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Cerium nitrate treatment prevents progressive tissue necrosis in the zone of stasis following burn

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ABSTRACT

Cerium nitrate (CN) was used as a topical antiseptic agent for the treatment of burn wounds and found to reduce the number of anticipated death in burn. This decreased burn related mortality cannot be explained by the control of wound infection alone. In the studies performed to elucidate the unexplained effects of CN treatment, it was shown that CN treatment reduced the alarm cytokine levels, decreased leukocyte activation, reduced macromolecular leakage and finally burn edema formation. We hypothesized that CN treatment prevents the conversion of the zone of stasis to progressive tissue necrosis by decreasing leukocyte activation and reducing macromolecular leakage and burn edema.

This was investigated on a well-described burn comb model in the rats. Fifty-four rats were randomly divided into control and CN treatment groups. Each rat in CN treatment group received 0.04 M CN bathing 30 min after burn whereas rats in control group received 0.09% saline bathing. Viability of zone of stasis is assessed with ^{99m}Tc-sestamibi scintigraphy. Nine rats in each group were scintigraphically evaluated at the 3rd and 7th day after burn and remaining 9 rats had macroscopic and histological examination at the 21st day after burn to confirm the scintigraphic results.

In CN treatment groups, the scintigraphic uptake ratios were higher both at post burn day 3rd and 7th when compared to that of control groups. This was statistically significant ($p \leq 0.05$). In the CN treatment group, the results of the average percentage of the re-epithelialization in the zone of stasis were higher than that of control groups. The difference between the groups was also statistically significant ($p \leq 0.05$).

These results were accepted that CN treatment prevents progressive tissue necrosis in the zone of stasis. This study further elucidates the unexplained effects of CN treatment on burn.

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1. Introduction

Thermal injury results in three zones of damage in the skin [1]. The zone of coagulation is the central zone which is directly and irreversibly affected by burn trauma. The zone of hyperemia is the outermost zone that remains vital. The zone of stasis is the intermediate zone consists of a layer of tissue 1–2 mm thick characterized by reduced blood flow and progressive necrosis, which can result in additional tissue death within 24–48 h after the occurrence of the burn. In addition to extension of the initial burn wound area, this process can result in the conversion of a partial-thickness

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burn into a full-thickness burn which takes longer time to heal and increase the probability of forming hypertrophic scars [2]. Since then saving the zone of stasis is an important issue to prevent the enlargement of the burned area by way of limiting the depth and width of burn wound and to reduce the mortality and morbidity of burn.

Various events could contribute the conversion of viable zone of stasis to progressive tissue necrosis and finally tissue death such as hypercoagulability, increased vascular permeability, blood flow reduction, systemic activation of white blood cells and plugging of capillaries [3]. Numerous antithrombotic, anticoagulants, anti-inflammatory agents have been studied for saving the zone of stasis with reports of favorable results [4–6].

Mainly increased vascular permeability and neutrophil activation and plugging of capillaries have been responsible in the pathogenesis of blood flow reduction in the zone of stasis. It has been shown that antibodies which block adhesion of neutrophil to endothelial cells administered 30 min following burn improved blood flow in the zone of stasis in comb model [3,7,8]. In another study, Poloxamer-188, non-unionic block copolymer surfactant found in artificial blood substitutes, improved capillary blood flow and reduced the zone of stasis.

CN was used as a topical antiseptic agent for the treatment of burn wounds and found to reduce the number of anticipated death in burn [9,10]. This decreased burn related mortality cannot be explained by the control of wound infection alone because treatment with 0.04 M CN bathing in the first 24 h still effective even if burn wound infection may occur later. In the studies performed to elucidate the unexplained effects of CN treatment it was shown that CN treatment reduced the alarms cytokine levels, modulate leukocyte activation by way of reducing rolling sticking and transmigrating leukocyte [11,12]. In a recently performed study, it was shown that CN bathing reduced macromolecular leakage and finally burn edema formation [13]. It was hypothesized that CN treatment may prevent the conversion of the zone of stasis to progressive tissue necrosis by modulating leukocyte activation and reducing macromolecular leakage and burn edema in which important issue in saving zone of stasis.

2. Materials and methods

This study was performed according to the procedures approved by the Animal Research Committee of Gulhane Military Medical Academy. All the animals were used in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the ethic council.

Fifty-four female Sprague-Dawley rats, weighing 300–350 g were provided by the Experiment Research Center. The animals were caged individually at room temperature with 12 h light and 12 h night cycle and free access to water and standard laboratory food for rats.

The rats were randomly divided into control and experiment groups. Each rat in experimental group received 0.04 M CN bathing following 30 min burn whereas rats in control group received 0.09% saline bathing was applied for the control group and was applied for the experiment group. Nine rats in each group were scintigraphically evaluated at the 3rd and 7th post burn day and remaining 9 rats had macroscopic and histological examination at 21st day after burn.

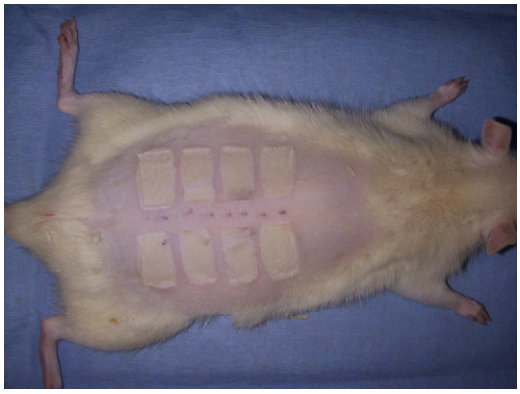

Fig. 1 – Burn comb model.

2.1. *Thermal injury*

General anesthesia was induced with intramuscular ketamine 10% (90 mg/kg) and xylasine 2% (10 mg/kg). Following the anesthesia, the entire dorsum of each rat was shaved. Burn wounds were established as in 'burn comb model', which was first described by Regas and Ehrlich [14]. A specially manufactured metal stamp, containing four rows (1 cm × 2 cm) and three interspaces (0.5 cm × 1 cm) was immersed into boiling water until thermal equilibrium was achieved between the comb and the water. The heated metal stamp placed on the dorsum of the rat 0.5 cm lateral and parallel to midline and held for 20 s without any pressure and finally we made a full thickness burn (Fig. 1).

2.2. *Preparation of cerium nitrate solution*

0.04 M cerium nitrate(3) hexahydrate (CN) solution was prepared with double distilled water in the Department of Pharmacology at Gulhane Military Medical Academy. No autoclaving or sterile filtration was recommended as CN has its anti bacterial properties.

2.3. *Procedure for bathing*

The basins with dimensions 20 cm × 10 cm were filled with 0.09% saline for the control group and 0.04 M CN solution for the experimental group. Rats were kept in supine position in the basins for 30 min immediately after thermal injury.

2.4. *Nuclear imaging*

Nine rats in each group were scintigraphically evaluated at the 3rd and 7th day. Each rat received an intravenous injection of 111 MBq (3 mCi) technetium-99m methoxyisobutylisonitrile ($^{99}Tc^{m}$-MIBI) via caudal vein under general anesthesia. 30 min after the injection, the rats were sacrificed and the entire dorsal skins including the panniculus carnosus were excised.

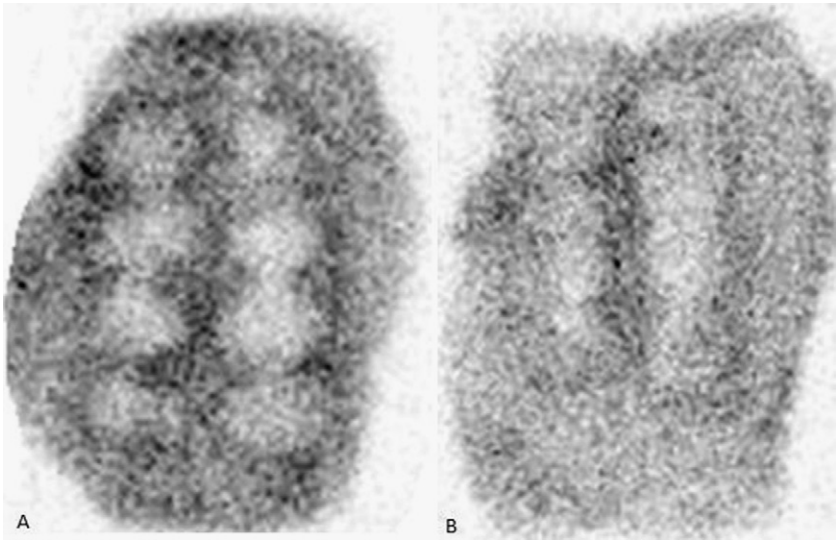

Fig. 2 – (A) Nuclear imaging of the rats at 3rd day in CN treatment group. The zone of stasis took more radioactive agent so the border of the zone of stasis is seen clearly. (B) Nuclear imaging of the rat at 3rd day in the control group. No significant difference is seen between the zone of stasis and the zone of necrosis.

Anterior planar imaging of each excised specimen was performed for 15 min.

At the 1st hour after injection of $^{99}Tc^{m}$-MIBI, the specimen were imaged with a 20% window centered at 140 keV, using 128 × 128 pixel matrix size and 1.33 zoom factor. Scintigraphic images were performed using large field of view gamma camera equipped with a low energy, high resolution collimator (Millenium, GE Medical Systems, Milwaukee, WI). The images of each specimen were evaluated by an experienced nuclear medicine physicians blinded to rats' group. Linear relatively increased activities between the burning zones were considered as the vital areas which were saved from burning. In a second step, by drawing regions of interests (ROI) equal in size in the center of burning zone and in the intersection area between the burning zones (stasis zone), an average ratio between the number of counts per pixel (cpp) in each region was calculated to show the extent of viable tissue (Figs. 2 and 3).

2.5. *Macroscopical and histological assessment*

At the 21st day, nine rats from each group were evaluated macroscopically and histologically. The growth of the hair follicles which is the indicator of the viability of the burned tissues was observed on the burned areas (Fig. 4).

After macroscopic assessment the rats were sacrificed under general anesthesia, then the entire dorsal skin including the panniculus carnosus was excised. The exact zones of stasis were sampled and fixed in %10 formalin solution for 24 h. The specimens were embedded in paraffin block, after that 4 micron sections stained with hematoxylin and eosin (HE). The specimens were evaluated for percentage

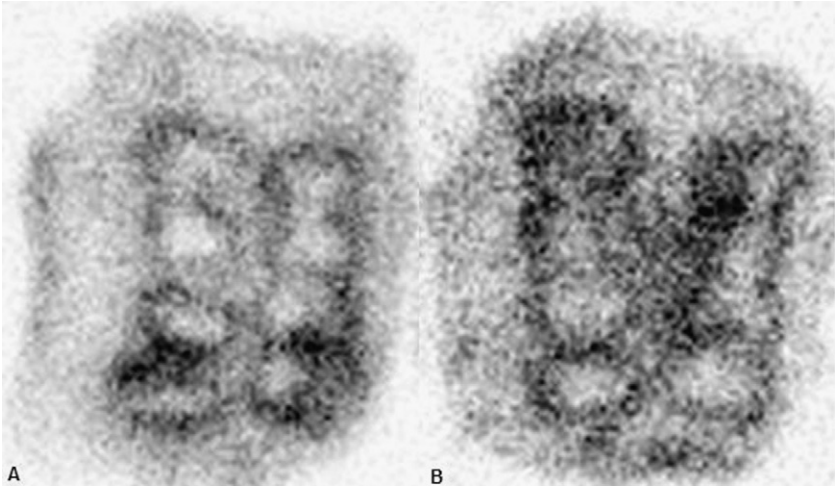

Fig. 3 – (A) Nuclear imaging of the rats at 7th day in the CN treatment group. The zone of stasis took more radioactive agent than the zone of necrosis and so the border of the zone of stasis is seen clearly. (B) Nuclear imaging of the rat at 7th day in the control group. No significant difference is seen between the zone of stasis and the zone of necrosis.

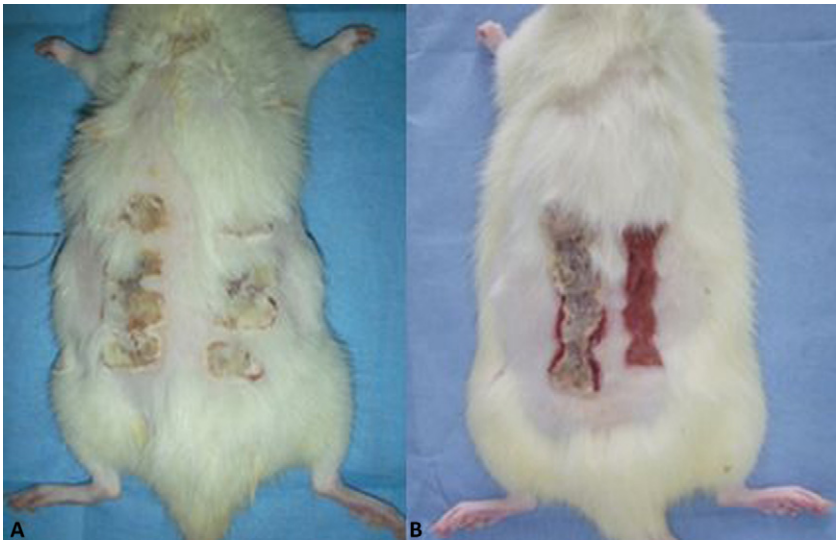

Fig. 4 – Macroscopic view of the rats at 21st day. (A) The growth of hair follicles are seen in the zone of stasis in the experiment group. (B) The hair follicles are not seen in the zone of stasis and the necrotic areas are relatively larger in the control group.

of re-epithelialization, neutrophils and vital cutaneous appendages. The neutrophils were counted and scored as 0–5 cells (+), 5–10 cells (++) and more than 10 cells (+++). The vital cutaneous appendages were counted and scored as 0–5 cutaneous appendages (+), 5–10 cutaneous appendages (++) and more than 10 cutaneous appendages (+++). At the zone of stasis, the re-epithelialization area measured and rate for total zone, and the result were evaluated as a percentage.

2.6. *Statistical analysis*

The results in the nuclear imaging assessment were analyzed graphically according to its normal distribution and with Shapiro–Wilk test. The Student's *t* test was used for comparison of the percentage of the re-epithelialization in the groups. Variance Analyze in the Repeated Measurements (Repeated Measures ANOVA) was applied so as to examine the change of the measurement variants in the groups over time. The interquartile range was used to show the illustrative statistical value for the parameter of the number of the neutrophils and the vital cutaneous appendages and the differences between the groups were shown with Mann Whitney test.

When a difference is found, so as to detect the source of the difference Bonferroni test was used for post hoc double comparisons. MS-Excel and SPSS for Win. Ver. 15.0 (SPSS Inc., Chicago, IL, USA) package programs were used for statistical analyses and calculations. In the statistical decisions $p \leq 0.05$ was accepted as the sign of significant difference.

3. Results

3.1. *Nuclear imaging*

The distribution of the viable counts at the central zones according to the control and the CN treatment groups is shown in Table 1 and Fig. 5. There were no statistically significant differences between control and CN treatment groups at the central zones ($F = 3.527$; $p = 0.087$). Also the effect of time and group, and time interaction effect were not statistically significant ($F = 0.049$; $p = 0.829$ and $F = 0.000$; $p = 0.988$).

Table 1 – The average counts of the viable tissues in the zone of stasis areas according to days and groups (with standard deviation sticks).

Group	3rd day		7th day	
	X	S.D.	X	S.D.
Cerium nitrate	7725.43	2485.66	8065.71	3649.74
Control	5920.00	2776.65	6310.67	4509.90

The average gamma counts of the necrosis in the zone of stasis were obtained from the rats at the 3rd and 7th days and the distribution according to the groups were shown in Table 2 and Fig. 6. The average count of the necrosis in the zone of stasis in the CN treatment group was greater than the control group both at the 3rd and 7th days. The difference between the

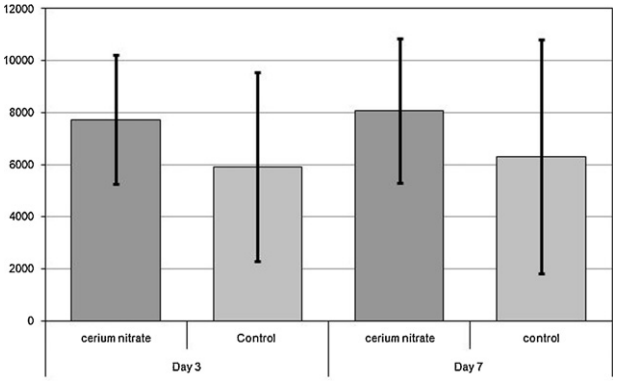

Fig. 5 – The distribution of average gamma counts (cpp) of the viable tissues at the zone of necrosis according to days and groups (cpp, counts per pixel).

Table 2 – The average percentage of the re-epithelialization results and standard deviations in the groups.

Group	3rd day		7th day	
	X	S.D.	X	S.D.
Cerium nitrate	14,465.50	4441.21	15,253.71	6618.13
Control	7958.56	4573.02	8909.83	6297.76

groups is also statistically significant ($F = 14.688$; $p = 0.003$). The effect of change over time in the groups and the group, and time interaction effect were not statistically significant ($F = 0.025$; $p = 0.876$ and $F = 0.004$; $p = 0.950$). The statistical results show that the zone of stasis is more preserved by the treatment in comparison to the control group.

3.2. *Macroscopical and histological assessment*

At the 21 st day, in the CN treatment group, 90% of the zone of stasis was viable and the growth of the hair follicles, the color and the texture of the skin was similiar to that of unburned skin whereas in the control group 10% of the zone of stasis was viable and few hair follicles were seen in this area.

In the histological assessment the average percentage of re-epithelialization in the zone of stasis was %64 in the CN treatment group and was %23 in the control group (Fig. 7). The results of the average percentage of the re-epithelialization in the CN treatment group were found higher than as in the control group statistically ($t = 5.075$; $p < 0.001$).

The median of the neutrophils was scored (+) (IOR = 1.0) in the CN treatment group and (+++) (IOR = 1.0) in the control group. The median of neutrophils in the control group was found higher than as in the CN treatment group statistically ($Z = 3.141$; $p = 0.001$).

The median of the vital cutaneous appendages was scored (+++) (IOR = 1.0) in the CN treatment group and (+) (IOR = 1.0) in the control group. The median of vital cutaneous appendages was found higher than that of the control group statistically ($Z = 2.693$; $p = 0.008$).

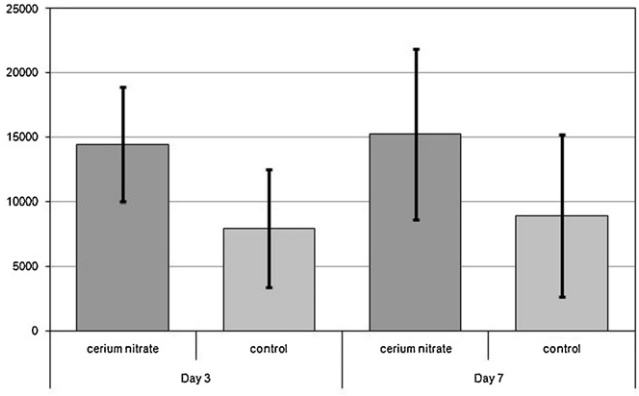

Fig. 6 – The average gamma counts of the viable tissues at the zone of necrosis according to days and the groups (with standard deviation sticks).

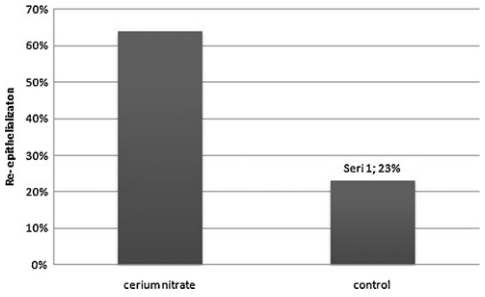

Fig. 7 – The distribution of the average counts of the viable tissues in the zone of stasis according to days and groups (cpp, counts per pixel).

4. Discussion

In this study we found that CN bathing prevented progressive tissue necrosis in the zone of stasis. This was assessed by both scintigraphic and histological examination short and long term period. In the critical period, viability of zone of stasis was scintigraphically evaluated. Viability of zone of stasis can be assessed by laser Doppler flowmetry, angiography, autoradiography, histological examination [5,15,16]. ^{99m}Tc-sestamibi scintigraphy was used in this study to show fate of zone of stasis.

The results of blood flow measurements with radionuclide are not always reproducible or reliable and determination of viability at the cellular level is doubtful with these methods [17].

^{99m}Tc-sestamibi imaging has been used for the assessment of the tissue viability in electrical burns, frostbite injuries and in many different diseases of extremities and monitorization of viability of buried flaps [17–20]. In an experimental study, a clear demarcation line between necrotic and normal tissue was demonstrated using ^{99m}Tc-sestamibi scan, and pathology confirmed these findings [21].

Cellular uptake and trapping of ^{99m}Tc-sestamibi are related not only to regional blood flow but also to mitochondrial metabolic conditions and viability [22]. The tracer is distributed across both plasma and mitochondrial membranes in response to progressively larger negative transmembrane potentials, and trapped within the mitochondrial layer [23]. The potential role of viability study of 3-dimensional stasis zone in burn with ^{99m}Tc-sestamibi is analogous to its use for myocardial scintigraphy. As with myocardial scintigraphy, necrotic, ischemic and viable tissues in zone of stasis can be determined by ^{99m}Tc-sestamibi scan. The problems of poorer counting statistics when using blood flow studies are avoided with ^{99m}Tc-sestamibi [24].

The zone of stasis is vital in the first 24–48 h and then progress to coagulation necrosis afterward. The zone of stasis could be saved with proper intervention during the first 72 h [5]. Also in clinical practice it was difficult to predict burn depth in first 24 h following burn however in the 3rd day progression of injury was complete and the zone of stasis is vital. Since then the post burn 3rd day was chosen in this study and performed previous study [5,6]. The post burn day 7 was

chosen to show the continuous effectiveness of the treatment modality.

In CN treatment group, it was shown that scintigraphic uptake ratio was higher both at post burn day 3rd and 7th and this was statistically significant when compared to that of control groups. This was accepted that CN treatment prevents progressive tissue necrosis in zone of stasis. Finally this scintigraphic results were confirmed by macroscopic and histological examination at post burn day 21st. In the histological assessment the average percentage of re-epitheliazation in the zone of stasis was %64 in the CN treatment group and was %23 in the control group. This is also a late clinical confirmation of our scintigraphic findings.

Monafo et al. [9] first used CN treatment in burned patients. CN treatment has been reported to increase the survival rates in high-risk burned patients [10,25,26]. The exact mechanism of CN treatment has not been known well. It is recently suggested that specific toxic material released from burn eschar, known as lipid protein complex (LPC), are the trigger agent and it is implicated in phagocyte activation that results in release of several cytokines following burn [26–29] has been reported that CN irreversibly denature the LPC in eschar tissue which causes phagocyte activation [28,30]. Thus, the prevention of phagocyte activation decreases the secretion of cytokines, which induce systemic inflammatory response. In our previous study we showed that treatment with CN bathing causes reduction of TNF-α level, which is known to induce neutrophil and endothelial cell activation [12]. Consequently, we showed that CN treatment reduced the rolling, sticking and transmigrating leukocytes following burn in an intra vital microscopic study in the rat cremaster muscle flap. We believe that modulation of leukocyte activation following CN treatment is effective in prevention of tissue necrosis in zone of stasis. In another study, it was shown that CN treatment significantly reduced macromolecular leakage and modulates micro vascular circulation following burn.

The prevention of phagocyte activation [28], modulation of leukocyte activation [11] and reduced macromolecular leakage and edema [13] are effective to prevent local tissue ischemia. Tissue ischemia is a combination of systemic and local reactions following burn. Systemic reactions causes increased vascular permeability. This third spacing decreases effective vascular volume and decreases perfusion. Local reactions such as vasoconstruction, coagulation (due to low flow and tissue damage), edema (due to increased local vascular permeability), and vascular plugging (due to inflammatory infiltrate) could accentuate local ischemia (Slalom A, Kramer E. Annu Plast Surg 2001) As we mentioned above CN treatment is effective to prevent most of the systemic and local reactions. These help us to explain effectiveness of CN treatment in zone of stasis.

We followed the CN treatment protocol, which is recommended in the current literature [10] and found it to be effective for prevention or modulation of neutrophil activation. From our data and earlier study, we inferred that the mechanism of the effect of CN treatment on the inflammatory response to burning might be as follows: first, LPC is fixed by CN treatment in eschar tissue and a physical barrier is constituted between eschar tissue and the underlying dermis, so the activation of phagocytes is prevented. As a result decreased activation of these cells leads primarily to depression of the level of alarm cytokines and other inflammatory mediators. The lack of appropriate stimulus for the leukocytes, as well as endothelial cells, by the decreased secretion of cytokines and metabolic products, results in prevention of in vivo activation of leukocytes and endothelial cells. Finally, this causes a decrease of the number of rolling, sticking and transmigrating leukocytes as shown in our intra vital microcirculatory study. This result may be interpreted as a modulation of systemic inflammatory response.

Disclosure

Cerium nitrate was made by the Department of Pharmacology in Gulhane Military Medical Academy. All authors will receive no benefit of any kind either directly or indirectly and have no commercial in any of research presented here.

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