Properties of methicillin activity with sensitized methicillin resistant Staphylococcus aureus in presence of human alpha-lactalbumin made lethal to tumor cells in infected wound healing in diabetes: An animal model study

Propiedades de la actividad de la meticilina con Staphylococcus aureus resistente a la meticilina sensibilizada en presencia de alfa-lactalbúmina humana letal para las células tumorales en la curación de heridas infectadas en la diabetes: un estudio en un modelo animal

Reza Ranjbar[1] Alireza Kabirian-Bajestani[2]*
[1]Molecular Biology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran
[2]Department of Theriogenology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.
Corresponding Author's: Reza Ranjbar, Molecular Biology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran
**Correspondence email: ranjbarre@gmail.com*

Abstract

Background: Wound healing and other tissue abnormalities are considered as major concerns in diabetes. The novel therapeutic options are urgently needed for treatment of infections in diabetes caused by multidrug resistant S. aureus. Reportedly, HAMLET could sensitize bacterial pathogens to conventional antimicrobial agents. The objective of the present study was to evaluate wound healing activity of methicillin in presence of HAMLET in methicillin resistant Staphylococcus aureus infected wounds in diabetic rats.

Materials & Methods: Fifty male diabetic rats were randomized into five groups of ten animals each. Induction of diabetes was achieved using 60 mg/kg streptozotocin. In group I, 0.1 mL sterile saline 0.9% solution was added to the wounds with no infection. In group II, the wounds were infected with MRSA and only treated with 0.1 mL the sterile saline 0.9% solution. In group III, infected wounds were treated with HAMLET (100 µg). In group IV, animals with infected wounds were treated with 0.1 mL topical application of 1 mg/mL methicillin. In group V, animals with infected wounds were treated with topical application of 0.1 mL solution of methicillin (1 mg/mL) and HAMLET (100 µg).

Results: Microbiological examination, planimetric, histological and quantitative morphometric studies and determination of hydroxyproline levels showed that there was significant difference between animals in group V compared to other groups (P=0.001).

Conclusions: HAMLET could make methicillin useful for treatment of MRSA infected wounds in diabetes.

Key words: MRSA, HAMLET, diabetic rat, infected wound

Resumen

Antecedentes: La curación de heridas y otras anomalías en los tejidos se consideran las principales preocupaciones en la diabetes. Las nuevas opciones terapéuticas son urgentemente necesarias para el tratamiento de infecciones en la diabetes causadas por S. aureus multirresistente. Según se informa, HAMLET podría sensibilizar los patógenos bacterianos a los agentes antimicrobianos convencionales. El objetivo del presente estudio fue evaluar la actividad de curación de la herida de la meticilina en presencia de HAMLET en heridas infectadas con Staphylococcus aureus resistentes a la meticilina en ratas diabéticas.

Materiales y métodos: Cincuenta ratas macho diabéticas se asignaron al azar a cinco grupos de diez animales cada uno. La inducción de la diabetes se logró utilizando 60 mg / kg de estreptozotocina. En el grupo I, se añadieron 0,1 ml de solución salina estéril al 0,9% a las heridas sin infección. En el grupo II, las heridas se infectaron con MRSA y solo se trataron con 0,1 ml de la solución salina estéril al 0,9%. En el grupo III, las heridas infectadas se trataron con HAMLET (100 µg). En el grupo IV, los animales con heridas infectadas se trataron con 0,1 ml de aplicación tópica de 1 mg / ml de meticilina. En el grupo V, los animales con heridas infectadas se trataron con una aplicación tópica de 0,1 ml de solución de meticilina (1 mg / ml) y HAMLET (100 µg).

Resultados: el estudio microbiológico, planimétrico, histológico y morfométrico cuantitativo y la determinación de los niveles de hidroxiprolina mostraron que había una diferencia significativa entre los animales en el grupo V en comparación con otros grupos (P = 0,001).

Conclusiones: HAMLET podría hacer que la meticilina sea útil para el tratamiento de las heridas infectadas por SARM en la diabetes.

Palabras clave: MRSA, HAMLET, rata diabética, herida infectada.

Introduction

In diabetes impaired wound healing and other tissue abnormalities are considered as a major concerns[1]. The biochemical mechanisms involved in the healing process are mainly associated with disorders in collagen production that consequently end up delayed re-epithelialization in wounds, compromised migration and proliferation of keratinocytes and fibroblasts[2]. Various treatments have been adopted to solve this complex clinical problem, however, only a few have been proven to be effective[3].

The prevalence of diabetes has become a major clinical problem and a serious issue for public health. The impaired wound healing in diabetic patients is one of the complications[4]. Lack of cellular and molecular signals required for normal wound repair process such as angiogenesis, granulation tissue formation, epithelialization, and remodeling are encountered in diabetic patients that contribute to the poor healing of diabetic wound. The normal healing process in healthy individuals occur at an optimal rate, however, it is usually delayed or even completely compromised in diabetic patients[4].

Open wounds are particularly prone to infection, especially by bacteria, and also provide an entry point for systemic infections. Infected wounds heal less rapidly and also often result in the formation of unpleasant exudates and toxins that will be produced with concomitant killing of regenerating cells. Consequently, there is a need to stimulate healing and restore the normal functions of the affected part of the body to ease the discomfort and pain associated with wounds, preventing infection, and activating tissue repair processes[5]. Staphylococcus aureus (S. aureus) is an important cause of nosocomial infections in most health centers[6].

Methicillin-resistant Staphylococcus aureus (MRSA) is the most widespread bacterial pathogen causing various infections ranging from skin and soft tissue infections to serious invasive infections, such as pneumonia, endocarditis, bacteremia and sepsis[7,8]. It is estimated that Multi-drug resistant Staphylococcus aureus infections leads to high mortality with an associated annual health care costs[9,10]. Despite this high mortality rate, there are relatively few new antibacterial agents in the pharmaceutical pipeline[11]. Instead, the majority of antibiotics developed in the last decade are molecules re-engineered from existing antibiotic classes for which underlying resistance mechanisms are already present[12]. Therefore, effective new therapeutic options for treatment of infections caused by multidrug resistant S. aureus are urgently needed.

Human alpha-lactalbumin made lethal to tumor cells (HAMLET) is a protein-lipid complex from human milk with both tumoricidal and bactericidal activities in vitro. HAMLET has been reported to be able to sensitize bacterial pathogens to traditional antimicrobial agents in vitro[13]. It has also been demonstrated that HAMLET in combination with existing antimicrobial therapies has worked against multi-drug-resistant staphylococci both in vitro and in vivo[14].

The present study was designed to assess wound healing activity of methicillin in presence of HAMLET in methicillin resistant Staphylococcus aureus infected wounds in rats and to the best knowledge of the authors this was the first in vivo study of this kind in the literature.

The assessments were based on excision wound model and planimetric studies, histological preparation and quantitative morphometric studies and determination of hydroxyproline levels.

Materials and methods

All antibiotics and reagents were research grade and purchased from Sigma-Aldrich, St. Louis, MO. and used without further purification. Methicillin stock was suspended in water. The Antibiotic stock was diluted at least 100-fold in phosphate buffered saline (PBS), pH 7.4, before use in the assays.

Induction of diabetes: For insulin-deficient diabetes, rats were fasted overnight before receiving a single intraperitoneal injection (60 mg/kg in 0.9% sterile saline) of streptozotocin (STZ). Hyperglycemia (15 mmol/l or greater) was confirmed 2 days later by measurement of tail-vein blood glucose concentration (Ames Glucostix; Myles, Elkhart, IN). The rats underwent the procedures three days after induction of diabetes.

Preparation of human alpha-lactalbumin made lethal to tumor cells: HAMLET prepared based on a method described by others[15]. In brief, it was prepared by converting EDTA-treated, partially unfolded alpha-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.

Wound creation and infection: Rats were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg of BW) and xylazine (5mg/kg of BW), the hair on their back was shaved and the skin cleansed with 70% alcohol solution. Following shaving and aseptic preparation, a circular excision wound was made 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 CFU of Staphylococcus aureus ATCC 43300. The methicillin-resistant S. aureus ATCC 43300 strain was commercially available. The pocket was closed by means of 4-0 nylon sutures and this procedure resulted in a local abscess af-

ter 24 h. The rats were returned to individual cages and they were examined daily. After 24 h, the wounds were opened, the gauze removed for quantitative bacterial cultures and treatment started.

Study design and animals: This study was carried out in strict accordance with the guidelines of the Ethics Committee of the International Association for the Study of Pain[16]. The protocol was approved by the Institutional Animal Care and Use Committee. All bacterial inoculations and treatments were performed under conditions to minimize any potential suffering of the animals. Fifty male rats were randomized into five groups of ten animals each. In group I, 0.1 mL sterile saline 0.9% solution was added to the wounds with no infection. In group II, the wounds were infected with MRSA and only treated with 0.1 mL the sterile saline 0.9% solution. In group III, infected wounds were treated with HAMLET (100 µg). In group IV, animals with infected wounds were treated with 0.1 mL topical application of 1 mg/mL methicillin. In group V, animals with infected wounds were treated with topical application of 0.1 mL solution of methicillin (1 mg/mL) and HAMLET (100 µg). All the test formulations were applied for 10 days, twice a day, starting from the first treatment.

Microbiological assessments: Briefly, 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, Darmstadt, Germany) superficially and duplicated. The cultured plates were incubated at 37 °C for 24 to 48 hours. After incubation, all colonies were counted and results described as CFU/g of granulation tissue[17].

Excision wound model and wound area measurements: Wound-healing property was evaluated by wound contraction percentage and wound closure time. Photographs were taken immediately after wounding and on days 6, 9, 12, 15, 18 and 21 post-wounding by a digital camera while a ruler was placed near the wounds. The wound areas were analyzed by Measuring Tool of Adobe Acrobat 9 Pro Extended software (Adobe Systems Inc, San Jose, CA, USA) and wound contraction percentage was calculated using the following formula: Percentage of wound contraction = $(A_0 - A_t) / A_0 \times 100$

Where A_0 is the original wound area and A_t is the wound area at the time of imaging. Animal houses were in standard environmental conditions of temperature (22±3°C), humidity (60±5%), and a 12h 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 were separated, excluded from the study and replaced.

Histology and morphometric studies: The tissue samples were taken on 7, 14, 21 days after surgery from periphery of the wound along with normal skin and fixed in 10% buffered formalin, drhydrated and embedded in paraffin wax, sectioned at 5 µm and stained with hematoxylin and eosin (H&E) and Masson's trichrome stains. Photomicrographs were obtained under light microscope to assess the predominant stage of wound healing. Three parallel sections were obtained from each specimen. Cellular infiltration including the number of mononuclear cells, poly morphonuclear cells and fibroblastic aggregation were quantitatively evaluated. Acute hemorrhage, congestion, vascularization, epithelialization, collagen production and density were also evaluated qualitatively. Morphological findings were scored using image analyzing software (Image-Pro Express, version 6.0.0.319, Media Cybernetics, Silver Springs, MD, USA). The histological parameters were classified according to the intensity of occurrence in five levels (- absence; + discrete; ++ moderate; +++ intense; ++++ very intense)[18].

Determination of hydroxyproline levels: On the day 21 after surgery, a piece of skin from the healed wound area was collected and analyzed for hydroxyproline content. As a major part of collagen, hydroxyproline has an essential role in collagen stability. The collagen is the major component of extracellular tissue, which gives support and strength. Tissues were dried in a hot air oven at 60–70 ◦C to constant weight and were hydrolyzed in 6N HCl at 130 ◦C for 4 h in sealed tubes. The hydrolysate was neutralized to pH 7.0 and was subjected to chloramine-T oxidation for 20 min. The reaction was terminated by addition of 0.4M perchloric acid and color was developed with the help of Ehrlich reagent at 60 °C and measured at 557 nm using UV-visible spectrophotometer.

Statistical analysis: Differences among groups were evaluated by Kruskal–Wallis variance analysis. When the P-value from the Kruskal–Wallis test statistics was statistically significant, multiple comparison tests were used to know differences. Comparison among days was assessed by Mann–Whitney U-test. The Bonferroni correction was applied for all possible multiple comparisons. SPSS 18 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. A P-value was set at 0·05.

Results

Microbiological assessments: In animals of group V whose infected wounds were treated with both methicillin and HAMLET, the counts of S. aureus cultured in the wound tissues were significantly lower than in the infected wounds in groups III and IV (P=0.001)

No animals died due to infection or anesthetics. The uninfected wounds treated with saline had no CFU/g of S. aureus count. Topical application of 0.1 mL solution of methicillin (1 mg/mL) and HAMLET (100 µg) significantly reduced the rate of total bacterial count on 7 and 14 days post-wounding compared in groups III and IV (P=0.001) (Table 1).

Table 1 – Wound bacterial count in experimental groups on tow time points of day 7 and day 14.

Groups	Wound bacterial count (CFU/g) of granulation tissue	
	On day seventh	On day fourteenth
Group I	0.00 ± 0.00	0.00 ± 0.00
Group II	1297.32 ± 262.15	1077.81 ± 229.19
Group III	1031.52 ± 285.27	979.34 ± 272.54
Group IV	1273.55 ± 229.85	981.65 ± 228.37
Group V	181.14 ± 47.21*	00.00 ± 0.00*

CFU: Colony-forming units. *P<0.05 vs. groups III and IV.

Reduction in wound area: Wound contraction percentage in different groups within the study period is shown in Table 2. The healing rate of wounds in group V was significantly different compared to groups III and IV (P= 0.001).

Table 2: Effects on circular excision wound contraction area (mm²). Values are given as mean ± SEM.

	Wound area in days (mm²)					
Groups	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21
Group I	236.16±4.92	104.33±5.12	86.75±3.67	41.78±3.31	23.20±2.10	7.80±3.31
Group II	255.12±4.60	207.11±4.13	180.15±3.20	145.70±3.90	95.60±3.40	73.10±3.70
Group III	220.25±4.10	190.71±4.70	170.83±3.20	125.50±2.15	70.64±2.16	60.35±2.80
Group IV	220.20±4.10	190.50±4.55	173.30±3.15	125.50±3.65	75.10±2.13	67.60±2.25
Group V	111.10±3.10*	71.60±2.10*	30.75±2.20*	13.30±1.50*	4.50±0.70*	0.00±0.00*

The treated groups are compared by Student t test with other groups*: The mean difference was significant at the .05 level vs. groups III and IV.

Histological and morphometric findings: There were significant differences in comparisons of group V and IV, particularly in terms of cellular infiltration, acute hemorrhage, congestion, edema, collagen production and density, reepithelialisation and neovascularization. During the study period, scores for reepithelialisation and neovascularisation were significantly higher in group V rats than groups III and IV (P =0.001). Polymorphonuclear (PMN) and mononuclear (MNC) cell count, fibroblast cell proliferation and also Mean Rank of the qualitative study of acute hemorrhage, edema and collagen production score in group V were significantly higher than those of groups III and IV (P =0.001) (Table 3) (Fig 1-4).

Table 3. Evaluation of Intensity of histological parameters in experimental groups.

Histological parameters						
Groups	Days	Acute Hemorrhage	Congestion	Vascularization	Epithelialization	Collagen
Group I	7	+++	+++	+	-	+
	14	++	+	++	+	++
	21	-	-	++	++	++
Group II	7	++++	+++	-	-	-
	14	+++	+++	+	+	+
	21	++	++	+	+	+
Group III	7	+++	+++	-	-	-
	14	+++	++	+	+	+
	21	++	++	+	+	+
Group IV	7	+++	+++	-	+	+
	14	++	+	+	++	++
	21	+	+	+	++	++
Group V	7	+*	+*	+++*	++*	++*
	14	-	-	++++*	+++*	+++*
	21	-	-	++++*	++++*	++++*

Classification of histological parameters according to the intensity of occurrence: - absence; + discrete; ++ moderate; +++ intense; ++++ very intense. Histopatological damages were assessed as explained under material and methods on days, 7, 14 and 21 of lesion.*P<0.05 vs. groups III and IV.

Figure 1: Box-and-whisker plots of number of polymorphnuclear cells (PMN) in excisional model of the rat's skin in experimental groups. Results were expressed as mean ± SEM.

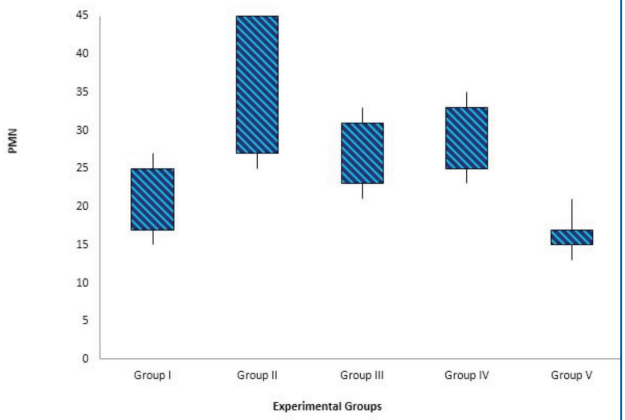


Figure 2. Line graph indicating number of mononuclear cells (MNC) in excisional model of the rat's skin in experimental groups. Results were expressed as mean ± SEM. * P < 0.05 vs groups III and IV.

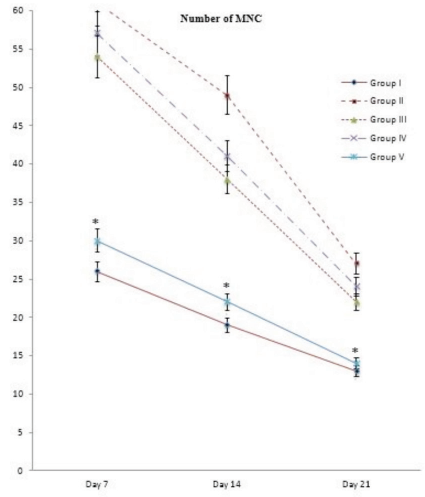


Figure 3. Box-and-whisker plots of number of fibroblasts in excisional model of the rat's skin in experimental groups. Results were expressed as mean ± SEM.

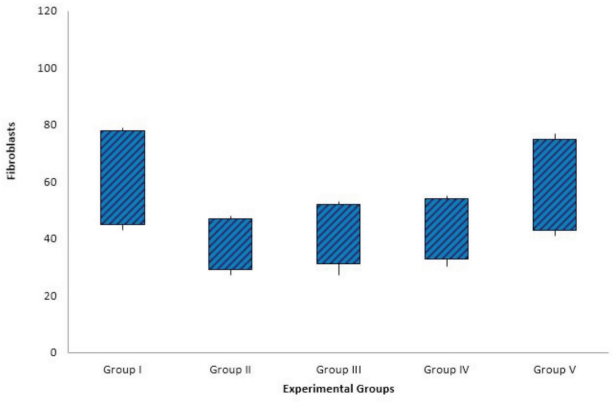
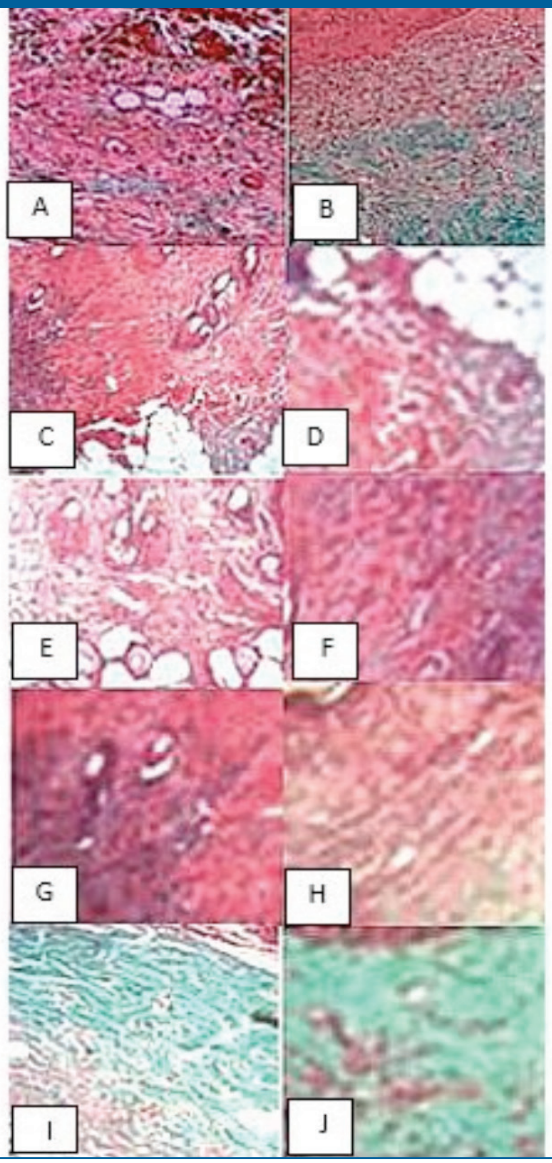


Figure 4. Histological characteristics of rat skin on the 7th (A,C,E, G,I) and 14th day (B,D,F,H,J) after wound creation in excisional wound model. A and B: Group I, C and D: Group II, E and F: Group III, G and H: Group IV, I and J: Group V. Wounds with surrounding skin were prepared for histological microscopic evaluation by Masson trichrome staining (×400).



Hydroxyproline content of wound: Proline is hydroxylated to form hydroxyproline after protein synthesis. Hydroxyproline contents in groups I to V were found to be 47.65±2.31, 63.47±2.82, 72.17±3.19, 70.17±2.16 and 99.78±3.36 mg g^{-1}, respectively. Hydroxyproline contents were significantly increased in the group V which implies more collagen deposition compared to groups III and IV (P =0.001).

Discussion: Wound healing process in diabetic patient is impaired and delayed due to high blood glucose levels. High blood glucose hampers proliferation of cells and decreases collagen production that result in decrease in chemotaxis and phagocytosis[19]. Elevated blood glucose level, a reduction in the levels of growth factors, and the inhibition of fibroblast proliferation have all been suggested to contribute to the observed impairment in wound healing[19]. STZ induced diabetes in rats is one of the most extensively studied models of diabetes. In this study, therefore, STZ induced diabetic rats were used as the model of diabetes to study diabetic wound healing.

Wound healing is characterized by reepithelialization, granulation tissue growth and remodeling of extracellular matrix. Although the wound healing process occurs by itself, spontaneously, and does not require much help, there are various risk factors such

as infection, supply of blood, nutritional status and other factors that influence the resolution of this process[20]. It is well known that attack by microbes, which invade the skin barrier, delays the natural wound healing process[21.] MRSA is increasing in infections and is a serious threat to patients in health care facilities and the community. There are many reports in the literature that researchers have been working on various agnets to combat MRSA related infections[22-32]. Resistance to common antibiotics makes treating MRSA costly and difficult. The main end point observed in this study, wound contraction and reduction in wound area, was accelerated by treating the wounds with methicillin in presence of HAMLET. All the parameters observed (presence of necrotic tissue, clotting and crust, re-epithelialization and granulation tissue growth, bacterial count) were affected; suggesting that methicillin in presence of HAMLET was effective against MRSA. Topical application of HAMLET at the wound site produced significant wound healing activity, indicating that it could have sensitized MRSA to methicillin.

In excisional wound model there was a significant decrease in wound area. This indicated improved collagen maturation by increased cross linking. The balance between synthesis and breakdown and so deposition of collagen is important in wound healing and development of wound strength[33]. Hydroxyproline is a major component of the collagen that permits the sharp twisting of the collagen helix. It helps on providing stability to the triple-helical structure of collagen by forming hydrogen bonds. Hydroxyproline is found in few proteins other than collagen. For this reason, hydroxyproline content has been used as an indicator to determine collagen content[34]. Increase in hydroxyproline content in group V indicated increased collagen content, since hydroxyproline is the direct estimate of collagen synthesis.

Our preliminary data showed that methicillin in presence of HAMLET significantly reduced tissue bacteria count and promoted the healing stages. Accordingly, the animals in group V showed shortened homeostasis and inflammatory phases and accelerated proliferation and maturation stages. Considering the importance of the bacterial infection as well as presence of pathogens in wound tissue, we analyzed the MRSA colonies count in wound area. The observations demonstrated that the infection was controlled after administration of the methicillin combined with HAMLET.

The inflammation phase is considered as a main step in order to eliminate cellular debris from tissue as well as extensive response for microbial infection[35,36]. Therefore, rapid inflammatory response is necessary to control the inflammation. Neutrophils, macrophages and lymphocytes infiltrate to the site of injury during inflammatory stage[35,37]. Light microscopic analyses showed that in group V mononuclear immune cell infiltration was significantly increased on day 8 post operation. This situation plays a critical role in eliminating the infection and provoking the healing process by considering the key role of inflammatory cells (especially macrophages) in organizing the granulation tissue. Therefore, the antibacterial impact of methicillin in presence of HAMLET may largely correlates with these agents.

The observations of our study showed that methicillin in presence of HAMLET resulted in enhanced cellular proliferation. The fibroblasts and fibrocytes distribution in one mm^2 of the wound site was significantly higher in comparison with other groups. Regarding the key role of fibroblasts and fibrocytes in synthesis of collagen, we could hypothesize that elevated collagen deposition in group V was attributed to high cellularity of fibroblasts and fibrocytes. Increased neovascularization on day 8 following wound induction showed that methicillin in presence of HAMLET could provoke the healing process via stimulating cells infiltration after 8 days.

Our histochemical findings for vascular distribution were in good accordance with these findings. The animals in group Vexhibited remarkably higher vascularization compared to groups III and IV. Increased neovascularization on day 8 following wound induction demonstrated that methicillin in presence of HAMLET could provoke the healing process via stimulating infiltration of cells after 8 days.

The key point to end of the inflammation is the apoptotic activity of immune cells. Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the

immune system, hormone-dependent atrophy, embryonic development, and chemical-induced cell death[38]. In the inflammation response, the mediators induce the infiltration of activated immune cells into inflammation site to protect the tissue against the pathogen infection. In the end of the inflammation, apoptosis of the immune cells and the apoptotic cells are cleared by macrophages. The clearance by macrophages of cells apoptosis is a key point phenomenon associated with actively tissue formation from wound inflammation[39-41].

There are several reports that the HAMLET has sensitized the bacterial pathogens to traditional antimicrobial agents[13,14,42-44]. The purpose of this study was to demonstrate that methicillin in presence of HAMLET could show antimicrobial activity against MRSA. This ability to increase the efficacy of methicillin to the degree that drug-resistant S. aureus could again become sensitive to this antibiotic in in vivo assays is reported for the first time in the literature. Thus, our results showed that HAMLET could make methicillin useful for treatment of MRSA infected wounds and had the potential to offer a return to this safer agent for topical use. Dose-response studies are needed to study various concentrations for the methicillin and HAMLET for determination of optimum dosages to achieve maximum effects.

Acknowledgments: We would like to thank from the "Clinical Research Development Center of Baqiyatallah hospital" for their kind cooperation. This study was financially supported in part by "Clinical Research Development Center of Baqiyatallah hospital.

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