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EVALUATION OF TOPICAL APPLICATION OF NANOCURCUMIN SUSPENSION IN TREATMENT OF ORAL CANDIDAL INFECTION (EXPERIMENTAL STUDY)

Sarah N. Abd Elmonaem[1]* *MSc,* Eglal Moussa[2] *PhD,* Aliaa G. Aboulela[3] *PhD,* Marwa M. Essawy[4,5] *PhD,* Souzy K. Anwar[6] *PhD*

ABSTRACT

INTRODUCTION: Oral candidiasis is the most common human fungal infection resulting from infection of the oral cavity by yeast-like fungus, candida. Its treatment by available topical or systemic antifungal drugs has several problems including toxicity and the development of drug resistance. Hence recent studies have given much attention to molecules from natural sources. Therefore, curcumin is considered one of the promising natural antifungal drugs. It has antifungal properties, but its poor solubility in aqueous solvents results in poor oral bioavailability. To improve the properties of curcumin, recent trails used nanotechnology. The size of nanoparticles can help them to gain entry into cells increasing its solubility and improving its bioavailability.

OBJECTIVES: To evaluate the use of topical application of nanocurcumin in treatment of oral candidiasis and compare it to curcumin and nystatin in a murine model.

MATERIALS AND METHODS: Thirty-nine female mice were randomly divided into three groups of 13 animals each after induction of oral candidiasis. Group 1 received nanocurcumin at 64 µg/ml. Group 2 received curcumin at 128 µg/ml. Group 3 received nystatin 100000 U/ml. All animals were received treatment topically twice daily for 10 days. The clinical evaluation and microbiological analysis were at baseline, day 5 and day 10.

RESULTS: There was no statistical differences between the three studied groups in the number of cured animals at the end of the treatment period (p=0.358).

CONCLUSION: Nanocurcumin has a good antifungal effect but further research is needed to get the maximum benefits of these nanoparticles.

KEYWORDS: Curcumin, Nanocurcumin, Nanotechnology, Oral candidiasis.

RUNNING TITLE: Evaluation of nanocurcumin in treatment of oral candidiasis

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1 Oral Medicine, Periodontology, Oral Diagnosis and Oral Radiology department, Faculty of Dentistry, Alexandria University, Egypt.
2 Professor of Oral Medicine, Periodontology, Oral Diagnosis and Oral Radiology. Department of Oral Medicine, Periodontology, Oral Diagnosis and Oral Radiology, Faculty of Dentistry, Alexandria University, Egypt.
3 Lecturer of Microbiology, Department of Microbiology, Medical Research Institute, Alexandria University, Egypt.
4 Oral Pathology Department, Faculty of Dentistry, Alexandria University, Egypt.
5 Center of Excellence for Research in Regenerative Medicine and Applications (CERRMA), Faculty of Medicine, Alexandria University, Egypt.
6 Lecturer of Oral Medicine, Periodontology, Oral Diagnosis and Oral Radiology. Department of Oral Medicine, Periodontology, Oral Diagnosis and Oral Radiology, Faculty of Dentistry, Alexandria University, Egypt.

* *Corresponding Author:*

E-mail: sarah.hafez92@gmail.com

INTRODUCTION

Oral candidiasis is the most common human fungal infection especially in early and later life. It is an opportunistic infection of the oral cavity resulting from an overgrowth or infection of the oral cavity by a yeast-like fungus, candida (1). Candida albicans is considered the most common species that can develop this fungal infection. It is normally present in oral cavity, but in the presence of some local or systemic risk factors such as hyposalivation, denture wearers, patients that use immunosuppressive drugs, broad-spectrum antibiotics, anti-diabetic mediations, anticancer therapies, and in patients with the acquired immunodeficiency syndrome (AIDS), it can change to a pathogenic hyphal form and cause infection to the oral cavity (2,3).

Oral candidiasis can lead to local discomfort, altered taste sensation, dysphagia resulting in poor nutrition, and slow recovery. There are different types of oral candidiasis including acute pseudomembranous, acute erythematous, chronic plaque-type, chronic erythematous, denture stomatitis, median rhomboid glossitis, and angular cheilitis (4).

Management of oral candidiasis is based on four principles. First principle is to make a definite diagnosis. Second one is correction of the predisposing factors. The third one is to establish the type of candidal infection and use the appropriate antifungal therapy. Finally, is to maintain good and proper oral hygiene (5).

Studies showed that the use of available topical or systemic antifungal drugs have several problems including toxicity and development of drug resistance (6,7). Accordingly, studies have searched for new strategies to control fungal species and much attention has been given to natural products (8,9). From these molecules, curcumin (CUR) is considered as a promising natural antifungal drug (10).

Curcumin is a yellow pigment present in the spice turmeric (*Curcuma longa*). It has antioxidant, anti-inflammatory, antiproliferative, anticancer, antiangiogenic, antidiabetic, antibacterial and antiviral effects in addition to its antifungal properties (11,12). However, its optimum performance is limited by its poor solubility in aqueous solvents, which results in its poor oral bioavailability leading to poor absorption, fast metabolism, and quick systemic elimination (13,14). Additionally, curcumin is only soluble in organic solvents. So, the biological uses of it are limited due to the reported toxicity of these solvents (15).

To overcome this problem, numerous approaches have been used. One of them is using nanotechnology for synthesis of curcumin nanoparticles. The advantages of these nanoparticles are their small size, which helps them to gain entry into cells, increasing its solubility in addition to improving its bioavailability, the ability to be delivered to specific target sites, and their efficient delivery of proteins, nucleic acids and other small molecules (16).

Therefore, it is worthy to evaluate the topical application of nanocurcumin in treatment of oral candidal infection in comparison to curcumin and conventional antifungal treatment.

MATERIALS AND METHODS

Study Design

This experimental study was conducted in the Oral Medicine Department, Faculty of Dentistry, Alexandria University in cooperation with Microbiology department, Medical Research Institute, Alexandria University and Physiology department, Faculty of Medicine, Alexandria University and Center Of Excellence for Research In Regenerative Medicine and It is Applications. The study design carried out following the ethical guidelines for conduct of research on experimental animals, by the Faculty of Dentistry, Alexandria University (IRB NO: 00010556 – IORG 0008839).

A total of forty-two female mice, about 6 to 8 weeks old, weighting approximately 20 to 25 g were selected. The animals were adapted to the standard laboratory conditions of temperature, humidity, and light/dark cycle (12 h/12 h) and were given a standard diet during the study period.

Sample size and randomization

Sample size was estimated based on assuming confidence level= 95% and study power= 80%. Dovigo et al (17) reported mean ± SD log 10 of Candida albicans after nanocurcumin = 0.26±0. 59 CFU/ml while Sakima et al (18) reported mean ± SD after Curcumin and Nystatin= 4.95±0.6 and 3.37±1.85 respectively. The minimum sample size was calculated to be 13 mice per group which was increased to 14 mice per group to make up for possible attrition of the sample. The total sample size = number of groups × number per group= 3 X 14= 42 mice. Sample size was based on Rosner's method (19) calculated by Gpower 3.0.10 software (20).

The animals were divided randomly into three groups of 13 animals after the death of 3 during the induction of oral candidiasis. They were randomly assigned using a computer-generated list of random numbers to one of the three groups (21). They were received treatment for 10 days. Group 1 received nanocurcumin at 64 µg/ml topically twice daily. Group 2 received curcumin at 128 µg/mL topically twice daily. Group 3 received nystatin 100000 U/ml topically twice daily.

Materials

Curcumin powder was purchased from Alpha Chemika (Mumbai, India). The coating agents; Polyvinyl pyrrolidone (PVP; Mw 40,000) were obtained from Sigma-Aldrich (St. Luis, MO, USA). Acetone and all other reagents were of analytical grade and used as received. The used deionized water (DIH2O) was ultra-purified from Millipore Milli-Q system (resistivity ~80 MΩ cm). Prednisolone acetate was obtained from Egyptian Pharmaceutical Industries Co. (Cairo, Egypt). Tetracycline hydrochloride was obtained from Chemical Industries Development (Cairo, Egypt).

Methods

Synthesis and characterization of curcumin nanoparticles

The nanocurcumin particles were synthesized by solvent antisolvent precipitation method following our optimized protocol. The curcumin powder (Alpha Chemika, Mumbai, India) was dissolved in acetone (10 mg/ml) (El Nasr Pharmaceutical Chemicals Company, Gesr El Suez, Cairo, Egypt). One ml of the prepared solution was added to 15 ml deionized water (DIH2O) (ultra-purified from Millipore Milli-Q system "resistivity ~80 MΩ cm") containing polyvinyl pyrrolidone (0.5 % w/v) (Sigma-Aldrich, St. Luis, MO, USA) in drop-wise manner under stirring at 500 rpm for 1 min (21).

The synthesized curcumin nanoparticles were characterized by UV-Vis Spectrophotometer to measure the absorption spectra between 200-800 nm. The mean particles' size as well as surface charge was measured by dynamic light scattering analysis and Zeta potential, respectively. Transmission electron microscope examination of the nanoparticles was conducted to assess the shape and distribution of the particles (22,23).

Preparation of curcumin suspension

Curcumin at concentration of 128µg/mL dissolved in acetone to form curcumin suspension. The suspension was prepared daily for the reason of evaporation of the solvent.

Induction of candidal infection

A total of forty-two female mice, about 6 to 8 weeks old, weighting approximately 20 to 25 g were used for the study. They were adapted to the standard laboratory conditions of

temperature, humidity, and light/dark cycle (12 h/12 h) and given a standard diet during the study period.

- Before any procedure, the tongues of the animals were swabbed and showed a negative culture to *Candida*.
- Oral candidiasis in mice induced following the methodology described by Takakura et al. (24) and Mima et al. (25). All animals were immunosuppressed with two subcutaneous injections of prednisolone (Egyptian Pharmaceutical Industries Co., Egypt) at a dose of 100 mg/kg body weight 1 day prior to and 3 days after infection with *Candida*. Tetracycline hydrochloride (Chemical Industries Development, Egypt) in drinking water at the concentration of 0.83 mg/ml was given to the animals beginning one day before inoculation.
- The reference strain *C. albicans* ATCC90028 was used in the investigation. It was reactivated in Sabureaud Dextrose Agar medium culture (SDA) at 37°C for 48 hours. Then the strain was suspended in sterile saline (107 CFU/ml).
- To produce oral infection, the animals were sedated with chlorpromazine hydrochloride 0.1 ml (2 mg/mL) and then small cotton pads were soaked in a *C. albicans* cell suspension and the dorsal surface of the tongues of the animals were swabbed for 1 minute.
- Within 3 days after inoculation, 3 mice died.
- After 4 days from the infection, a cotton swab was rolled twice over the tongue of the 39 mice and used to inoculate in Sabouraud Dextrose Agar plates supplemented with chloramphenicol. After 48 hours of inoculation, the detected growth of candida confirmed the successful induction of candida infection in the oral cavity before the beginning of the treatment.

Study groups

The animals were randomly divided into three groups of 13 animals each after confirmation of the presence of oral candidal infection. All groups were received topical treatment using oral dropper and oral brush. The mice were immobilized during dosing in a supine position for 1 min till the suspension topically applied all over their tongues.

- Group I: received nanocurcumin at the concentration of 64 µg/ml twice topically daily (26).
- Group II: received curcumin at the concentration of 128 µg/ml topically twice daily (26).
- Group III: received nystatin 100000 U/mL topically twice daily (27).

The animals were received treatment for 10 days. The experiment was terminated, and all animals were sacrificed with an intramuscular injection of a lethal dose of ketamine and then discarded by incineration according to the guidelines of faculty of medicine.

Follow up

- Clinical evaluation

Clinical evaluation was done at baseline, day 5 and day10 by inspection and taking photographs to evaluate the progression of lesions on the tongue of the animals.

- Microbiological analysis:

Samples were collected at: baseline, day 5 and day 10 of treatment by rolling sterile cotton swab over the dorsal surface of the tongue of all animals. The end of the cotton swab was then cut off, placed in a tube containing 1 ml of sterile saline and vortexed for 1 min to resuspend the yeast cell then used to inoculate in Sabouraud Dextrose Agar plates supplemented with chloramphenicol for 48 hours. The evaluation of the antifungal effect was done according to the presence or absence (positive or negative culture) of C. albicans in the cultures at the evaluated time points.

Statistical analysis

The statistical software SPSS for Windows was used for data analysis (28). The comparison of the results between the three studied groups was done using **Monte Carlo** test**. Fisher's Exact** test was used to compare between each two groups separately.

RESULTS

Characterization of curcumin nanoparticles.

The synthesized curcumin nanoparticles were characterized by UV-Vis Spectrophotometer and the absorption spectra was between 200-800 nm with the absorbance peak at 419 nm. The average particles' size was 122.0 ± 2.704 nm and Zeta potential was -20.2 ± 4.48 mV when measured by dynamic light scattering analysis.

The curcumin nanoparticles exhibited regular spherical shapes with the particle size ranging from 17.97 nm to 87.61 nm when examined with transmission electron microscope.

Clinical evaluation

The immunosuppression of the mice rendered them susceptible to oral candidiasis. Oral candidiasis was established with a positive candida culture 4 days after candida inoculation. All infected animals with C. albicans showed red or/and white patches/pseudomembranes on the tongue dorsum, upon examination at day 4 after the inoculation.

Five days after treatment with topical nanocurcumin, one animal showed total remission of the tongue lesion and ten animals showed decreasing in the size of the lesions. The other two animals died before evaluation. Five animals in curcumin group showed total remission of the lesions upon clinical examination and five animals showed partial remission of the lesions. The other three animals died through 5 days of treatment. Dryness of the tongue and oral cavity was observed only in all animals treated with topical curcumin. Also, animals became ill, weight loss, anorexia and decrease in activity were observed. Animals treated with topical nystatin showed faster enhancement of the lesions with total remission in nine animals and smaller-sized lesions in four animals.

After 10 days of treatment, another four animals of nanocurcumin group showed total remission of oral lesions and the other animals maintained with smaller-sized lesions. All the remaining six animals in the curcumin group showed total remission of lesions with signs of dryness of the oral cavity and severe body weight loss. Four animals died before last clinical and microbiological evaluation. Finally, seven animals died from curcumin group throughout the 10 days of treatment. Concerning nystatin group, one animal showed total remission of the lesion and the other three animals

maintained with remaining small lesions. So that ten animals in nystatin group presented total remission of the lesions clinically at the end of the study.

Microbiological evaluation

Concerning the number of cured animals (negative cultures) among the three study groups, there was no statistical difference at day 10 of treatment (p=0.358). After 5 days of treatment, there was a statistical difference only between nystatin and curcumin group (P=0.046). The percentage of the number of cured animals in nystatin group was 46.15% (6 mice) at the end of the treatment period which is the highest percentage among the three groups. However, the percentages of the nanocurcumin and curcumin groups were (18.2%) (2 mice) and (33.3%) (2 mice) after 10 days of treatment (Table 1).

Table 1: Comparing the Number of cured animals (negative cultures) at 5 and 10 days among the study group according to microbiological assessment.

	Group I	Group II	Group III	^{MC}p	$^{FE}p_1$	$^{FE}p_2$	$^{FE}p_3$
Number of cured animals							
5 days	1/11 (9.1%)	0/10 (0.0%)	5/13 (38.5%)	0.042*	1.000	0.166	0.046*
10 days	2/11 (18.2%)	2/6 (33.3%)	6/13 (46.2%)	0.358	>0.05	>0.05	>0.05

MC: **Monte Carlo** FE**: Fisher Exact**

p: p value for comparing between the three studied groups

p_1: p value for comparing between **Group I** and **Group II**

p_2: p value for comparing between **Group I** and **Group III**

p_3: p value for comparing between **Group II** and **Group III**

*: Statistically significant at $p \leq 0.05$

Group I: Nanocurcumin, **Group II:** Curcumin,

Group III: Nystatin

DISCUSSION

Curcumin is as a natural compound widely used in herbal medicine. It has a potent antifungal effect (10). However, the maximum benefits of curcumin are limited due to its poor pharmacodynamic action in vivo. This is on account of its poor water-solubility which results in its poor oral bioavailability leading to poor absorption, fast metabolism, and quick systemic elimination (13,14). Nanotechnology overcomed these stability and bioavailability problems associated with poorly soluble drugs (16). In the current study we aimed to evaluate the in vivo antifungal effect of topical nanocurcumin in treatment of oral candidiasis of mice.

Nanocurcumin was prepared by solvent-antisolvent precipitation method which is considered a suitable technique for synthesis of poorly soluble curcumin nanoparticles (22). In our study, the average particles size of nanocurcumin was 122.0 ± 2.704 nm. These particles of the nanocurcumin synthesized are considered as nanoparticles as they fall into the nanoscale range of 1–300 nm according to the National Organic Standards Board guidance (NOP, 2010) (29). The particle size of nanocurcumin is considered the most important physical characteristic which is responsible for its efficacy when compared to free curcumin. This allows nanocurcumin to reach the organs which are inaccessible for curcumin (30).

In this study, female mice were the animal model for induction of oral candidiasis which provides a useful tool to evaluate the therapeutic activity for antifungal agents under experimental conditions. White patches were clinically observed on the dorsal surface of the tongues of immunosuppressed mice. When these white patches were mechanically removed, the tongue showed a reddish and irregular surface. In accordance with Takakura et al (24); these observations indicate that the clinical presentation of the mice model closely mimics the pathological condition seen in patients with oral candidiasis.

All mice were negative to oral candida before starting the study. This was confirmed by swabbing the oral cavities of the mice, and then these swabs were used to inoculate in culture plates and were given negative cultures to candida. This procedure was done to exclude the presence of oral candidiasis in the animals before starting the induction of candida infection.

The clinical evaluation was done in this study by inspection and taking photographs to evaluate the progression of lesions on the tongue of the animals through the treatment period.

All animals, in the three groups, showed better clinical presentation of the lesions after the 10 days of treatment when compared to the result before treatment.

Mice treated with topical nystatin showed the best clinical presentation after treatment with ten animals showed total remission of the tongue lesions and only 3 animals maintained with partial remission with clear observed decrease in the size of the lesions. In our study, we used the antifungal nystatin in a positive control group as a comparison parameter because it is considered the most commonly used topical antifungal for the treatment of oral candidiasis (31).

In another study used topical nystatin once daily, all animals showed partial remission of lesions after 5 days of treatment (32). In contrary, 69% of the animals showed total remission of the lesion after 5 days of treatment. This may be because we used nystatin twice daily so that our results were better.

The other group was treated with curcumin at 128 µg/ml. This concentration was prepared following the minimum inhibitory concentration (MIC) of curcumin determined by Baiji Xue et al (26) against the reference strain *C. albicans* ATCC90028 which is the same strain used in our study. The MIC was defined as the lowest concentration of the agents that inhibited visible fungal growth by 100% compared with drug-free control.

In our study, all remaining mice treated with topical curcumin showed total remission of the oral lesions clinically at the end of treatment period and this supports that curcumin is a good antifungal compound.

Though the effectiveness of curcumin clinically, dryness of tongue and oral cavity was observed only in all animals in this group. Besides, animals became ill, weight loss, anorexia and decrease in activity were observed along the treatment period.

Curcumin is water insoluble and highly soluble in organic solvents. These solvents have side effects on mucosa including dryness and cytotoxicity, which may give explanation for the illness and death of the animals in this group (15).

Studies evaluated the antifungal effect of curcumin in vitro due to its low pharmacokonitics in vivo. All these studies showed that curcumin is effective against a lot of fungi including candida species (33-35) but in our study we evaluated the topical antifungal effect curcumin in vivo and compare it with the nanosized curcumin.

To our knowledge, this is the first study to evaluate the effectiveness of topical nanosized curcumin in the treatment of oral candidiasis in vivo. Several studies evaluated nanocurcumin in vivo using dendrosomal nanocurcumin or Curcumin-Silk Fibroin nanoparticles systemically for treatment of systemic candidiasis and compare it with curcumin (25,36). All the results of these studies showed that nanocurcumin had more potent antifungal properties than native curcumin.

In contrary, the nanocurcumin group in our study showed clinical enhancement after 10 days with complete remission of the lesions for 5 animals and partial remission for 6 animals. So nanocurcumin was needed more time than curcumin and nystatin to show enhancement clinically.

Concerning the microbiological evaluation, there was no significant difference between the three studied groups in the number of cured animals (negative cultures) at the end of the treatment. Nystatin group has the highest percentage (46.15) of cured animals (6 animals) at the end of the treatment. This percentage is almost close to the result of the study by Bassiri-Jahromi et al. (37) with 40% of the animals cured after 10 days of treatment with nystatin although it was applied once daily.

There was no statistically significant difference between nanocurcunin and nystatin groups at the two evaluation time points. Thus nanocurcumin has antifungal effect like nystatin and can be used to solve the resistance problems associated with nystatin and other antifungal drugs.

Also, there was no statistically significant difference between nanocurcunin and curcumin groups in the microbiological results through the treatment period. Furthermore, nanocurcumin did not show the dryness and illness problems as seen with curcumin. Thus, nanocurcumin can be considered a new treatment modality to enhance the solubility of the native curcumin and avoid the toxicity of its organic solvent.

Hence, for the first topical application of nanocurcumin in vivo, further studies are recommended to modulate the concentration and time needed for the treatment of oral candidiasis.

CONCLUSION

Nanocurcumin has a good antifungal effect as curcumin and nystatin but further research are needed to get the maximum benefits of these nanoparticles.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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