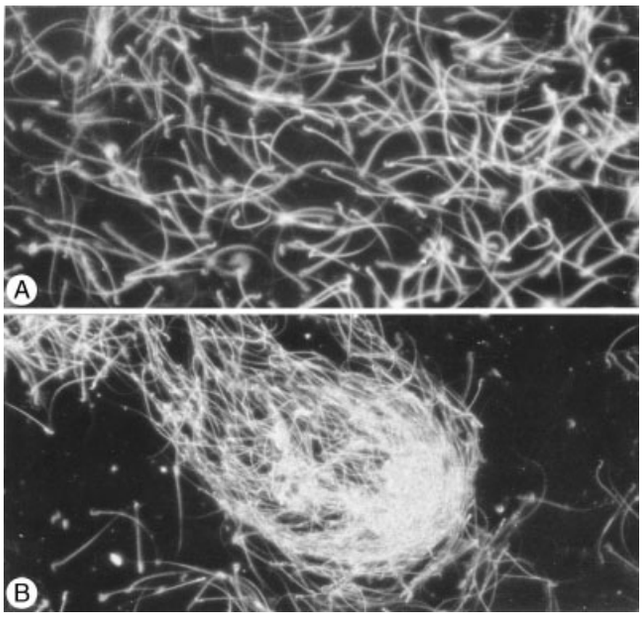

Figure 2. *In vitro* activity of the NACE on rat epididymal cauda spermatozoa. A. In the absence of the extract (control). B. In the presence of ***Kalanchoe gastonis bonnieri*** NACE, 100% agglutination and immobilization effect on spermatozoa was observed. (Enlarged from 200X)

CONCLUSIONS

1. Sublethal doses used to produce the effects were 33 and 66 fold lower than the LD_{50} indicating the low toxicity of the extract.

2. There was a statistically significant decrease in carnitine and sialic acid in the cauda epididymal plasma which drastically affected the motility, viability and sperm density.

3. It is clear from our investigations that the NACE of the plant studied possesses a non-toxic substance that could be used in future to regulate male fertility. Further research is required.

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