# Role of Inflammatory Cell Responses in Stimulating Fibroblasts in Diabetic Oral Ulcer after Treatment with Liquid Smoke of Coconut Endocarp: A Histological Assessment

Diah Savitri Ernawati[1] Meircurius Dwi Condro Surboyo[1] Nurina Febriyanti Ayuningtyas[1]
Ayu Anggraini Broto Nagoro[2]

[1]Department of Oral Medicine, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia
[2]Bachelor of Dental Surgery Program, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia

**Address for correspondence** Meircurius Dwi Condro Surboyo, Drg, MKes, Department of Oral Medicine, Faculty of Dental Medicine, Universitas Airlangga, Surabaya 60132, Indonesia (e-mail: meircurius-2015@fkg.unair.ac.id, meircurius@fkg.unair.ac.id).

Eur J Dent:2021;5:71–76

## Abstract

**Objective** The liquid smoke of coconut endocarp (LS-CE) contains high antioxidants that promote oral ulcer healing in diabetics. This study reveals the profile of inflammatory cell responses to oral ulcer healing in diabetics under treatment with LS-CE.

**Materials and Methods** A diabetic model was induced with alloxan. Treatment with LS-CE was performed on oral ulcer at a dose of 1 µL/g weight for 3, 5, and 7 days. The anti-inflammatory effect was tested on animal's oral ulcer model by measuring the inflammatory cell responses of the neutrophils, macrophages, lymphocytes, and fibroblasts through histological assessment.

**Results** The LS-CE stimulated the healing by simultaneously increasing the inflammatory cell responses. The numbers of neutrophils, macrophages, and fibroblasts after treatment for 7 days are higher than that after 3 days and 5 days ($p < 0.01$), but not for neutrophils. The LS-CE shows increase in the fibroblasts by hastening responses of macrophage recruitment by five times, but not neutrophil and lymphocyte recruitment. The higher phenolic compounds in LS-CE are responsible for increase in the proliferation of fibroblasts, as it hastens cellular responses of macrophages.

**Conclusions** The application of LS-CE enables hastening of the healing of diabetic oral ulcer by stimulating the macrophages.

**Keywords**
- liquid smoke
- coconut endocarp
- inflammatory
- oral ulcer
- macrophages
- fibroblast

## Introduction

The oral ulcer is a wound in oral cavity. Each drug that is applied on the oral ulcer has to be able to stimulate and promote complete healing. The healing of oral ulcer is a complex process that includes the cellular and tissue responses.[1] Cellular response, as part of inflammation, is the second response after hemostasis in wound-healing cascade,[2] which includes the recruitment of inflammatory cells such as neutrophils and macrophages into the ulcer area.[3,4] Many mediators such as cytokines and growth factors are released by macrophages and lymphocytes to proceed the cell proliferation to stimulate the tissue response to form the new epithelial.[5] The recruitment of inflammatory cells is very important to define healing of oral ulcers. On the other hand, fibroblast is the cellular component responsible for synthesis of collagens to complete the new epithelial, as a consequence of responses in inflammation.[6]

The diabetic condition causes problems in activating and recruiting the cellular response, especially the inflammatory

**DOI** https://doi.org/10.1055/s-0040-1715913
**ISSN** 1305-7456.



Thieme Medical and Scientific Publishers Pvt. Ltd., A-12, 2nd Floor, Sector 2, Noida-201301 UP, India

cells. Prolonged inflammation followed by delayed healing occurs in diabetics. Activities like delayed neutrophil recruitment, impaired macrophage function, continuous release of proinflammatory cytokines, impaired keratinocytes, and fibroblast proliferation are the key factors in delayed healing in diabetics.[7] Neutrophils and macrophages are slowly added to the wound after injury in diabetes, but it stays in the wound bed for an extended time in a large number. It creates a surrounding that is particularly enriched by reactive oxygen species (ROS) and proinflammatory cytokines, which further obstructs the proliferation of fibroblasts and keratinocytes while damaging the tissue.[8] Macrophages are also shown to have altered functions. It increases the number of inflammatory profile and apoptosis, while fails to stimulate tissue repair by exhibiting reduced phagocytic capacity.[9,10] The delayed recruitment of neutrophils caused by increased amount of advanced glycation end products (AGEs) deposition directly inhibits the chemotactic activity of neutrophils.[11] The higher number of AGEs also mediates the activation of ROS that causes impaired keratinocyte and fibroblast migration and proliferation, blocking the wound healing. Moreover, the diabetic wound fibroblast also has abnormal morphology, decreased adhesion, decreased response to growth factors and cytokines, and decreased production of collagens and fibronectin, resulting in abnormal extracellular matrix structure and composition.[8,12] The activation of ROS also induces lymphocyte apoptosis that inhibits the healing cascade.[13]

Liquid smoke of coconut endocarp (LS-CE) contains phenolic compounds like phenol, guaiacol, and 4-ethyl-2-methoxyphenol (2-EMP). These compounds show capability to inhibit the free radicals. LS-CE in treatment of oral ulcer in diabetic condition modulates the ulcer healing by decreasing the tumor necrosis factor α (TNF-α) and nuclear factor kappa B (NF-kB)[14] and increasing the number of collagens,[15] thus increasing the healing process.[16] The decrease in TNF-α and NF-kB will hasten and fasten the inflammatory responses. The first inflammatory response in the wound healing is recruitment of inflammatory cells such as neutrophils, macrophages, and lymphocytes.[17] Based on that finding, we need to confirm the profile of inflammatory cells responses in diabetic oral ulcer under treatment with LS-CE by histological assessment.

## Materials and Methods

### Liquid Smoke

The clean and dry *Cocos nucifera* L. endocarp was used to produce a liquid smoke. A total of 5 kg endocarp was obtained from *Cocos nucifera L*. Pyrolysis process was conducted to produce the liquid smoke. The pyrolysis of endocarp takes 4 hour and 30 minutes, with the final temperature being 400°C. The heating rate was constant at 3.33°C/min. After 72 hours, the liquid smoke was distilled at 120–150°C to obtain the pure liquid smoke.[14]

### Animals

The protocol of this research was approved by the Ethical Clearance Committee, Faculty of Dental Medicine, Universitas Airlangga (number: 536/HRECC.FODM/VIII/2019).

Twenty-one male *Rattus norvegicus*, weighing 120–160 g, were used as diabetic animal models induced with alloxan (Alloxan monohydrate, Sigma Aldrich., St. Louise, USA) with a dose of 0.15 mg/g, where the condition itself confirmed as presenting fasting glucose >200 mg/dL about 72 hours later. Further, 10 mm of oral ulcer was created in the labial fornix incisive inferior, using a round stainless blade, after being anesthetized using combination of ketamine and xylazine. After 24 hours, the oral ulcer appears as white color surrounded by erythematous arc. At this point, treatment with LS-CE was performed at a dose of 1μL/g weight once a day on the oral ulcer for 3, 5, and 7 days.[16]

### Neutrophils, Macrophages, Lymphocytes, and Fibroblasts

The neutrophils, macrophages, lymphocytes, and fibroblasts were histologically analyzed with staining using hematoxylin-eosin. All parameters were evaluated and performed in a blinded manner by a single calibrated operator under light microscopy with magnification 400×.

### Data Analysis

Statistical analysis was performed using Statistical Package for the Social Sciences 25.0 software for Windows. Analysis of variance was then performed on data obtained regarding the number of neutrophils, macrophages, lymphocytes, and fibroblasts. Then post-hoc test was conducted with $p < 0.01$ in cases of any discrepancy between the treatment groups. To analyze the effect LS-CE on neutrophils, macrophages, and lymphocytes in stimulating fibroblasts, linear regression analysis was done with $p < 0.05$.

## Result

The oral ulcer condition was indicated by the disintegrated labial epithelia (►**Fig. 1**). The microscopic presentation of neutrophils, macrophages, lymphocytes, and fibroblasts in the oral ulcer tissue was done using hematoxylin-eosin staining for each day as presented in ►**Fig. 2**.

The inflammatory cell responses after treatment with LS-CE simultaneously increased after 3–7 days of treatment. The number of neutrophils after treatment for 7 days is higher than that after 3 days ($p = 0.001$) but lower than that after 5 days ($p = 0.004$; ►**Fig. 3**).

The number of macrophages after treatment for 7 days is higher than that after 5 days ($p = 0.009$) and 3 days ($p = 0.000$). The number of macrophages after treatment for 5 days is higher than that after 3 days ($p = 0.004$; ►**Fig. 3**).

The number of lymphocytes is no different after treatment for 3, 5, and 7 days ($p = 0.068$; ►**Fig. 3**).

The number of fibroblasts after treatment for 7 days is higher than that after 3 days ($p = 0.006$) and 5 days ($p = 0.000$; ►**Fig. 3**).

The effect of LS-CE on fibroblast was 90% ($R = 0.900$; $p = 0.000$). The LS-CE affected the increase in macrophages ($a = 0.586$; $p = 0.004$) but not in the number of neutrophils ($p = 0.075$) and lymphocytes ($p = 0.506$; ►**Table 1**).

## Discussion

The wound-healing mechanism in diabetes mellitus is seen mostly with abnormalities, namely delayed recruitment of inflammatory cells, prolonged inflammation, impaired neovascularization, decreased collagen synthesis, increased protease rates, and disordered macrophage function.[18] Wound-healing process starts from hemostasis until remodeling.[6] These phases must happen regularly; some abnormalities can lead to delayed wound healing.[19]

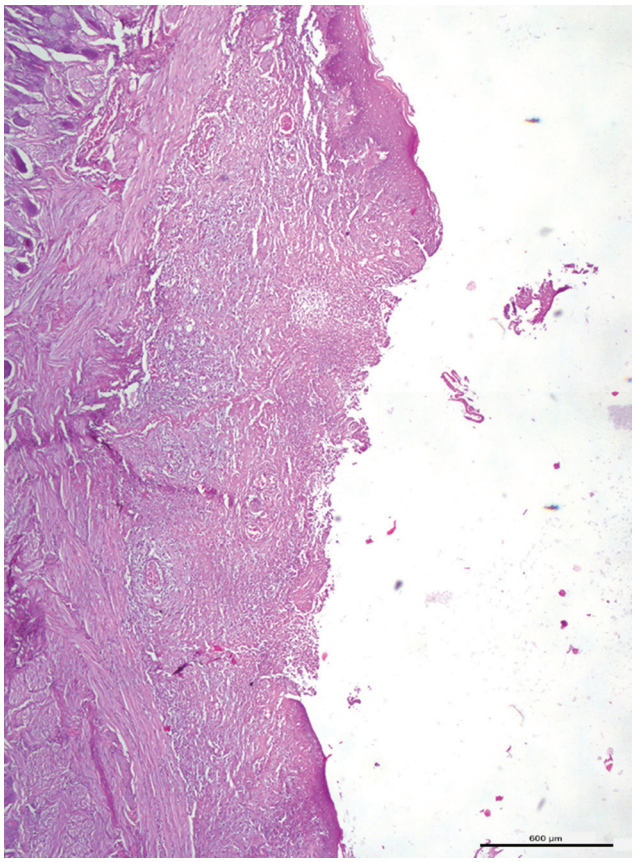


**Fig. 1** Histology of oral ulcer (magnification at 100×).

In this study, we used LS-CE to treat diabetic oral ulcer in animal model. The treatment resulted in increase in the number of fibroblasts, macrophages, and lymphocytes continuously after 3 days up to 7 days, except the number of neutrophils that increased after 3 days up to 5 days but then decreased by the 7th day.

LS-CE is identified to have seven groups of components: phenol, guaiacol, furan and pyran, carbonyl, ketone, syringol, and alkyl aryl ether. The phenol content in LS-CE reached to 43.6%.[14] The highly effective phenolic compounds in LS-CE that have antioxidant properties are 2-methoxyphenols (guaiacol), phenol, and 2-EMP.[15] Phenolic compounds are among the most important ingredients of free radical terminators and key antioxidants.[20] In the inflammatory process, LS-CE phenols play a role during wound healing by scavenging free radicals or ROS.[15,18] During inflammatory phase, neutrophils, macrophages, fibroblasts, and endothelial cells produce ROS as a result of compensation from inflammatory responses and protection from microorganisms' invasion. The overproduction of ROS can decrease the rate of healing.[21]

The role of neutrophils in the ulcer area is to cleanse the bacteria and foreign materials to provide a good environment for ulcer-healing process. Neutrophils cause other inflammatory cells such as mast cells and macrophages to activate, which secrete chemokines and cytokines.[22] Neutrophils and macrophages work together to enhance the inflammatory response against pathogens. In diabetic condition, the time that neutrophils take to be recruited to the wound site is prolonged while maintaining a longer

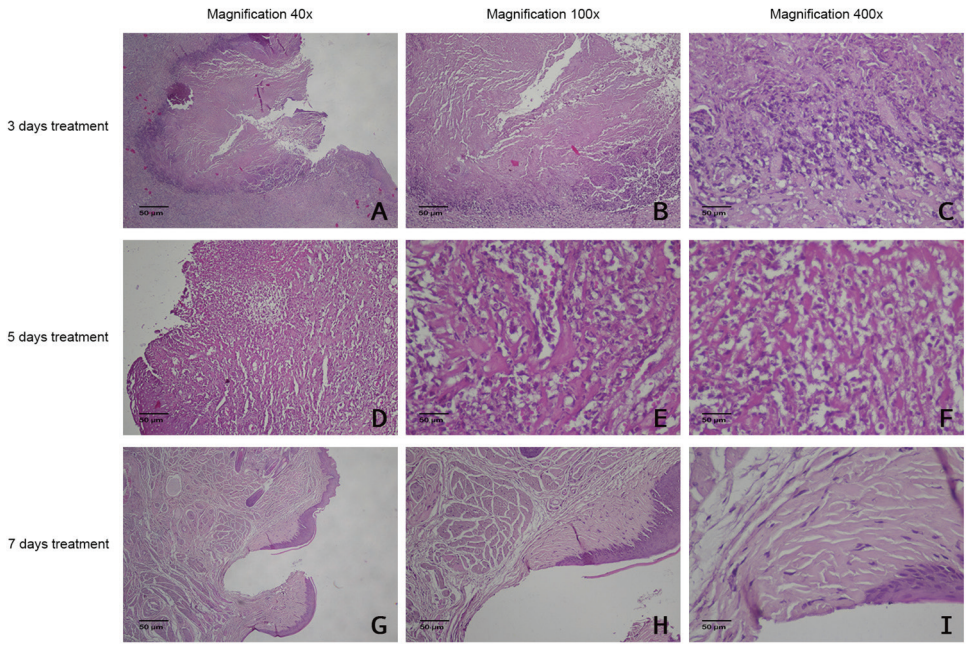


**Fig. 2** Histological analysis of neutrophils, macrophages, lymphocytes, and fibroblasts.

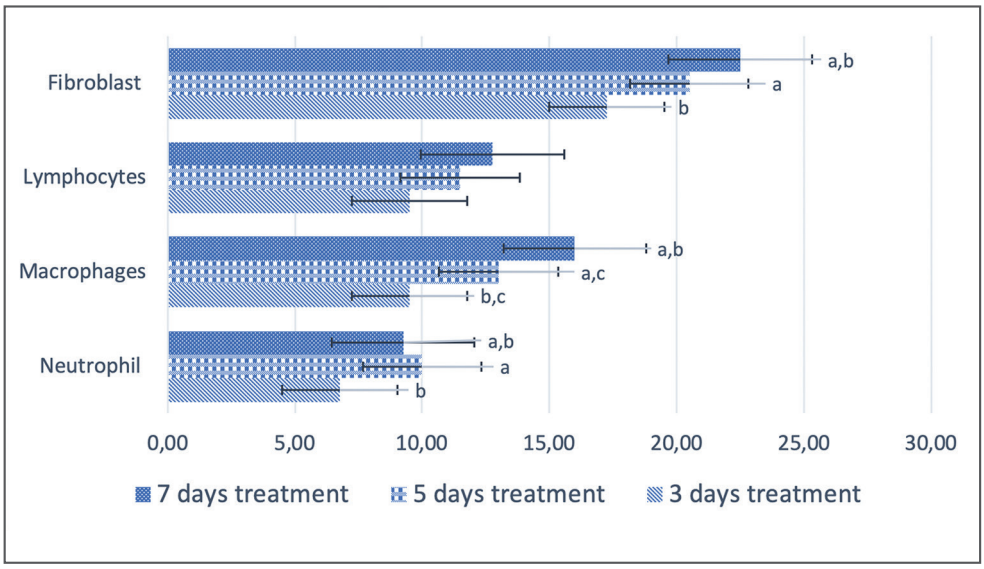


**Fig. 3** Cellular responses after treatment with liquid smoke of coconut endocarp. The same superscript at the top of each bar indicates the differences with each other ($p < 0.001$).

**Table 1** The effect of liquid smoke of coconut endocarp on inflammatory cell responses in stimulating fibroblasts

| Cellular component | *P* | *R* | α | *a* | sig |
|---|---|---|---|---|---|
| Neutrophil | 0.000 | 0.900 | 6.011 | 0.557 | 0.075 |
| Macrophages | | | | 0.586 | 0.004 |
| Lymphocytes | | | | 0.162 | 0.506 |

Abbreviations: α, constant; a, coefficient of regression; *P*, value of analysis of variance significance; *R*, coefficient of correlation; sig, value of significance ($p < 0.05$, consider as significant value).

time in the wound.[23] The longer stay of the neutrophil in the wound can cause prolonged inflammatory responses that can possibly contribute to worsening of the wound.[24] The increase in the number of neutrophils after 3 days up to 5 days and then decrease by the 7th day is a good sign because the neutrophils need to recruit for stimulating the fibroblast proliferation in oral ulcer healing. The neutrophils release extracellular neutrophil traps while under normal inflammatory stimuli, to facilitate fibroblast differentiation and function (►**Fig. 4**).[25] In diabetic conditions, however, a large number of neutrophils binds outside the vascular tissue with AGEs, thereby inhibiting their migration while releasing a large number of inflammatory cytokines that cause oxidative stress, resulting in delayed wound healing.[26] The role of LS-CE in affecting the functioning of neutrophils may be attributed to the fact that the phenolic compounds inside could inhibit the development of AGEs and subsequent protein crosslinking. The antiglycation capacity of the phenolic compound itself could be related to the antioxidant feature.[27] The decreased number of AGEs can restore the chemotactic ability of the neutrophils, and effectively increase neutrophils' recruitment to the wound site (►**Fig. 4**).

The LS-CE treatment on oral ulcer also affected the macrophages. The number of macrophages increased after 3 days up to 7 days. The LS-CE was also able to stimulate the fibroblasts by increasing the macrophage recruitment.[28] The correlation between the increased number of fibroblasts and macrophages is due to the macrophage function that promotes the proliferation of fibroblasts.[29] The macrophage is mainly divided into regulatory macrophages and wound-healing macrophages.[30] The regulatory macrophages release anti-inflammatory cytokines, such as interleukin 10 and transforming growth factor β,[31] enabling to downregulate the inflammation, leading to the mesenchymal transition from the endothelial and increased number of the fibroblast.[32] The wound-healing macrophages are the kind of M2 macrophages that is derived through interleukin 4 induction that could secrete chemokine ligands, such as CCL2, CCL17, CCL18, and CCL22.[29,33] In abnormal conditions such as diabetes, the high level of ROS can decrease the number of macrophages that could infiltrate the ulcer area. It causes the persistence of apoptotic cells, neutrophils, and wound debris that create a constant inflammatory process while continuing to release proteases that degrade the wound microenvironment nonspecifically, and as a result it prolongs the wound inflammation.[9,24] The application of LS-CE may affect the regulatory macrophages. The phenolic compounds in the LS-CE are able to decrease the M2 macrophages to produce the proinflammatory cytokine like TNF-α,[14] by inhibiting the activation of NF-kB.[14]

Meanwhile, in the wound-healing process, lymphocytes have a modulatory role. Lymphocytes infiltrate the

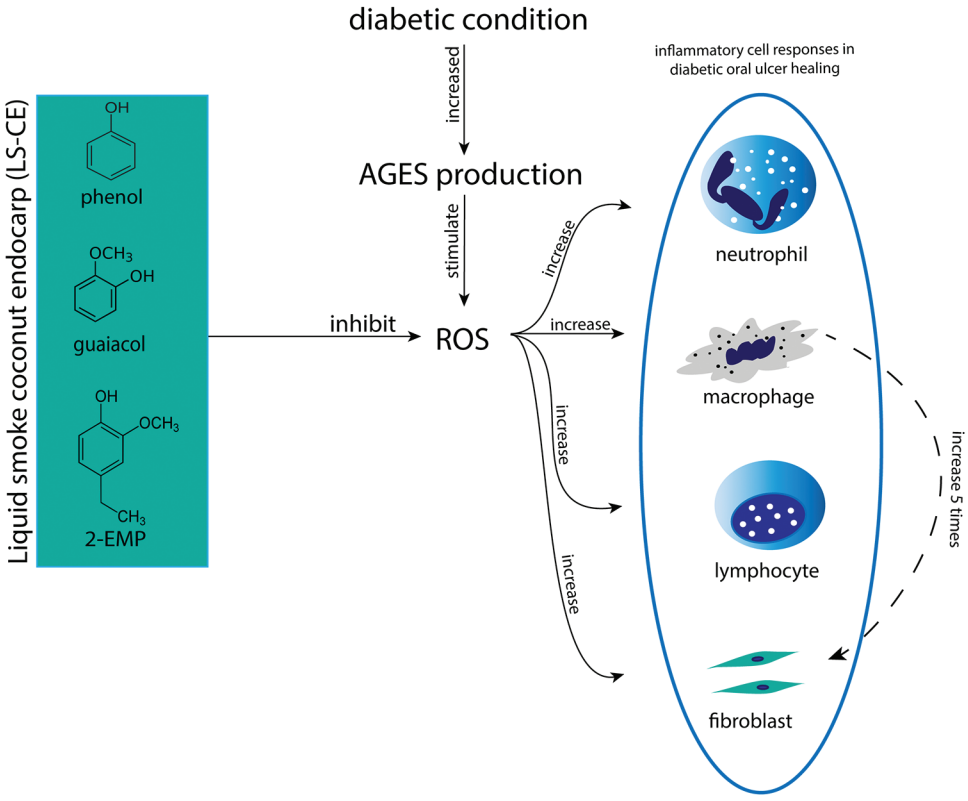


**Fig. 4** The mechanism of LS-CE to stimulating fibroblast.

wound area following the inflammatory cells and macrophages, and become higher in number for the next phase.[1] Treatment with LS-CE showed no effect on lymphocytes. The explanation may be that the phenolic compounds inside LS-CE can produce $H_2O_2$ that may modulate inflammatory responses while regulating the production of proinflammatory cytokines released from lymphocytes. But the generated number of $H_2O_2$ produced by phenolic compounds may vary, so it could have little to no effect on the number of the lymphocytes.[34]

The fibroblasts generate collagen during the proliferative process, which play a major role in the formation of extracellular matrix.[1] In the remodeling phase, the granulation tissue, which is from blood vessels, capillaries, fibroblasts, macrophages, and collagen fibers, becomes mature and has an increased tissue tensile strength.[35] Treatment with LS-CE will have an effect on the number of fibroblasts, as a result of controlled inflammation and stabilization of inflammatory cell profile in the ulcer area. LS-CE in the previous researches showed to increase the fibroblasts, caused faster wound closure[36] and oral ulcer healing,[16] and increased the synthesis of collagen.[15]

This study is a corroborating evidence that LS-CE can affect the healing of diabetic oral ulcer by affecting the inflammatory cell responses. The result of this study also confirmed the two previous studies that LS-CE also affects the macrophages by directly inhibiting the inflammatory process[28] and increasing the collagen density,[15] thus increasing the ulcer healing.[16] The limitation of this study lies in the single dose of LS-CE. Further research needs to isolate the phenol compounds present in the LS-CE to decrease the acidity.

In conclusion, the higher phenolic compounds in LS-CE are responsible for increased proliferation of fibroblasts by hastening inflammatory cell responses such as that of macrophages. Treatment with LS-CE is able to hasten healing of diabetic oral ulcer by stimulating macrophage recruitment.

**Funding**
This work was supported by Kementerian Riset Teknologi Dan Pendidikan Tinggi Republik Indonesia, Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) 2019 with grant number 6/E/KTP/2019.

**Conflict of Interest**
None declared.

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