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Research Report

Topical application of 1% $ZnSO_4$ on oral ulcers increases the number of macrophages in normal or diabetic conditions of wistar rats

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ABSTRACT

Background: *Therapy for chronic ulcer in diabetic patient is by modifying local inflammation response using drugs that acts as immunomodulator, neuromodulator and growth factors stimulator. Topical zinc is one of drug that can modifiy local inflammation response, immunostimulation or immunosuppresion.* ***Purpose:*** *This study was to prove about the number of macrophage in oral ulcer between normal and diabetes microscopically and the difference if treated by 1% $ZnSO_4$ gel topically.* ***Method:*** *Ulcer in lower labial mucosa was made in normal and diabetic Wistar rats (induced by STZ), then applied 1% $ZnSO_4$ gel and CMC-Na gel as control. They were decapitated in third and fifth day and specimen was made by processing lower labial mucosa* ***Result:*** *Microscopically, the result showed the number of macrophages in oral ulcer in diabetic condition was significantly higher than normal and the application of 1% $ZnSO_4$ increased the number of macrophages in fifth day.* ***Conclusion:*** *The number of macrophages was higher in diabetic than normal condition, and was proven that topical application of 1% $ZnSO_4$ increased the number macrophages of oral ulcer diabetic and normal condition.*

Keywords: *macrophage; diabetes; $ZnSO_4$ 1% gel*

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INTRODUCTION

Chronic ulcers is one of complications experienced by diabetic patients. It is caused by a deviating local inflammatory response which is marked by the number of persistent inflammatory cells. This condition is caused by a decreased chemokine expression which, in turn, leads to a slower production of growth factor and a slower inflammatory cell infiltration.[1,2] The amount of persistent macrophages in a diabetic condition influences the healing process of ulcers, resulting in a continuous cell deterioration and increasing expressions of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and matrix metalloproteinases (MMPs).[3-5]

A study proposed a calculation of macrophages in ulcers both in normal and diabetic conditions using a mathematical formula, concluding that the amount of macrophages in the diabetic condition keeps increasing and thus becomes more persistent than the normal condition's. The model study, however, the formula has not been proven.[6]

Polyuria in diabetic patients causes excessive Zn excretion (hyperzincuria),[5,7-11] resulting in Zn deficiency.[12,13] The topical Zn application on ulcer of a diabetic patient helps the healing process more than the systemic Zn application.[11] The topical Zn application on ulcer will increase the concentration bioavailability of Zn approximately 1.000-3.000 μmol/L. Zn may help autodebridement process, influencing MMP to eliminate any necrotic tissues and facilitate keratinocyte migration in the re-epitelisation process.[8]

Zinc sulphate ($ZnSO_4$) is one of active substances that can be topically absorbed by oral mucosa and recommended to heal oral ulcers and prevent recurrent ones.[14-16] The topical application of 0,5% $ZnSO_4$ for oral suspension is

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effective in reducing oral lesions in patients with oral herpes simplex.[17] The gels of 1% $ZnSO_4$ may reduce perioral lesions and pains of any patients who infected by herpes simplex virus (HSV),[18,19] and speed up the healing process of post dental extraction scars by reducing the amount of macrophages and increasing fibroblast poliferation as well as capillaries.[20]

This study aimed to microscopically observe differences in the amount of macrophages in both normal and diabetic conditions and the influences of topical 1% $ZnSO_4$ on the amount of macrophages in the diabetic rats' oral ulcers.

MATERIALS AND METHOD

The method employed in this research was the true experimental post test only control group design, using Wistar rats as the laboratory animals. The rats were divided into two groups, 24 normal Wistar rats and 24 normal Wistar rats which later were made diabetic. The preparation of the laboratory animals, treatment application and tissues retrieval were performed in the animal house of the Biochemistry laboratory in the Faculty of Medicine, Universitas Airlangga. The tissues processing and the creation of histological preparation were performed in the laboratory of Anatomic Pathology at the regional public hospital of Dr. Soetomo, Surabaya. The identification and the calculation of the amount of the macrophages were performed in the laboratory of Anatomic Pathology at the hospital of Islam Sultan Agung Semarang.

The Wistar rats which were made diabetic had been fasting for 4 hours before being induced with streptozotocin (STZ) (bioWORLD, Dublin, Ohio, USA) dissolved into citric buffer at pH 4.5, in order to empty their stomachs and reduce the risks of aspiration. The amount of STZ needed was 150mg/kg of the weight of a Wistar rat, dissolved with a concentration of 22.5mg/ ml STZ in citrate buffer solutions. The STZ solutions then were injected through intraperitoneal according to the appropriate dose for each rat. The induction process was only performed once. In order to avoid sudden hypoglycemic post injection**,** the rats received 10% sucrose or dextrose during the first night. The blood sugar level of the rats during fasting hours was observed every morning. The fasting rats did not receive food and the cages were devoid of husks for six hours. Any meaningful hyperglicemia condition would be found two days post induction.[21]

A scar was made in the lower labial mucosa using the tip of round burniser in 2 mm diameter which had been heated for ±15 seconds with a bunsen burner. The tip of burnisher then was touched the lower labial mucosa of each Wistar rat for one second in 2 mm deep. We spread some gels of 20% benzocaine as a topical anesthesia in the lower labial mucosa of each rat 5 minutes before and after ulcer was made. The observations were performed at the 24th and 48th hours after the scars being made. At the 24th hour observation, a decay in the lower labial mucosa with a thin, white base with 3 mm diameter was visible. In the 48th hours post-trauma, a deep ulcer with a yellowish base in the lower labial mucosa was visible (Figure 1).

The applications of gels of sodium carboxymethyl cellulose (CMC Na) and 1% $ZnSO_4$ were performed after the ulcers were formed in the oral mucosa of the rats. The gels were applied using cotton buds in the morning and the evening until the days when the animals' tissues would be retrieved, which were the 3rd dan 5th days. The rats did not receive food or drink for 30 minutes after the gel applications.

Several Wistar rats were randomly selected from each group according to the predetermined amount of samples. The selected rats were then terminated on the 3rd and 5th days. Their lower lips were cut and used in the tissues processing to produce histological preparations and calculated their macrophages cells. The microscopic preparations were observed using **a** microscope with a camera DS Fi2 300 megapixel (Nikon H600L, Konan, Minota-ku, Tokyo, Japan), while the tissues were calculated using software (Optilab.Image Raster v21, Sleman, Yogyakarta, Indoneisa).

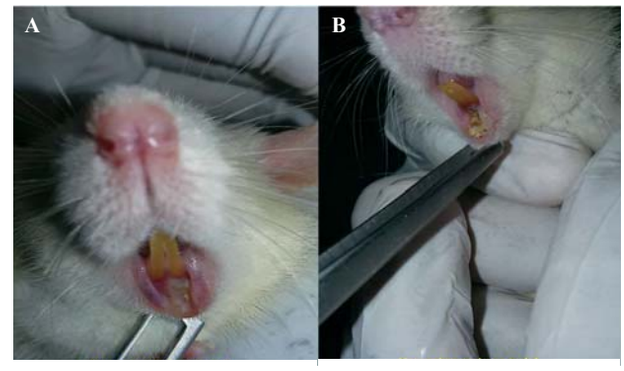

Figure 1. The illustration of a macroscopic ulcer in the oral mucose of a rat with a scar made by the tip of burnisher in 2mm diameter. In the 24th hours after trauma, a scar of the mucose with a thin, white base in 3mm diameter was observed (A). In the 48th hour post-trauma, a deep ulcer in the mucose with a yellowish base was observed (B).

Table 1. The average amount of macrophages in the treatment groups on the 3rd and 5th days

	3rd day	5th day
Normal + CMC Na	464	144
Diabetes + CMC Na	352	183
Normal + Zn SO_4	414	303
Diabetes + Zn SO_4	194	324

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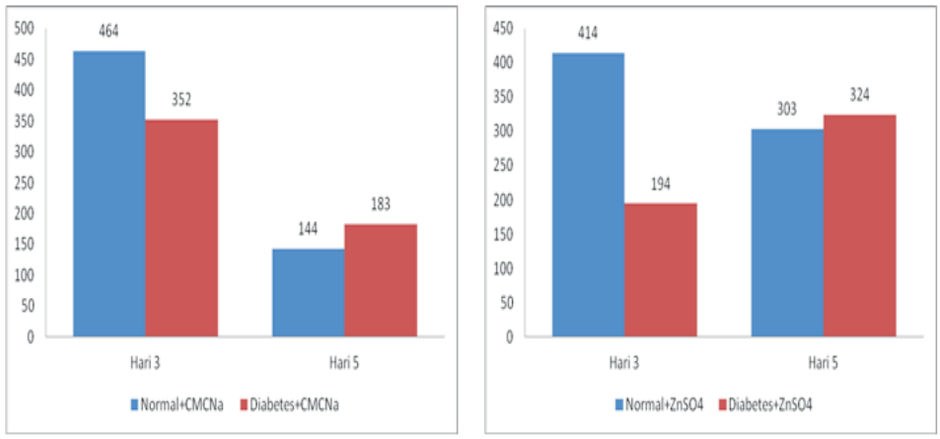

Figure 2. The average amount of macrophages in the treatment groups on the 3rd and 5th days.

RESULTS

The Kolmogrov Smirnov (one-sample K-S) test showed that the data from each variable of each treatment group on the 3rd and 4th days were normally distributed. The next data testing employed the independent sample t test by comparing the data on the 3rd and 5th days from the normal and diabetic groups with CMC Na therapy and from the normal and diabetic groups with $ZnSO_4$ therapy.

On the 3rd day, the amount of macrophages of the diabetic group applied with CMC showed a less meaningful distinction from the normal one's, while on the 5th day, the amount of macrophages showed a meaningful distinction although it was larger than the normal group's. In the comparison between the amount of macrophages with $ZnSO_4$ and omitted the one with CMC Na both in the normal and diabetic conditions, the amount of macrophages on the 5th day increased after the application of $ZnSO_4$.

DISCUSSION

The macrophages in the healing process of ulcers manage inflammatory and angiogenesis processes. The macrophage proliferation responds to pathogen, hemostasis process in tissues, inflammation, resolution, and repair processes. The macrophages remove cytokines and growth factor which influence various cells and induce cytokines (anti-inflammation), glucocorticood, and glucose metabolism and lipid.[5,22-24] During an inflammation in normal condition, the macrophages increase shortly between 3rd and 5th days.[25]

The macrophages coming from monocytes that migrate to the tissues will increase in numbers for two days (48 hours) around an ulcer and stay there for the next five days. Once the inflammation can be controlled by the macrophages, the healing process will begin from the 3rd day until the 5th day, marked by the migration and increase of fibroblast, proliferation of endothelial cells and then the emergence of granulation tissue. [2,5,11,26,27]

Diabetes reduces the amount or quality of insulin receptor or makes it resistant to peripheral tissue, resulting in insulin binding, or afinity, or insulin's decreased sensitivity.[5,7,28] The reduced quality of insulin receptor will disturb the functionality of mitochondria in producing ATP as the source of cell energy. ATP is a product of glucose metabolism in the intercells which enters through FLUT-4 in the cell membranes. GLUT-4 is activated by the signal transduction sent by the insulin receptor which is linked to insulin.[28] The disturbed mitochondria may cause disturbances in the neutrophil apoptosis during the autophagocytosis process which is supported by the macrophages, disturbances in the macrophages migration to the tissue, and increase pro-inflammatory cytokine (IL-6 dan TNF-α) which is produced by pro-inflammatory macrophages (M1).[1,2,4,5,22-24,28,29]

A normal person's body contains 2-3 grams of zinc which influences cell metabolisms. In the cell membranes, there is a transporter in which ionic zinc may enter (ZIP=Zirt-Irt-Protein) and exit (ZnT=Zinc transport) to maintain the amount of ionic zinc in cytoplasm. Ionic zinc inside the cytoplasm serves as a pro-antioxidant which helps zinc superoxide dismutase enzyme to scavenge ROS which is a sideline product of glucose metabolism (in the cells).[30,31]

In a normal condition, the amount of zinc will increase 15-20% in the edges of ulcer on the 24th hour after inflammation phase. During a formation of granulation tissue and epitherial proliferation, the amount of Zn will increase up to 30%.[8] In diabetic patients, the amount of Zn in blood (hypozincemia) decrease which in turn will reduce the amount of Zn in the tissues.[8,9,11,13,16]

During an inflammation in diabetic patients, there is an increase in the amount of neutrophil that results in an imbalance between the amount of neutrophil and of macrophages. In fact, the balance between the two are necessary in the ulcer healing process.[2] Neutrophil will increase dramatically on the 48th hour of the inflammation[26] and increasingly stimulate the amount of macrophages. After that, neutrophil will experience apoptosis.[2] Deficit

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zinc will influence the amount and function of leukocyte (neutrophil-granulocyte/PMN and monosit) and reduce neutrophil chemotaxis which, in turn, will reduce the performance of phagocytosis.[30]

Therapy on chronic ulcers towards diabetic patients may be performed by modifying the deviating local inflammatory response[2] with the use of several drugs which serve as imunomodulator, neuromodulator, and growth factors stimulator, during one or more phases of ulcer healing.[32] Zinc is one of the topical immunomodulators used to modify local immune responses which may stimulate immune response or suppress immune response.[31,33]

Zinc serves to manage proliferation process, differentiation and cell apoptosis. During an inflammation phase, zinc helps the functions of neutrophil and macrophages. Zinc affects the production of pro-inflammatory cytokine (IL-1 β, IL-6 and TNF-α) which helps chemotaxis process and phagocytosis of neutrophil.[8,16,18,19,31,34] Ions of topical zinc applied to ulcers will pass epitelium after an hour, enter the sub-epitelium and then be absorbed in the blood circulation. The deeper the damage in epitelium is, the more zinc will enter blood circulation.[8]

Zinc influnces chemokine phorbol myristate acetate (PMA) which serves a role in the attachment of monocyte in the endothelial cells and monocyte chemoattractant protein-1 (MCP-1) which serves in the monocyte migration to the tissue, becoming macrophages. Zinc in the monocyte-macrophages serves as a pro-inflammation or anti-inflammation, depending on its concentration in the cells,[34,35] but not an antioxidant and does not take part in phagocytosis.[31] Diabetic patients are recommended to use topical zinc application to heal their ulcers.[8,11]

In conclusion, the amount of macrophages in the diabetic condition was greater than the one in the normal condition microscopically. The application of 1% $ZnSO_4$ increased the amount of macrophages in both normal and diabetic conditions, and clinically the application of 1% $ZnSO_4$ showed a faster ulcer recovery process in the oral mucosa.

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