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Antioxidant effect of green synthesized silver nanoparticles on moderate local heat burn injury

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By penetrating the damaged blood vessels walls, the free radicals formed in the heat burn wound field lead to oxidative stress-induced distant organs failure, latter being the most serious consequences of the burn injury. The oxidative stress (OS) may be successfully diminished by administering scavengers of free radicals along with substances preventing bacterial superposing. Silver is a well known highly efficient antimicrobial agent against antibiotic-resistant bacterial strains, with no registered allergic reactions. Recently silver nanoparticles on carriers (AgNPs) are preferred in wound treatment. In the present work, glycerol was used as a medium for AgNPs synthesis from $AgNO_3$ and green tea extract. The resulting AgNPs suspension was applied on local moderate rat's heat burn wound for 1 to 5 days. The OS in the rat blood serum was monitored for 5 days. The topical treatment with green synthesized AgNPs resulted in a decreased OS level in the serum than those for the untreated burned animals.

Keywords: Heat burn, Systemic circulation, Oxidative stress, Silver nanoparticles, Green nanosynthesis, Rat's blood serum.

INTRODUCTION

The heat burn injury results in local oxidative stress (OS) and immediate and long lasting inflammatory response, producing toxins, inflammatory cytokines and free radicals which penetrate through the damaged blood vessels walls [1-3]. Once in the systemic circulation, they simultaneously induce OS and generalized inflammatory response in which the OS is a component, resulting in distant organs damage [4]. Under strict and efficient control by the antioxidant defense of the patient, the local OS is involved in wound healing [1,5,6], but if this control fails, the systemic inflammation and generalized OS prevail [1,3]. The heat burn-inflicted OS is clinically controlled by co-treatment with antioxidants and silver-containing bactericidal compositions [1,7-9].

Most of the topical compositions for burn treatment contain silver salts or silver nanoparticles (AgNPs) [10-12], the biological action of the latter being intensively investigated [13-15]. Despite the controversial data (mostly *in vitro*) about the toxicity [16,17] and efficacy of AgNPs in wound healing [10,18], the green synthesized AgNPs have been found to be promising for possible application in patients [19-24].

The bactericidal properties of glycerol [25,26], green tea extract [27,28] and green tea-synthesized AgNPs [29,30] grounded our attempt to produce green tea-synthesized AgNPs in glycerol medium and to investigate their effect on heat burn-induced OS in the systemic circulation in a rat model of moderate local heat burn injury.

MATERIALS AND METHODS

Materials and solutions

All chemicals were of finest grade, (Sigma-Aldrich). Distilled water, 96% ethanol and pure glycerol were used as solvents. Dried green tea leafs (Ahmad Tea, London) were used in this investigation. Several aqueous solutions were prepared at room temperature: 50 mM K,Na-phosphate buffer of pH 7.45 (PBS), 3 mM xanthine (X), and 3 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The following ice-cold aqueous solutions were prepared immediately before being used: one containing 3mM $FeCl_2$, 3 mM H_2O_2, and 0.4 mM EDTA; one containing 4 mg/ml ascorbate; one of 3 mM $FeCl_2$ and one of 3 mM H_2O_2. Glycerol was used to prepare 1 mM $AgNO_3$ solution.

Preparation of the green tea extract

Dried green tea leafs were kept in glycerol (100 mg dried leafs/ml) at 60°C for 10 min, followed by filtration using yellow and blue filters.

Preparation of the AgNPs

One ml of 1mM $AgNO_3$ was vigorously mixed for 15 min (Vortex) with 0.250 ml of the green tea extract, then sonified for 15 min and left at room

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temperature for 1 h. After 24 h rest in dark at room temperature, it was used for topical wound treatment in a rat model of moderate local heat burn. The UV spectra and transmission electron microscopy (TEM) micrographs proved that this was AgNPs suspension.

Animal models

21 male Wistar albino rats (200±30 g) were housed in individual standard cages, kept at room temperature (25±0.5 °C), standard humidity (60±1 %) and light/dark (12/12 h) cycle, receiving standard rodents food, and tub water *ad libitum*. All animals were treated in agreement with the general regulations for treatment of experimental animals, established by the Ethics Committee of the Medical University of Sofia, in agreement with EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

After 4 days of adaptation, the rats were separated into 7 groups (n=3). The negative control group (Control) wasn't injured or treated. All other animals were anesthetized by injecting 1mg/100g BW xylasine and 7.8 mg/ 100g BW ketamine, and shaved on the back. Area of 1 cm^2 was burned with stainless steel rod heated to 100°C as described by Cai *et al.* [31]. Half of the injured rats were separated in groups named "1 day", "3 days" and "5 days", depending on the number of days after infliction of the heat burn injury. The wounds of the rest ones were treated locally with 0.04 ml/cm^2 of AgNPs suspension once per day for 1, 3 or 5 days after the injury, forming groups "1 day+AgNPs", "3 days+AgNPs" and "5 days+AgNPs". After 1, 3 or 5 days, the corresponding groups were exterminated under anesthesia (100 mg/ 100g BW of ketamine).

Blood serum isolation

Briefly, the total blood was left at room temperature to coagulate and further centrifuged at 4000 rpm at 297K for 10 min (Zamezki K-24) [32]. The amount of proteins in the serum was determined [33].

UV spectral and spectrophotometric measurements

UV spectral and spectrophotometric measurements were performed using Shimadzu 1600 UV-VIS spectrophotometer equipped with software program package and connected to a PC.

Free radicals accumulation (FRA)-assay

FRA in the serum was initiated by the Fe(II)/H_2O_2/EDTA/ascorbate model system, in PBS medium. The formation of MTT-formazan from MTT was used as a marker. The relative increase of the absorption at 578 nm (characteristic for MTT-formazan) was monitored for 10 min using the kinetic software of the spectrophotometer. FRA was evaluated using the formula:

$$FRA = \frac{\Delta A_{blank} - (\Delta A_{sam.} - \Delta A_{contr.})}{\Delta A_{blank}} * 100 .$$

For the blank measurement the cuvette contained 0.05 ml Fe(II)/H_2O_2/EDTA, 0.05 ml ascorbate, 0.2 ml MTT, and PBS to 2 ml., for the control - 0.2 ml MTT, serum containing 1 mg/ml proteins and PBS to 2.0 ml, and for the sample measurement - 0.05 ml Fe(II)/H_2O_2/EDTA, 0.05 ml ascorbate, serum containing 1mg/ml proteins, 0.2 ml MTT, and PBS to 2.00 ml. The FRA of a group was presented as a percentage of that for group C.

Xanthine oxidase (XO) activity assay

Small amount of 3mM xanthine (X) introduced in the serum produced uric acid (UA) by reacting with the endogenous XO. The sample cuvette contained serum corresponding to 1 mg/ml proteins, 0.01 ml X, and PBS to 2 ml. For the control measurement the X was omitted, and for the blank measurement the serum was omitted. The relative change of the absorbance at 293 nm was measured for 10 min. One unit of XO activity was defined as the amount of XO needed to transform 1 μm of X to UA for 1 min. The XO activity for each group was presented as a percentage of that for group C.

Assay for MDA

The MDA accumulation in the serum was estimated by measuring the relative increase in absorption of the MDA characteristic band, A(245 nm), due to $OH^{\bullet}$-initiated lipid peroxidation in presence of the Fe(II)/H_2O_2 model system. The MDA accumulation was estimated using the formula:

$$MDA = \frac{\Delta A_{sample} - (\Delta A_{control} - \Delta A_{blank})}{\Delta A_{blank}},$$

ΔA being the relative change of the A(245 nm) for 10 min. The index "sample" corresponded to ΔA in presence of serum containing 1mg/ml proteins, 0.05 ml $FeCl_2$, 0.01 ml H_2O_2 and PBS to 2 ml. For the "control" measurement the $FeCl_2$ was omitted, and for the "blank" measurement the serum was omitted. The results for MDA were presented as percentage of the MDA content in the serum of group C.

UV spectra

The UV spectra were collected in the range of 600- 200 nm in a quartz cuvette at a very low speed

against glycerol. The Shimadzu instrumental limits for absorption detection were ±3.999 a.u. If the absorption of a band in a spectrum of a compound exceeded ±3.999 a.u, the compound was diluted until its spectrum appeared within the instrumental limits. The instrumental error was estimated by recording the spectrum of solvent against solvent and was found to be ± 2 nm in position, and ± 0.005 a.u. in absorption.

TEM

A drop of AgNPs suspension was fixed on a standard Cu grid covered with amorphous carbon and dried at room temperature in pure atmosphere for 24 h. Then the grid was mounted on the holder and introduced in the microscope for visualization of the particles morphology with TEM bright field mode. The TEM study of AgNPs particles was performed on the transmission electron microscope JEOL JEM 2100 at 200 kV accelerating voltage. The particle-size distribution was determined by measuring the linear diameter D of particles at different magnifications, and was based on a general population of 576 measured particles.

Data management

Each experimental point in the biochemical experiments was presented by average value and standard deviation of 5 parallel measurements. The statistical analysis was performed using One way ANOVA and Bonferoni post-test.

RESULTS

Fig.1a displays the UV-VIS spectra of the reactants (1 and 2) and that of the reaction product (3), recorded between 600 and 200 nm against glycerol. Below 300 nm the absorption in the spectrum of the green tea extract (Fig.1a, spectrum 1) was above 3.999 a.u.. The green tea extract was diluted 10 times in glycerol and its spectrum is presented in Fig.1b. The UV spectra were resolved in agreement with literature data for UV spectra of green tea [34-36] and AgNPs [16,17,37-43]. In the UV spectra of the green tea extract (Fig.1a, spectrum 1, Fig.1b) there are characteristic bands resulting from superposition of characteristic bands for epigallocatechin (EGC) at 340 and 240 nm, and those for epigallocatechin gallate (EGCG) at 272 and 212 nm.

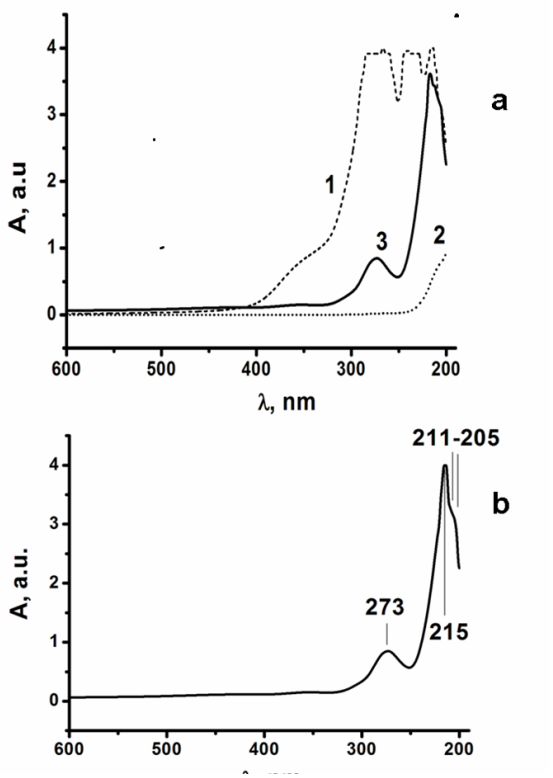

Figure 1. UV spectra of: (a) the reactants and the reaction mixture after 24 h, and (b) of green tea alone, diluted with glycerol 1:10 (v/v): a,1- 0.25 ml green tea extract in glycerol and 1 ml glycerol; a,2- 1.00 ml $AgNO_3$ solution in glycerol and 0.25 ml glycerol, a,3- 0.25 ml glycerol extract of green tea and 1.00 ml 1mM glycerol solution of $AgNO_3$.

The spectrum of the AgNPs suspension (Fig.1a, spectrum 3) was much more similar by intensity and bands to that of the 10-fold diluted (Fig. 1b), than to the spectrum of the undiluted green tea reactant (Fig.1a, spectrum 1). This might be explained with a decrease in concentration of the green tea extract in the suspension. The UV spectrum of the AgNPs suspension against green tea extract is shown in Fig. 2a. Similar to other studies [44-46], the characteristic band at 222 nm may be associated with $\pi \rightarrow \pi^*$ transitions of isolated C(OH)=O groups, while that at 248 nm is associated with $\pi \rightarrow \pi^*$ transitions in conjugated double bond systems in the organic molecules, or/and of phenolic O-H bond vibrations strongly affected by the solvent. The wide and intensive band with maximum at 431 nm is typical for the plasmonium resonance spectrum of AgNPs. The existence of AgNPs in the suspension was proved by the TEM micrograph (Fig. 1b). The size distribution of the AgNPs (Fig. 1b) showed monodispersity with average D=4.74±0.13 nm.

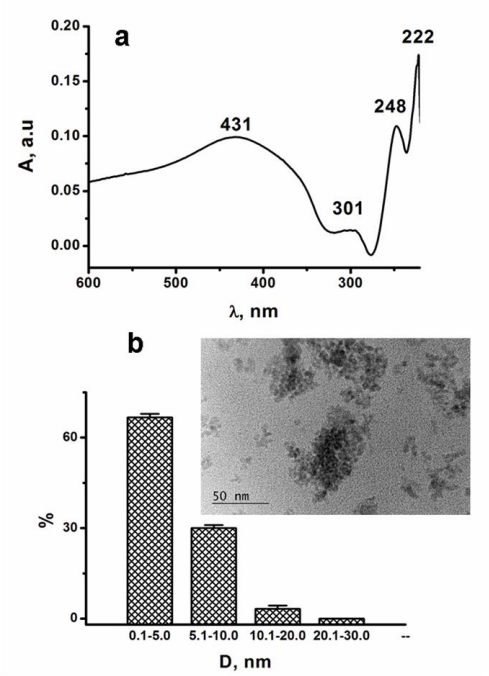

Figure 2. UV (a) and TEM (b) characteristics of Ag in the reaction mixture 24 h after the reaction.

Data in Fig. 2 confirmed the synthesis of AgNPs from green tea and $AgNO_3$ in glycerol, while Fig. 1 suggested a possible involvement of the green tea extract in capping of the AgNPs.

The effect of the moderate local heat burn injury on the OS level in the rat systemic circulation is illustrated in Fig. 3.

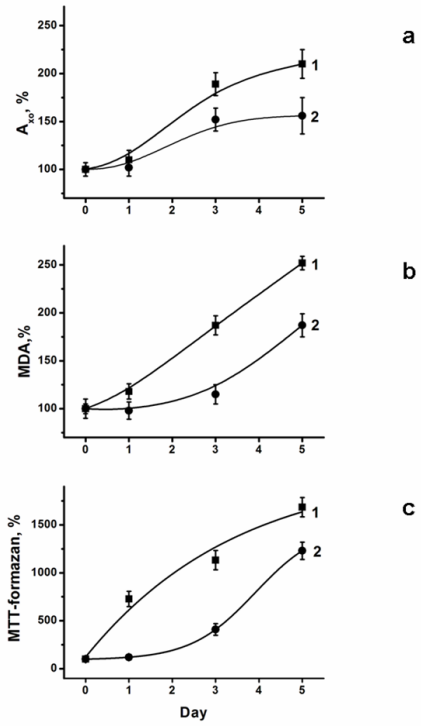

Figure 3. OS level in the blood serum of the untreated (curves 1) and treated (curves 2) positive controls as a percentage of that for the negative control group: (a) activity of XO, (b) MDA- accumulation, (c) free radicals accumulation.

All OS markers increased with time after the burn, in agreement with other studies [1-4]. The XO activity (Fig. 3a), the MDA levels (Fig. 3b) and FRA (Fig. 3c) were higher in the serum of the untreated (curves 1) than in the serum of the corresponding treated (curves 2) burned groups.

DISCUSSION

We experimentally proved the green synthesis of small AgNPs (4.74±0.13 nm) in glycerol. The UV spectra suggested a possible involvement of the green tea extract in capping of the AgNPs. Topical wound treatment with AgNPs suspension led to a decreased blood serum OS level in a rat model of moderate local heat burn injury. This allowed to suppose that the newly synthesized AgNPs suspension could diminish the risk of burn-induced OS in the systemic circulation, in this way decreasing the rate of OS-induced distant organs damage. In agreement with the literature data on the effects of any of our reactants and glycerol medium, and based on our research it might be assumed that the good control over the OS level in the rat blood serum resulted from the combined effects of the green tea extract, glycerol and AgNPs.

CONCLUSIONS

1. Small AgNPs (4.74±0.13 nm) were synthesized using green tea extract in glycerol medium.
2. Topical application of the AgNPs suspension significantly diminished the OS in the blood serum in a rat model of moderate local heat burn injury, in this way decreasing the risk of systemic OS.

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АНТИОКСИДАНТЕН ЕФЕКТ НА СРЕБЪРНИ НАНОЧАСТИЦИ, ПОЛУЧЕНИ ЧРЕЗ ЗЕЛЕН СИНТЕЗ, ПРИ ЛОКАЛНО ИЗГАРЯНЕ ОТ СРЕДНА СТЕПЕН

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(Резюме)

Проникващи в системното кръвообращение през увредените кръвоносни съдове, свободните радикали от раната при термично изгаряне достигат до отдалечени органи и предизвикват там оксидативни увреди. Това е едно от най-тежките усложнения при изгарянията. Оксидативният стрес може да бъде понижен при едновременното прилагане на радикалови скевинджъри и антибактериални препарати. Среброто е добре познат антибактериален агент с висока ефективност по отношение на резистентни на антибиотици щамове, без регистрирани алергични реакции към него. Напоследък при лечението на рани все по-предпочитани са материали и консумативи, съдържащи сребърни наночастици (СНЧ). В настоящето изследване СНЧ са синтезирани от екстракт на зелен чай и $AgNO_3$ в среда от глицерол. Получената суспензия на СНЧ бе приложена за третиране на термично кожно изгаряне при модел на плъх, за период от 1 до 5 дни. Бе оценено нивото на оксидативния стрес в кръвния серум. Третирането на раната със суспензията от СНЧ доведе до намаляване на оксидативния стрес в серума на третираните в сравнение с този при нетретираните животни с рани от изгаряне.