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A study of the treatment of cutaneous fungal infection in animal model using photoactivated composite of methylene blue and gold nanoparticle

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ABSTRACT

Background: Onychomycosis is a widespread public health problem, in which *T. rubrum* and *T. mentagrophytes* is the commenest causative organisms. Current medical therapy has many drawbacks and side effects. Methylene blue (m.b) photodynamic therapy (pdt) proved efficacy but with lengthy sessions.
Objectives: Optimizing methylene blue photodynamic therapy by combination of methylene blue photosensitizer and gold nanoparticles (aunps) in a composite as gold nanoparticles are efficient delivery systems and efficient enhancers of photosensitizers for antifungal photodynamic therapy.
Materials and methods: Eighty newzealand rabbit (*Oryctolagus cuniculus*) were used and categorized in eight equal groups as follows; healthy and infection control, composite photodynamic therapy and five comparative groups. Photodynamic therapy was initiated at day three to five post inoculation, for four sessions forty eight hours apart. Each group divided and light exposure at two fluencies; 80 J and 100 J. All groups were investigated macroscopically and microscopically (histopathology and scanning electron microscope) also flowcytometry assessment for cell death and X-ray analysis for gold nanoparticles accumulation in brain and liver tissues were determined.
Results: Recovery from infection approaching 96% in gold nanoparticles + light group, around 40% in methylene blue photodynamic therapy and 34% in composite photodynamic therapy. The observed findings confirmed by apparent decrease of apoptosis, however small amounts of gold nanoparticles detected in brain and liver.
Conclusion: Light stimulated gold nanoparticles is a promising tool in treatment of onychomycosis.

1. Introduction

Onychomycosis is a widespread public health problem [1]. Most onychomycosis are caused by dermatophytes of which *T. rubrum* and *T. mentagrophytes* are the first and second most frequent aetiologic agents worldwide [2]. Oral and topical treatment of onychomycosis has long course, multiple side effects, several drug interactions and drug resistance [3]. Photodynamic therapy (PDT) may have the potential to treat onychomycosis locally without adverse systemic effects [4]. *T. rubrum* and *T. mentagrophytes* are susceptible to *in-vitro* Methylene Blue (M.B) photodynamic therapy [5] and to *in-vivo* methylene blue photodynamic therapy using solar stimulator [6]. Methylene blue photodynamic therapy proved increased efficacy in comparison to fluconazole in treating onychomycosis but the drawbacks of lenghthy sessions and duration of treatment course remains unsolved [7]. In a trial to overcome the drawbacks and to augment the action of methylene blue photodynamic therapy, we used combination of methylene blue dye and Gold NanoParticles (AuNPs) in a composite as gold nanoparticles are efficient delivery systems [8] and efficient enhancers of photosensitizers for antifungal photodynamic therapy [9]. Also Light stimulated gold nanoparticles can provide *in-vitro* inhibitory effect on dermatophyte spore germination [10]. The purpose of the study is to assess the effect of combining methylene blue photo-

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sensitizer and gold nanoparticles in a composite in photodynamic therapy of cutaneous fungal infection in rabbits caused by one of the commonest organisms in onychomycosis which is *Trichophyton mentagrophytes*.

2. Materials and methods

2.1. Photosensitizers

2.1.1. Preparation of methylene blue (M.B)

A stock solution of 1.0 g in about 25 ml of distilled water was prepared and kept at 2–8 °C. M.B was used at a concentration of 10^{-7} mg/ml. All chemicals were purchased from sigma (USA).

2.1.2. Preparation of gold nanoparticles (AuNPs)

Gold nanoparticles were synthesized in colloidal form by a modified Turkevich-Frens method [11]. First, 1 mM $HAuCl_4$ (Sigma-Aldrich, St. Louis, MO) was mixed with 20 ml distilled water and kept on a hot plate in stirring condition at about 80 °C. A 1% of trisodium citrate dehydrate ($Na_3C_6O_7{\cdot}2H_2O$; Sigma-Aldrich) reducing agent was added to the solution in stirring condition. Gradually, the color of the solution changed from transparent to red. After 10 min it changed to a deep red wine color, indicating colloidal gold nanoparticles formation. After the change in color, the solution is refluxed for an additional 15 min, then the heater is turned off and the solution is stirred until it reached room temperature to control the particle size and thus achieving a narrow particle size distribution. The nanoparticles made in this way typically have average size around (13 ± 2) nm as determined by transmission electron microscope imaging.

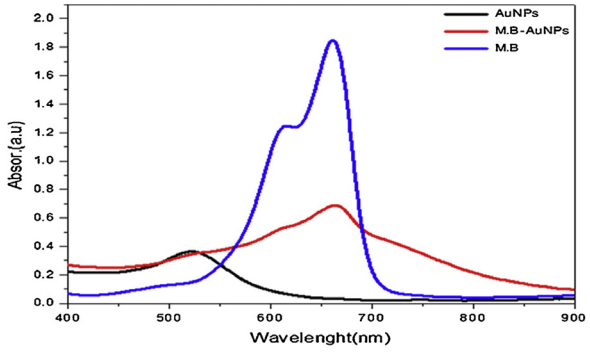

Fig. 1. The absorption spectrum of methylene blue (M.B), gold nanoparticles (AuNPs) and the spherical methylene blue gold-nanoparticles composite (M.B-AuNPs).

2.1.3. Preparation of methylene blue-gold nanoparticles composite (M.B-AuNPs)

Methylene blue loaded gold nanoparticles was prepared by adding 0.001 ml aqueous solution of M.B to 100 ml of the synthesized AuNPs, and stirred under ultra-sonication to enhance the interaction between the M.B and AuNPs. The color changed from red to violet and blue according to the amount of M.B added. The absorption spectrum curve of M.B, AuNPs and M.B-AuNPs composite were characterized *via* absorption spectra and Transmission Electron Microscope imaging (Figs. 1 and 2a and b). According to our experimental section, 0.001 M of M.B has been loaded into the same concentration of the AuNPs (1×10^{-3} M).

2.1.4. Spectroscopic characterization of synthesized goldnanoparticles

UV/visible spectrophotometer (SHIMADZU, Japan, model UV21650 PC) was used to monitor synthesis of nanomaterials and detect their absorption bands. Transmission Electron Microscope (TEM) analysis (JEOLTEM-2100) at 200 kV was carried out AuNPs and M.B-AuNPs composite.

2.2. Light sources

Light was delivered topically using a non-coherent Light Emitting Diode (LED) (Photon Scientific, Cairo, Egypt) that provides spectrum of visible light in two peaks one in red (650 nm), the other in green (530 nm). Emission spectra and light power as supplied by manufacturer.

2.3. Inoculum preparation

A clinical isolate of *T. mentagrophytes* was obtained from mycology unit, Clinical Pathology Department of Mansoura University, Mansoura, Egypt and maintained on Sabouraud dextrose agar (Difco) containing 0.05 mg/l chloramphenicol at 30 °C for 14–21 days. Fungal colonies were covered with 5 ml of sterile saline solution (NaCl 0, 85% w/v). The surface gently scraped with a sterile loop and this resultant mixture of fungal units was transferred to a sterile tube. The turbidity of the final inoculum was standardized according to McFarland scale 0.5 tubes and adjusted for presenting the fungal population of 10^6 colony former units (CFU). The confirmation of the inoculum quantification was made by plating 0.01 ml of inoculum suspension in Sabouraud dextrose agar (SDA). The plates were incubated at 28 °C and were examined daily for the presence of fungal colonies till growth became visible [12]. At the end of the incubation period, cellophane tape preparations were used to quantitate the conidial formation microscopically. The numbers of conidia were determined in five viewing fields. The average numbers of conidia were calculated. The data were expressed as conidial formation as a percentage of *T. mentagrophytes* isolates forming conidia on different agar media.

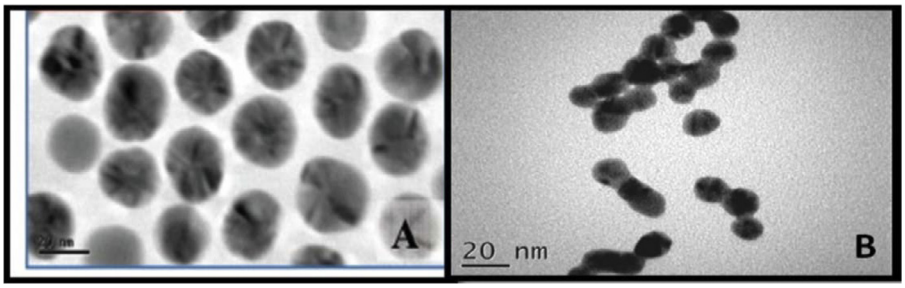

Fig. 2. (A) Transmission Electron Microscope (TEM) images of gold nanoparticles. The size and shape of the gold nanoparticles were measured by TEM imaging. The particles show mostly spherical or elliptical shapes with highly uniform in size and shape. (B) TEM images of methylene blue-gold nanoparticles composite.