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Original Article

Effect of Cinnamon Nanoparticles in Presence of HAMLET on Healing of Wounds Infected with *Pseudomonas aeruginosa*: An Animal Model Study

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ABSTRACT

The objective was to evaluate the ability of cinnamon nanoparticles (CNPs) in the healing of wounds with *Pseudomonas aeruginosa* (PAE) infection as well as HAMLET sensitization in rats. Fifty healthy male Wistar rats were used in this study. The rats were divided into 5 groups (n = 10), randomly. In the NORMAL group, no infected wounds were treated with a sterile solution of saline 0.9% (0.1 ml). In the PAE group, the wounds with *Pseudomonas aeruginosa* infection were only treated with a sterile solution of saline 0.9% (0.1 ml). In the PAE-HMLT group, HAMLET (100 µg) was used to treat infected wounds. In the PAE-CNM group, 1 mg/ml CNPs (0.1 ml) were applied topically to treat PAE-infected wounds. In the PAE-HMLT-CNM group, HAMLET (100 µg) and 1 mg/ml CNPs (0.1 ml) were applied topically to treat PAE-infected wounds. Microbiological examination, planimetric and biochemical showed a significant difference between rats in the PAE-HMT-CNM group in comparison with other groups ($p < 0.05$). In conclusion, CNPs could offer the potential to pay more attention to this harmless and easily available agent to be topically applied in wounds with infection.

Introduction

Antibiotics are used to manage wounds; however, common serious issue is drug resistance. Hence, it is vital to design novel treatments to minimize wound healing process.[1-4]

Pseudomonas aeruginosa is still considered as chief cause of nosocomial infection that could end up septicemia and mortality in patients with wounds.[5] Local anti-bacterial agents and disinfectants were demonstrated to be effective against infection, however, the incidence of allergic reactions and skin irritations to these agents have diminished the rate of skin regeneration and lead to a rise within the recovery period.[6,7] It has been demonstrated that HAMLET also bears bactericidal activities *in vitro*. It has been indicated that in *in vitro* and *in vivo* conditions.[8,9] Nowadays, products from various fragments of the plants (aromatic types) have been widely applied for medical intentions.[10] Like nanomaterials of metallic origin, the nanoparticles of organic origins, natural herbs, are preferred because of the existence of activated agents with biocompatible properties that are abundant, easily stabilized and safely handled.[11] Cinnamon cassia (bearing polyphenol and cinnamaldehyde compounds) have been utilized extensively all over the world as because of bearing

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healthy nutrition components. Irreplaceable qualities like safety, bioactivity, nontoxicity and anti-microbial ability have made cinnamon compounds appropriate for various purposes.[12] Cinnamon nanoparticles (CNPs) were confirmed to be operational as antibacterial. The sensitivity of structure, morphology, and optical and antibacterial characteristics of CNPs to the variation of laser ablation energy have already been demonstrated. Systematic characterizations of the CNPs have demonstrated the practicability of monitoring their morphology in an appropriate method that is profitable for medical purposes.[13]

In our investigation we aimed to evaluate accelerative effect of cinnamon nanoparticles as well as HAMLET on healing of wounds infected with *Pseudomonas aeruginosa* in rats.

Materials and Methods

Research Materials

The cinnamon nanoparticles were kindly gifted from Faculty of Pharmacy, Urmia University of Medical Sciences, Urmia, Iran. Transmission electron microscopy (TEM) of the nanoparticles is shown in Figure 1.

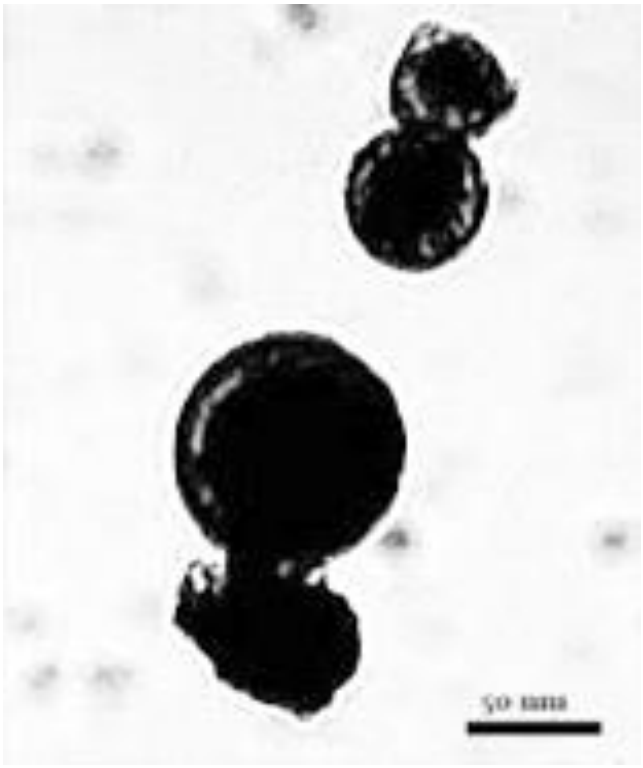

Figure 1. Transmission electron microscopy (TEM) of the synthesized cinnamon nanoparticles (CNPs).

Ethical Considerations

The Guide of the National Academy of Sciences published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985) were followed in the present study. The Institutional Ethical Committee of the University approved the procedures in order to minimize any potential pain of the animals under ethical code: IR.IAU.URMIA.REC.1400.023.

Preparation of HAMLET

Preparation of HAMLET was according to others.[14] It was prepared by converting ethylenediaminetetraacetic acid (EDTA)-treated, partially unfolded α-lactalbumin in the presence of oleic acid (C18:1) on an anion exchange matrix to a stable protein-lipid complex and was resuspended in PBS for all experiments as previously described.

The Procedures for Wound Creation and Infection

The processes for creation of wound and infection were done based on methods of others.[14] Intraperitoneal injection of ketamine (70 mg/kg) and xylazine (5 mg/kg), were used to anesthetize the animals. The area on their back was shaved, cleansed with 70% alcohol solution and after aseptic preparation, a circular excision wound was created by cutting away approximately 115 mm^2 full thickness of predetermined area on the anterior-dorsal side of each rat. Small gauze was placed over each wound and then inoculated with 5×10^7 colony-forming units (CFU) of toxigenic strains of *P. aeruginosa* (PA 103) strain was commercially available. The rats were returned to individual cages, monitored and after 24 hours, the gauze was removed for quantitative bacterial cultures, and the treatment was started.

Randomization and Grouping of Animals

Fifty male rats were divided into 5 groups (n = 10), randomly. In Normal group, no infected wounds were treated with sterile solution of saline 0.9% (0.1 ml). In PAE group, the wounds with *Pseudomonas aeruginosa* infection were only treated with sterile solution of 0.9% saline (0.1 ml). In PAE-HMLT group, HAMLET (100 µg) was used to treat infected wounds. In PAE-CNM group, 1 mg/ml CNPs (0.1 ml) were applied topically to treat PAE infected wounds. In PAE-HMLT-CNM group, HAMLET (100 µg) and 1 mg/ml CNPs (0.1 ml) were applied topically to treat PAE infected wounds.

Application of test formulations was lasted for 10 days and twice a day. Animal houses were in standard environmental conditions of temperature (22 ± 3° C), humidity (60 ± 5%), and a 12-hour light/dark cycle. The animals were maintained on standard pellet diet and tap water. All rats were closely observed for any infection; and if they showed signs of infection, they were separated, excluded from the study, and replaced.

Microbiological Tests

For total bacterial count on days 7 and 14 of treatment after wound creation, the granulated tissues were excised aseptically. Then, 0.1 g of sample was crushed and homogenized in sterile mortar containing 10 ml of sterile saline. The homogenized sample was serially diluted in tube containing 9 ml of sterile saline to 10−5. The diluted samples were cultured on plate count agar (Merck KGaA) superficially and duplicated. The cultured plates were incubated at 37° C for 24 to 48 hours. All colonies were counted following incubation and findings were described as CFU/g of granulation tissue.

Planimetric Studies in Excisional Wound Model

The process of the healing was investigated via wound closure time represented as wound contraction percentage. Photographs were taken immediately after wounding and on days 6, 9, 12, 15, 18, and 21 by a digital camera with a ruler as a scale. The wound areas were analyzed by Measuring Tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc.), and wound contraction percentage was calculated using the following formula:

Percentage of wound contraction = $(A_t - A_1) / A_t \times 100$

Where A_0 is the original wound area and A_t is the wound area at the time of imaging.

Biochemical Investigations

The tissue samples of wounds were kept at −80° C for 3 days, and then enzyme activities were determined in the tissues. The tissues were ground with liquid nitrogen in a mortar. One half gram was weighed for each group and then treated with 4.5 ml of PBS. This mixture was homogenized on ice with use of an ULTRA-TURRAX homogenizer (IKA Werke) for 15 minutes. Homogenates were filtered and centrifuged by using a refrigerator centrifuge at 4° C. Then the supernatants were used to determine the enzymatic activities. All assays were carried out at room temperature. Antioxidant activities including superoxide dismutase (SOD), malondialdehyde (MDA), myeloperoxidase (MPO), glutathione peroxidase (GPO) was performed.[14] The Cinagen kit (Tehran, Iran) was used for biochemical assessments.

Statistical Analysis

Kruskal-Wallis variance analysis was adopted for differences among groups. Where the *p* value (from the Kruskal-Wallis test statistics) was statistically significant, multiple comparison tests were utilized to get the differences. Mann-Whitney U test was used for comparison among days. For retrieving possible multiple comparisons, the Bonferroni correction was utilized. We utilized SPSS 11.5 (SPSS Inc) for the analyses and considered p value < 0.05 as significant level.

Results

Microbiological Examinations and Healing Rate

Significantly lower numbers of PAE inoculated in the wounds were observed in PAE-HMLT-CNM group compared to PAE-HMLT and PAE-CNM groups (p < 0.05). None of animals were died due to infection and anesthesia. No count of CFU/g of PAE was observed in uninfected wounds in Normal group. Application of CNPs and HAMLET significantly diminished the rate of total bacterial count in PAE-HMLT-CNM group compared to those of PAE-HMLT and PAE-CNM groups following days 7 and 14 after wounding (p < 0.05; Table 1). Table 2 shows values of percentage of contraction of wound in experimental animals throughout the experiment. The rate of healing of wounds in PAE-HMLT-CNM group was significantly higher than those of PAE-HMLT and PAE-CNM groups (p < 0.05).

Biochemical Findings

Topical usage of cinnamon nanoparticles resulted in significant augmentation in the activity of SOD in PAE-HMLT-CNM group in comparison with activity of SOD in other groups (p < 0.05). The activity of tNOS was declined in PAE-HMLT-CNM animals with a significant decrease compared to other groups (p < 0.05). Cinnamon nanoparticles significantly diminished MDA level in PAE-HMLT-CNM group in comparison with other experimental groups (p < 0.05). The cinnamon nanoparticles resulted in significant diminution level of MPO in tissues of PAE-HMLT-CNM animals (p < 0.05). Levels of GPO in PAE-HMLT-CNM animals were significantly increased in comparison with other experimental groups (p < 0.05, Figure 2).

Table 1. Wound bacterial count (mean ± SEM) in experimental groups on tow time points of day 7 and day 14.

Groups	Wound granulation tissue bacterial count (CFU/g)	
	Day 7	Day 14
Normal	0.00 ± 0.00	0.00 ± 0.00
PAE	1237.70 ± 261.15	1259.77 ± 273.49
PAE-HMLT	1386.89 ± 271.10	996.12 ± 237.23
PAE-CNM	1278.23 ± 239.10	957.30 ± 229.20
PAE-HMLT-CNM	567.25 ± 78.15*	260.40 ± 76.16*

CFU: Colony-forming units; PAE: *Pseudomonas aeruginosa*; HMLT: Human α-lactalbumin made lethal to tumor cells; CNP: Cinnamon nanoparticles. * $p < 0.05$ versus PAE-HMLT and PAE-CNM groups.

Table 2. Impact of application of cinnamon nanoparticles as well as HAMLET on circular excision wound contraction area (mm^2). Values are given as mean ± SEM.

Groups	Wound area (mm^2)					
	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
Normal	251.50 ± 4.70	105.30 ± 5.10	85.74 ± 3.60	45.75 ± 3.30	27.25 ± 2.50	6.10 ± 2.15
PAE	257.45 ± 4.50	200.75 ± 4.10	170.15 ± 3.20	140.70 ± 3.90	90.60 ± 3.70	70.10 ± 3.70
PAE-HMLT	240.23 ± 4.30	200.73 ± 4.50	180.54 ± 9.20	140.50 ± 7.10	80.70 ± 2.50	70.30 ± 2.80
PAE-CNM	220.30 ± 4.70	190.50 ± 4.70	160.60 ± 3.63	100.75 ± 3.55	60.10 ± 3.18	40.66 ± 2.20
PAE-HMLT -CNM	100.25 ± 3.95*	70.12 ± 2.70*	30.70 ± 2.60*	10.55 ± 1.70*	5.80 ± 3.56*	0.00 ± 0.00*

The treated groups are compared by Student t-test with other groups. * $p < 0.05$ *versus* PAE-HMLT and PAE-CNM groups.

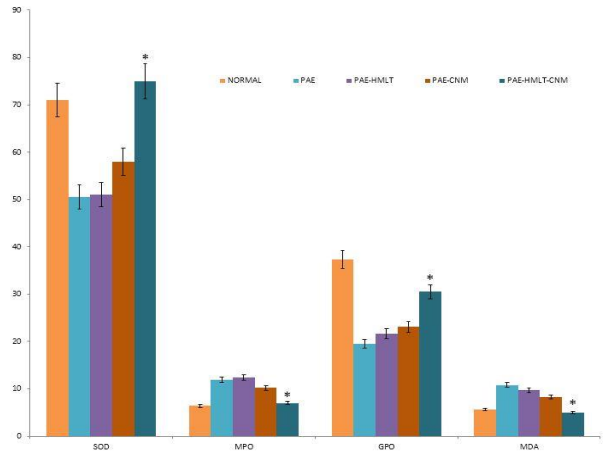

Figure 2. Bar graph shows comparison of the activities of biochemical parameters and a DNA damage product of 8-OHGual/Gua in the experimental groups. Results were presented as mean ± SEM. *$p < 0.05$ versus PAE, PAE-HMLT-CNM and PAE-HMLT groups. Abbreviations: SOD, superoxide dismutase; MDA, malondialdehyde; MPO, glutathione; GPO.

Discussion

Infection potentiates impairment of wound healing results in extensive amount of morbidity and mortality all over the world.[15] Deficiency in signals of cells and molecules, required for regular process in wound healing like neovascularization, generation of granulation tissue, epithelialization, and tissue remodeling are present in infected wounds and end up impairment in wound healing. The process of wound repair in infected wounds is compromised and delayed as a result of infection. The wound repair process takes place spontaneously without further help, but, numerous risk factors such as diseases, infection, blood supply and nutritional status may deteriorate the normal process.[16]

PAE induced infection is growing and becoming a severe hazard in the hospitals and the public. Common antibiotics resistance makes managing PAE induced infections expensive and problematic. Contraction of wound and subsequent lessening in the area of wound, a chief end point in our investigation, was hastened by treating the PAE induced infected wounds with topical use of cinnamon nanoparticles as well as HAMLET. A substantial diminution in wound area was observed in excisional wound model. This showed enhanced maturation of collagen by augmentation in cross-linking. The equilibrium between production and degradation, and collagen deposition is crucial in wound repair and improvement of strength of wound.[17] Hydroxyproline, a chief constituent of the collagen, allows the collagen helix sharp twisting. It supports firmness to unique structure of collagen via hydrogen bonds formation. Hydroxyproline is rarely located in proteins other than collagen and that is why quantity of hydroxyproline has been considered as a scale to approximate content of collagen.[18] Escalation in quantity of hydroxyproline in the PAE-HMLT-CNM group specified augmented collagen quantity because it is a straight estimation of collagen production. Nanoparticles of cinnamon have been shown to signify a novel local antibacterial and wound healing adjuvant for cutaneous damages and infected burn wounds.[19] Our findings revealed that cinnamon nanoparticles as well as HAMLET considerably decreased tissue bacterial count and encouraged the repair phases in infected wounds. Hence, PAE-HMLT-CNM animals revealed condensed phases of inflammation and homeostasis, and augmented phases of proliferation and maturation. Regarding the significance of the

bacterial infection and existence of pathogens in wound, counting the colonies of PAE in area of wound was performed in the present study. The findings revealed that the infection was managed after topical use of cinnamon nanoparticles as well as HAMLET.

Radicals are frequently connected to oxidative stress, that ends up to peroxidation of lipid and compromised wound repair.[20] Diminished oxidative stress augments the inflammatory reaction, and our results revealed that cinnamon nanoparticles could be able to remove radicals.[21] Therefore, In the present study the biochemical indices were meaningfully improved in PAE-HMLT-CNM animals in comparison with those of other experimental groups. The crucial fact to inflammation termination is activity of apoptosis in the immune cells.[22] The mediators promote infiltration of activated immune cells into inflammation spot to help defend the tissue in contradiction of infection in the inflammation response. At termination of the inflammation, apoptosis of the immune cells takes place and macrophages clear the apoptotic cells. The removal of apoptotic cells by macrophages is correlated with termination of wound inflammation and onset of active tissue formation in wound.[23,24]

The aim of the present work was to indicate that cinnamon nanoparticles as well as HAMLET could display antibacterial activity in contradiction of PAE. This is the first record of the literature on efficiency to upsurge the effectiveness of cinnamon nanoparticles in a way that drug-resistant PAE could become sensitive to this antibiotic in *in vivo* assessments. Hence, our results demonstrated that HAMLET could make cinnamon nanoparticles valuable for management of wounds infected with PAE and could offer potential to pay more attention to this harmless and easily available agent to be topically applied in wounds with infection. Studies in dose-response manner are required to assess different concentrations for the cinnamon nanoparticles as well as HAMLET to determine optimal dosages to accomplish extreme efficacy.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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