

In vivo* Antimicrobial Activity of Ozonized Theobroma Oil Ovules against *Candida albicans

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Abstract

Title: Effects of ozonated theobroma oil and ketoconazole ovules on rats infected with *Candida albicans*.

Background: Candidiasis is an infection caused by a yeast shaped fungus which includes *Candida* gender. *Candida albicans* is an opportunistic microorganism, which causes more than 80% of vaginal infections. The aim of this study was to determine the effect of ozonized theobroma oil vaginal ovules in the treatment of induced vaginal candidiasis compared to Ketoconazole ovules in Sprague Dawley female rats.

Methods and Findings: Animals were ovariectomized and injected with a hormonal treatment after 14 days in order to know the oestrus cycle. After 48 hours rats with keratinous cellulose were vaginally infected with an inoculum of 10⁶-10⁷ *Candida albicans* in 0.1 mL of phosphate buffer solution. Five animals groups were studied: group I (without treatment), group II (treated with unozonized theobroma oil ovules), group III (treated with ketoconazole ovules), group IV (treated with 10% ozonized theobroma oil ovules), and group V (treated with 20% ozonized theobroma oil ovules). Exudates were made before beginning the treatment, 5 and 10 days during the treatment and 48 hours after the end of treatment. Results demonstrated a decrease of 0.7 log of the number of rats with infection after 5 days of treatment with 20% ozonized theobroma oil ovules; however, it was not observed infection in rats after 10 days. A similar result was obtained with ketoconazole ovules.

Conclusions: Due to antimicrobial activity of 20% ozonized theobroma oil ovules, it can be recommended their use for treatment of Candidiasis

Keywords: *Candida albicans*; Candidiasis; Ketoconazole; Ovules; Ozonized theobroma oil; Sprague-Dawley female rats

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Introduction

Candidiasis is an infection caused by a yeast shaped fungus of family Cryptococacea, which also includes several types as *Torulopsis*, *Trichosporum*, *Cryptococcus* and *Candida*. *Candida albicans* is a saprophyte germ of normal microbiota of humans and other hot blood animals. It is an opportunistic microorganism, which causes more than 75% of the vaginal infections [1,2]. Treatment of these fungal infections is fundamentally based on polyenes and some other imidazol derivative drugs. However,

the prevalence of vaginitis by *Candida* is increasing around the world [3]. The short-term treatment has demonstrated an increase in the germ resistance to these medications and search of new treatment alternatives results happens to be of immense importance [4,5].

Several antifungal agents have been developed in the last years, but only a small quantity, *in vitro* and *in vivo*, showed antimicrobial activity [6,7]. Previous studies have demonstrated

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that some ozonized vegetable oils, as sunflower, olive and theobroma oils showed antibacterial activity *in vitro* against several microorganisms [8,9] and peroxidical compounds present in ozonized vegetable oil were responsible for antimicrobial activity [10-12]. On the other hand, preliminary studies with ozonized theobroma oil *in vivo* demonstrated satisfactory results [13].

Theobroma oil or cocoa butter is a solid fat with a melting point ranged between 32 and 35°C. This product is highly demanded because of its suitable properties for chocolate manufacture. At room temperature, it is hard and brittle giving chocolate its snapping characteristics, but it also has a steep melting curve with complete melting at human body temperature. This property gives a cooling sensation and a smooth creamy texture; for example, the content of solids falls from 45 to 1% between 30 and 35°C. The hardness of theobroma oil is related to its solid fat content at 20 and 25°C [14]. This melting behavior is related to the chemical composition of theobroma oil. This oil is rich in palmitic (24-30%), stearic (30 to 36%), and oleic acids (32 -39%) and its major triacylglycerol are of the kind SOS, where S represents saturated acyl chains in the 1 and 3 positions and O represents an oleyl chain in the 2 positions. Cocoa butter has a high content of saturated acids that raises health risks, but it has been argued that most of these saturated acids is the noncholesterolemic stearic acid [15]. According to composition and physical characteristics of the theobroma oil, suitable properties for preparing some pharmaceutical formulations from ozonized theobroma oil could be expected [16]. The aim of this study was to determine the effect of ozonized theobroma oil vaginal ovules in the treatment of the induced vaginal candidiasis compared with Ketoconazole ovules in Sprague Dawley female rats.

Materials and Methods

Forty female Sprague Dawley rats (180-200 g) were purchased from the National Centre for Laboratory Animals Production (Cuba). Animals were maintained in quarantine in an air-filtered and temperature-conditioned room (20 ± 2°C) with a relative humidity of 50 ± 5%. Rats were fed with standard laboratory chow and water ad libitum and were kept under an artificial light/dark cycle of 12 hours [17]. This was carried out according to the ethical guidelines for the research with laboratory animals and was approved by the Ethical Committee of Animal Experiments of the National Centre for Scientific Research, Havana City, Cuba.

Starting from *Candida albicans* ATCC 10231 on Sabouraud Dextrose Agar plates, it was prepared a suspension of 0.1 mL with 10⁶-10⁷ CFU/mL concentration on phosphate buffer sterile (PBS). Vaginal swabs were performed by washing with 0.2 mL of PBS. Subsequently 0.05 mL of fluid obtained were seeded on Sabouraud Dextrose Agar. Incubation was for 48 h at 30°C.

Sprague-Dawley female rats were ovariectomized. Vaginal swabs were performed after 14 days to determine oestrus cycle and the presence of *Candida albicans*. All rats had an injection with hormonal treatment. After 48 hours only the rats with keratinous cellules were vaginally infected by an inoculum of 10⁶-10⁷ *Candida albicans* in 0.1 mL of phosphate buffer solution. Four days later, a new vaginal swab was carried out to determine the infection

level. They were considered as infected those rats with more to 10² CFU/mL of infection.

Five groups of infected animals were studied: Group I (without treatment); Group II (treated with unozonized ovules); Group III (treated with ketoconazole ovules); Group IV (treated with 10% ozonized theobroma oil ovules); Group V (treated with 20% ozonized theobroma oil ovules).

One daily ovules was applied. Exudates were made before beginning of the treatment, at fifth day of the treatment, at tenth day of the treatment and 48 hours after the end of treatment.

Theobroma oil was supplied by "Rubén David Suárez Abella" company of Cuba. Ovules with theobroma oil and ozonized theobroma oil at 10 and 20% with vehicle in enough quantity for a 100%, were elaborated in the Ozone Research Centre with approximate values of peroxide index between 110 and 220 mmol-equiv of active O₂/kg of sample, respectively.

Ketoconazole ovules 400 mg L-0705, was supplied by "Roberto Escudero" pharmaceutical laboratory, Havana, Cuba.

Estradiol of Depósito 10 mg/1mL Lote 07001 and Sódico Tiopental 500 mg. Liofilizado. L-6004 were supplied by Quimefa Company of Cuba.

For each group of rats studied, the logarithm in base 10 of the number of microorganisms (Log N) as a function of time (0, 5, 10 and 12 days) was calculated to normalize the distribution. These values were summed and averaged for each group and normalized to be able to correctly interpret the results.

Results and Discussion

Four animals perished during surgery. Thirty-three rats were selected to carry out the experience (*Candida albicans* inoculation), because the vaginal mucous showed estro characteristics and did not present *Candida albicans* infection.

During infection process, 30 animals achieved infection levels superior to 10² CFU/mL (**Table 1**), thus it is considered that these animals are infected, which allowed the establishment of the effectivity in a 90%, which is a higher value than the 80% obtained by an experimental model published by Lezcano et al. [13].

Five days after the beginning of the treatment, it could be observed that in group I (without treatment), a single animal showed *Candida albicans* concentrations lower than 10² CFU, while in group II (treated with unozonized formulation) two animals exhibited this very same behavior. Sobel et al. [18] reported the spontaneous elimination of the disease in 10% of infected rats, thus these results can be attributed to the characteristics

Table 1 Infection Process.

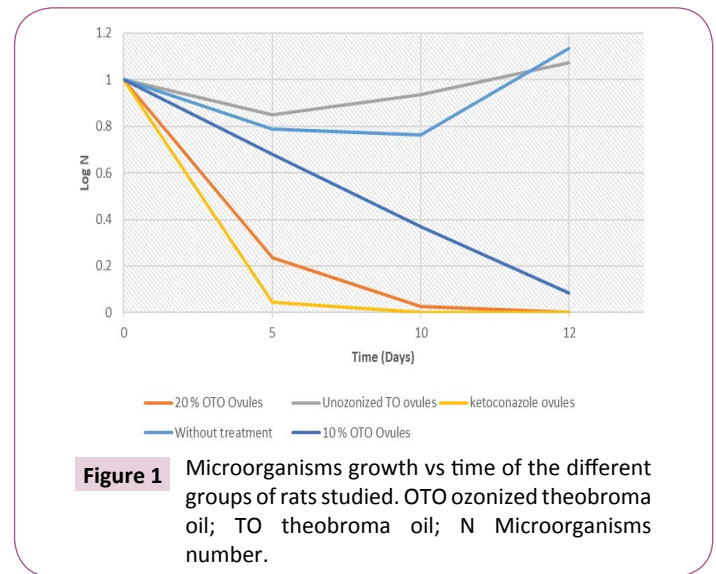
Infected rats	<i>Candida albicans</i> (CFU/mL) ^a	(%)
3/33	10 ⁴ -10 ⁵	9.1
10/33	10 ³ -10 ⁴	30.3
17/33	10 ² -10 ³	51.5
3/33	10-10 ²	9.1

^aCFU Colony forming units.

of the model as well as defense mechanisms inherent to vaginal mucous. Regarding group III treated with ketoconazole, it can be observed that at fifth day none of the animals is infected, since three of them showed no *Candida albicans* at all and two animals exhibited *Candida albicans* concentrations lower than 10^2 CFU. In group IV (treated with 10% ozonized theobroma oil ovules), they were found three animals with *Candida albicans* concentrations lower than 10^2 CFU, in addition number of microorganisms decreased in a 31%. On the other hand, in group V (treated with 20% ozonized theobroma oil ovules), none of the animals showed *Candida* concentrations superior to 10^2 CFU, thus, it can be claimed that the 20% ozonized theobroma formulation removed the infection in every treated animal of this study.

Ten days after the treatment begun group I (without treatment) and group II (treated with unozonized formulation) showed a decrease of the number of microorganisms lower than 25%, plus, every animal in both groups remained infected but a single animal of group II, which showed *Candida* concentrations lower than 10^2 CFU (3 CFU). On the other hand, group III (treated with ketoconazole) none of the animals were infected since every animal showed 0 CFU, but one that exhibited 1 CFU. Regarding group IV (treated with 10% ozonized theobroma formulation) infection was removed in every subjects; one of the animals showed 0 CFU.

Two days after the treatment concluded, groups I and II showed a number of microorganisms even higher than the initial value of this parameter. On the other side, in every animals of group III (treated with ketoconazole) infection was removed, since all the animals showed 0 CFU of *Candida albicans*. In group IV (treated with 10% ozonized theobroma oil ovules), all of the animals presented *Candida* concentrations lower than 10^2 CFU, nevertheless there were three animals showing *Candida* concentrations superior to 2 CFU. Animals of group V (treated



with 20% ozonized theobroma oil ovules) showed a behavior exactly alike than animals of group III (**Figure 1**).

These results evidence that 20% ozonized theobroma oil ovules is the most effective one, since it was observed a very similar behavior compared to the group treated with ketoconazole, which is the first choice drug for the treatment of this disease.

Conclusions

Results demonstrated that a decrease of 0.7 log of the number of microorganisms occurs after 5 days of treatment with 20% ozonized theobroma oil ovules; however, there were not found signs of infection in rats after 10 days. This result was very similar to the one obtained with ketoconazole. Due to the antimicrobial activity of (20%) ozonized theobroma oil ovules, it can be recommended its use for the treatment of vaginal Candidiasis infection.

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