Eugenol biologic activity in immunosuppressed rat females with \textit{Candida albicans} genital infection: histocytological changes

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Abstract: From our prior studies concerning eugenol’s biologic activity we observed the very good in vitro antifungal efficiency of this natural compound. Those positive results generated the need to supplement the available information with a comparative in vivo animal model. In this context, our current study was proposed to ascertain and compare the effects of eugenol with nystatin with a placebo control group (saline solution) by evaluating the cytohistological alterations in immunosuppressed Wistar female rats genitally infected with \textit{Candida albicans}. The observed histological alterations were most apparent in the placebo group. Candidiasis affected the liver, ovaries, kidneys, and spleen of the infected female rats. When compared to the groups treated with eugenol and nystatin, these alterations were either less discernible or were not reported at all, except for the liver degenerations, which were attributed to the immunosuppressive activity of drugs used and not to the \textit{Candida} infection. The efficiency in the in vivo experimental candidiasis exerted by eugenol upon the tissues’ histoarchitecture allows us to seriously consider it as a promising antifungal active substance, useful therapeutically in genital candidiasis and, in our opinion, with comparable biologic activity to that of nystatin.

Key words: \textit{Candida albicans}, eugenol, genital infection, female rat model

1. Introduction

Although the range of \textit{Candida albicans} infections is generally considered to be well known, its various manifestations are still commonly reported both in animals and humans and are studied by many researchers. Candidiasis infections still remain, to this day, a problem to be dealt with (Henriques-Contente, 2004; Marlete, 2005).

Since fungal infections can have strong and extensive action and at the same time be highly resistant to treatment, candidiasis remains a current topic because of its medical importance, frequency, and recurrence of emerging infections. For example, vulvovaginal candidiasis is a fungal infection that affects, even today, about a third of all women at least once in their life. Approximately 5% of these women are subject to recurrent episodes of vaginal candidiasis (Fidel and Sobel, 1996; Fındık and Tuncer, 2002).

This fungal infection is often associated with other conditions such as diabetes, treatments with antibiotics or corticosteroids, pregnancy, and resistance to antifungals (Dupont et al., 1996; Hata et al., 1996; Heseltine et al., 2003; Jadhav and Pal., 2006), and many treatment strategies and new antifungal agents are being developed against this mycosis (Jones et al., 1976; Ghaly, 2009; Hanafi et al., 2010).

These results generated the need to supplement the existing information with an animal model experiment. In this context, we deemed necessary the development of candidiasis in laboratory animals, as a preface for studies aiming to confirm or refute the effectiveness of different...
regimens of eugenol. In this respect, our study proposes to compare the effects of eugenol with a classic compound, namely nystatin, and a placebo (saline solution) control group, by evaluating the cytohistological organ alterations in immunosuppressed Wistar female rats genitally infected with Candida albicans.

2. Materials and methods
Research was performed in compliance with good laboratory practice; in accordance with the European Convention principles for the protection of vertebrate animals used in experimental and other scientific purposes, adopted in 1986 in Strasbourg (ECPVÆOSP, 1986), and the 2010/63/EU Directive of the European Parliament and of the European Council adopted 22 September 2010 on the protection of animals used for scientific purposes (European Parliament, 2010); in accordance with Romanian law for animal experimentation; and with the acceptance of the Scientific Ethics Committee of the Faculty of Veterinary Medicine of Timisoara (Romanian Government, 2002). Euthanasia of rats respected the procedures advocated by SVH AEC SOP 26 (Pierce, 2006).

2.1. Animals
The study involved 27 healthy young Wistar female rats (divided into three, with experimental and negative control groups), aged between 39 and 41 days, purchased from the authorized biobase of the University of Medicine and Pharmacy "Victor Babeş", Timișoara. All females were in the peripubertal period and of approximately the same weight (average weight: 190 ± 10 g). The groups were kept in facilities especially built for this purpose, in cages with controlled temperature and light cycles (22 °C, light cycle of 12 h starting at 0800 hours), with access to food and water ad libitum.

2.2. Immunosuppression and genital infection scheme
To assist in the rapid progress of Candida albicans infection, immunosuppressed female rats were used for the study, according to the protocols developed by Sobel et al. (1985) and improved by Chami et al. (2004).

To induce immunosuppression, all female rats from the experiment were initially treated with dexamethasone (Dex) (Cortamethasone, Vetoquinol), and a 4% tetracycline soluble powder (Tc) (Laprophan) was also administered. All rats females were immunosuppressed for a week-long preinfection period. All rats were given 0.5 mg/L of Dex and Tc (0.1%) via drinking water.

During the infection period, the dose of Dex was increased to 1 mg/L while the Tc concentration was reduced to 0.01%, which was maintained throughout the experiment.

All female rats were infected genetically twice at intervals of 24 h (days 0 and 1), with 0.1 mL of saline suspension that contained 3.1 × 10³ (3108 viable cells) Candida albicans, strain ATCC 10231, provided from the fungi collection of the Department of Mycology of the Faculty of Veterinary Medicine, Timișoara.

2.3. Antifungal assay scheme
For comparative efficacy testing, natural eugenol was used (≥98%, FG) (C₁₀H₁₂O₂) along with nystatin (C₄₇H₇₅NO₁₇) (syn., Fungicidin, Mycostatin) (N4014, Sigma Nystatin), purchased from Sigma Aldrich (Germany). The experimental series consisted of three groups composed of fifteen rat females each:

- ICAE: Immunosuppressed females infected with C. albicans and treated with eugenol (nine female rats);
- ICAN: Immunosuppressed females infected with C. albicans and treated with nystatin (nine female rats);
- ICA: Immunosuppressed females infected with C. albicans and subjected to a placebo (saline solution), used as a negative control group (nine female rats).

The rats were infected on day 0 and day 1 of the experiment and treated over a 7-day period per group. Rats (three from each group/day) were euthanized successively at days 3, 6, and 9 after infection and fresh tissue samples were collected in order to carry out the comparative cytohistological investigation. Eugenol, nystatin, and saline solution administrations were done orally, diluted in 0.8% agar solution (CLSI, 2009), with syringe, twice a day for 8 days as follows:

- The ICAE group received 0.5 mL (~4 mg/animal).
- The ICAN group received a suspension of 0.5 mL (~0.054 mg/animal).
- The ICA group received saline solution at 0.5 mL/animal.

The entire experimental process is presented schematically in Figure 1.

2.4. Sample collections and cytohistological technique
The main cytohistological alterations were identified mainly in the following organs: liver, spleen, kidneys, and ovaries. For the intestines, lungs, uterus, and nervous system, changes were not relevant to this study. Fresh tissue fragments for the cytohistological investigation were fixed in 80% alcohol for 7 days, after which they were washed, dehydrated, and embedded in paraffin.

Paraffin blocks including fragments of tissue were sectioned using a microtome, yielding sections of 5 μm in thickness, after which they were mounted on glass slides with Mayer albumin following the technique described by Şincai (1996). The coloration was the classical hematoxylin-eosin (H&E) and microscopy was performed at 200× and 400× magnifications, the images being processed with an Olympus CX 41 microscope with image capture software and data interpretation.
3. Results
The results after treatments with eugenol were compared to those obtained following the administration of nystatin. The alterations observed resulting from immunosuppression and infection were reduced to moderate at the beginning phase of the experiment (days 3 and 6) and were eventually eliminated, according to our following analysis on day 9 of the study, probably due to the studied rats' healing resources.

The main histologic changes were observed on days 3 and 6, as presented in Figure 2. For the immunosuppressed infected females that were treated with eugenol and nystatin, the results were quite similar and did not indicate any major changes in organ histology; only representative histoarchitecture changes are presented.

3.1. Ovary
The histological analysis performed on ovaries (Figure 2A) sampled from the immunosuppressed, infected rat females treated with placebo revealed oocytes and degenerative lesions, accompanied by ovarian follicular edema in the cortical zone. For females that were treated with eugenol (Figure 2B) and nystatin, ovarian follicular edema in the cortical zone was observed. The similarity between the results from the experimental ICAE and ICAN groups can most likely be accredited as a result of the immunosuppression phenomenon and the degeneration of interstitial cells responsible for the secretion of sexual hormones in all groups.

3.2. Liver
In the liver (Figures 2C and 2D), on days 3 and 6, degenerative processes of the hepatocytes could be observed, together with hydropic dystrophy, karyopyknosis, and karyolysis, which affected the liver function and its cellular integrity in the immunosuppressed infected animals that were treated with eugenol or nystatin. These changes were more evident in the placebo group.

This reaction could be attributed to the hepatic immunosuppressive drugs in the case of the experimental groups treated with eugenol or nystatin. These alterations, however, manifested only for a short period of time and were not present after the sixth day of treatment, when the cytoplasmic and nuclear structure of hepatocytes had returned to normal as a consequence of the regenerative healing, an observation justified by the return to normal cytoplasmic and nuclear structure of hepatocytes and thus the liver's refunctionalizing.

These observations, although not backed by laboratory tests, show that although infection with Candida albicans causes a major effect on the liver within 6 days, this organ has remarkable regenerative capacity, as in our case after the sixth day and especially in the case of the eugenol group.

3.3. Kidneys (fig. 2 E-F)
After 3 days, in the immunosuppressed infected animals that were treated with placebo (Figure 2E), the emergence of large zones of degenerative phenomena was observed, manifested mainly by tumescence of convoluted tubule nephrocytes and Bowman's capsule destruction, followed by the disappearance of uriniferous spaces. This can lead to glomerulonephritis, the alterations being found irreversible after days 6 and 9. We observed that in the case of the immunosuppressed, infected specimens that were treated with eugenol or nystatin (Figure 2F), these alterations were much more reduced in intensity and extent.

3.4. Spleen
A dramatic reduction of the areas of leucopoiesis could be observed (Figures 2G and 2H). This had a major effect on the formation of white blood cells, but the erythropoiesis areas remained unchanged. It is also worth mentioning that spleen lymph nodes remained present in the form of lymphocyte cords, the C. albicans infection blocking the...
Figure 2. The main observed histologic changes in immunosuppressed female rats infected with *C. albicans*. A) Ovary from placebo group: degenerative lesions of oocytes [a], ovarian follicular edema [b] in the cortical zone, H&E 200×. B) Ovary from eugenol group: edema of ovarian follicles [a] in the cortical region, H&E 400×. C) Liver from placebo group: degenerative process of hepatocytes, degenerative process of hepatocytes, hydropic dystrophy [a], karyolysis and karyopyknosis [b], H&E, 400×. D) Liver from eugenol group: normal structure at 6 days, H&E, 200×. E) Kidney from placebo group: extensive degenerative phenomena manifested by tumescent nephrocytes of the distal and proximal tubule [a] and destruction of Bowman’s capsule [b], H&E 400×. F) Kidney from eugenol group: extensive degenerative phenomena manifested by slightly tumescent nephrocytes of the distal and proximal tubules [a], H&E 400×. G) Spleen from placebo group: dramatic reduction of the first phase areas of leucopoiesis [a], splenic lymph nodes remain or are present only in the form of cord lymphocytes [b], H&E, 200×. H) Spleen from eugenol group: reduction in the first-phase areas of leucopoiesis [a], H&E, 400×.
progress of reactivity and proliferation of leukocytes, as is known in infectious diseases.

4. Discussion
The antimicrobial and antifungal effects of the essential oils and specific activity of plants provided from spontaneous flora have been noted in numerous studies to date (Hammer et al., 1998; Giordani et al., 2004; Donaldson et al., 2005; Cavaleiro et al., 2006; Neves et al. 2009; Baykan Erel et al., 2012).

From the numerous compounds found in these oils, with certain studied antifungal activity, eugenol (4-allyl-2-methoxyphenol) is a phenolic compound present in many plants with proven healing bioabilities (Tan et al., 1998; Neves et al. 2009; Baykan Erel et al., 2012).

Knowing that candidiasis is a current topic in medicine (C. albicans being able to show an important deleterious activity, often followed by relapse), a great number of active structures were tested and are still being testing (including the natural compounds here), proving the importance of this fungal disease. Following our previous studies concerning microbiological and GC/MS analysis of plant compositions from Artemisia spp., it emerged that eugenol is a potent antifungal biocompound and thus an ideal candidate for further testing to develop novel ant-Candida phytotherapeutic means (Obistoiu et al., 2014). Following the experimental infection with C. albicans and after the therapy, the results showed that eugenol was highly effective in the treatment of genital candidiasis, probably due to its antioxidant/prooxidant activities. According to our results about eugenol activity, we agree with the findings of Özkan and Erdoğan (2013), who ascertained that incubation of mitochondria with eugenol resulted in the uptake of significant quantities of eugenol, which inhibited subsequent lipid peroxidation by acting as a chain-breaking antioxidant.

In another study, Nagababu et al. (2010), after liver damage was induced by administration of CCl4 in rats, proved that eugenol’s inhibitory activity was about five-fold higher than that observed for α-tocopherol and about ten-fold less than that observed for BHT. Eugenol also significantly inhibited the rise in SGOT activity and cell necrosis. The protective action of eugenol has been ascertained to be due to interception of secondary radicals derived from ER lipids rather than interference with primary radicals of CCl4 (respectively: CCl3/CCl3OO).

The efficiency in experimental genital candidiasis showed by eugenol allows us to consider it as a potent antifungal agent for candidiasis, with an activity comparable to that of nystatin. The cytohistological alterations observed by us in the immunosuppressed infected rats that were treated with placebo were the most apparent. We consider that eugenol, as a natural compound, has the advantage of volatile molecules that can penetrate areas inaccessible to other antifungal substances also having a low toxicity.

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References


