Original Article

Neovascular Pattern in Wound Healing after Zinc Oxide and *Curcuma longa* Rhizome Extract Dressing Application

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Abstract

Introduction: Zinc oxide-eugenol dressing is widely used in the dentistry field. Eugenol in wound dressing acts as an antibacterial and analgesic agent but known to cause hypersensitivity reaction and allergies. *Curcuma longa* rhizome extract could perform as a substitute of eugenol as its active compounds have an anti-inflammation, antioxidant, anticancer, and antibacterial properties. It has also been shown to have proangiogenic and promote wound healing. This study aimed to discover the number of neovascular in the wound healing process after the application of zinc oxide and *C. longa* rhizome extract wound dressing. **Materials and Methods:** Full-thickness excision wound of 6 mm × 6 mm was made in the dorsal of 32 Wistar strains Rattus norvegicus, before being equally divided into eight groups (n = 4): four control groups (C3, C5, C7, and C14) without any dressing and 4 treatment groups (T3, T5, T7, and T14) were dressed with zinc oxide and *C. longa* extract wound dressing. The rats consecutively sacrificed on day 3 (C3, T3), day 5 (C5, T5), day 7 (C7, T7), and day 14 (C14, T14) to observe the neovascular pattern and number using H and E staining. Obtained data were analyzed using ANOVA. **Results:** The neovascular growth in both control and treatment groups have the same pattern, while the number of neovascular in treatment groups significantly higher than the control groups ($P < 0.05$). **Conclusions:** Wound dressing combination of zinc oxide and *C. longa* extract can increase the number of neovascular in the wound healing process, while the optimum duration of application is 14 days.

Keywords: *Neovascularization, periodontal dressings, turmeric extract, wound healing, zinc oxide*

Introduction

Wound is a disruption in the incontinuity of the epithelial lining of the skin or mucosa resulting from physical or thermal damage.[1] The body response to trauma or injury was to conduct a wound healing process.[2] Physiologically, the wound healing process the whole body has a similar principle only differing in the number of cells involved.[3]

Wound dressing is a physical barrier to protect the wound site from possible interventions of healing.[4] Wound dressing has three classifications, zinc oxide-eugenol, zinc oxide-noneugenol and other types.[5] The first type of wound dressing introduced by AW Ward is zinc oxide-eugenol.[6]

Eugenol is used as a wound dressing material because of its pharmacological properties as antibacterial and analgesic.[6] Clinical use of eugenol has some negative effects because of the risk of hypersensitivity, irritation, and allergic reactions. One case is contact stomatitis allergic in the use of eugenol as a cement ingredient.[7]

The negative effect of eugenol is the reason for finding an alternative wound dressing. One of the alternative is a combination of wound dressing zinc oxide and *C. longa* extract. Previous experiments have proven that the combination of zinc oxide wound dressing and the *C. longa* extract can hasten the wound healing process by decreasing the expression of toll-like receptors-2, nuclear factor κB (NF-κB), and tumor necrosis factor α in the inflammatory phase.[8] The nature of zinc oxide as antibacterial, disinfectant, and drying agent is the reason of zinc oxide use in medicine field. The previous experiments showed that the nature of zinc oxide can hasten the wound healing.[9]

C. longa or turmeric is herbs used as spices, dyes, and medicines in India and China. *C. longa* can be used to treat diabetes mellitus, cough, liver disease, sinusitis,

How to cite this article: Faiga NN, Rachmadi P, Meizarini A. Neovascular pattern in wound healing after zinc oxide and *Curcuma longa* rhizome extract dressing application. Contemp Clin Dent 2018;9:S337-41.

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Access this article online

Website: www.contempclindent.org

DOI: 10.4103/ccd.ccd_435_18

Quick Response Code:

rheumatism, and swelling due to injury.[10] *C. longa* has an active substance called curcuminoid. Curcuminoid consists of curcumin, demetoxycurcumin, and bisdemetoxycurcumin.[11]

Curcumin is also known to have a role in regulating neovascular.[12] Curcumin can increase the expression of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β), and fibroblast growth factor-2 (FGF-2) which important for angiogenesis process.[12-15]

The negative effects of eugenol, the pleiotropic properties of *C. longa.* and the role of *C. longa* rhizome extract on neovascular become the background of the study. This study aimed to discover the number of neovascular in the wound healing process after the application of zinc oxide and *C. longa* rhizome extract wound dressing.

Materials and Methods

Ethical approval

All procedures performed in this research is ethically approved and legalized by Ethical Committee of Faculty of Dental Medicine, Universitas Airlangga. The ethical clearance certificate number of this research is 139/HRECC.FODM/VIII/2017.

Animal subjects

The experimental design of this study was post test only control group design. The subjects of this study were 32 healthy, 3 months' old, male Wistar strain Rattus norvegicus weighing around 200–300 mg, which were randomly divided into eight groups (n = 4): four control groups (C3, C5, C7, and C14) and four treatment groups (T3, T5, T7, and T14). The rats were kept for seven days to adapt in the cage, which placed in a closed room sufficient with light to avoid humidity and predator reach, away from noise and not exposed to direct sunlight. Food provided is corn-based food (PT. Charoen Pokphan, Mojokerto, Indonesia) and tap water *ad libitum*. The cage of each group was labeled based on the treatment given.

Experimental design

Before the treatment, all rats were intramuscularly anesthetized using ketamine (KEPRO, ZA, Denmark) and xylazine (Interchemie werken, Venray, Holland) with 1:1 ratio (0.1 ml/rat body weight) in the right upper leg. The dorsal area of vertebral thoracic of the rats was shaved and wiped with cotton roll drenched in 70% ethanol solution, before a full thickness excision of 6 mm × 6 mm with 2 mm in depth was made using scalpel handle no. 3, scalpel blade no. 15 (Swann Morton, England, UK), surgical scissor, and chirurgic tweezers. After the excision, the wound site was cleaned by wiping a cotton roll drenched in 0.9% NaCl solution.

The wound of the four control groups was covered with hypoallergenic tape (Hypafix, Hamburg, Germany) and maintained until the observation time while the wound of the treatment groups was dressed with zinc oxide and *C. longa* rhizome extract wound dressing before being covered by hypoallergenic tape. The dressing was made by mixing zinc oxide powder (Merck KGaA, Darmstadt, Germany) and *C. longa* rhizome liquid extract (Balai Materia Medica, Batu, Indonesia) with 1:1 ratio (0.3:0.3 g) on the mixing pad with circular movement using stainless steel cement spatula until become a homogenous dough in 60 s. A wide excision was made on the wound site, and the excised tissue was soaked in sterile aquadest before being wrapped with filter paper to avoid folded tissue, then immersed in 10% neutral buffer formalin for 2 days.

Immunohistochemistry examination

The collected tissue was blocked by paraffin before being further processed for histopathological anatomy microscope slides and stained with H and E (Santa Cruz Biotechnology, Dallas, USA) to observe the neovascular formation. Neovascular observations were performed by manually counting the five fields of view with × 400 magnification under Nikon E100 light microscope (Nikon, Tokyo, Japan) that connects directly to the monitor, which conducted by two observers. The results of both observers were added and averaged. The object to be counted was a small lumen surrounded by endothelial cells and contains red blood cells and resides around existing blood vessels. The picture of field of view taken with camera SONY ILCE α6000 (SONY, Tokyo, Japan).

Statistical analysis

The data obtained was statistical analyzed with SPSS 23.0 (IBM, New York, NY, USA) using Kolmogorov–Smirnov continued by Levene test to find data distribution and homogeneity, before being calculated using one-way ANOVA followed by Tukey's *Post hoc* test to determine the significance of each group. Significant value used was $P < 0.05$.

Results

The result of H and E staining on each group was shown in Figure 1 while the mean and standard deviation could be summarized in Table 1. The statistical analysis was performed using Kolmogorov–Smirnov test has value of 0.956 ($P > 0.05$), and Levene test has a value of 0.507 ($P > 0.05$), showed that all data are normally distributed and homogeneous. The one-way ANOVA statistical test has value of 0.00 ($P < 0.05$) which was followed by Tukey's *Post hoc* test which was presented as the superscript on each group.

From the data obtained, the lowest number of neovascular was found on C3 group while the highest was found on T14 group. In control groups, while the C3 groups were significantly lower and C14 was significantly higher compared to other groups, there was no significant

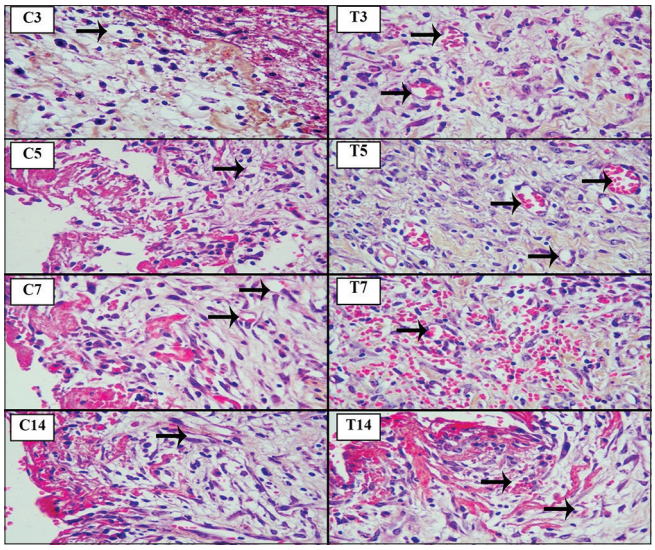

Figure 1: Neovascular observation result of H and E staining on both control and treatment groups (×400). Black arrows denote the neovascular formation

Table 1: Mean and standard deviation of the neovascular number on each group

Group	Mean±SD
Control	
C3	3.25^{a}±1.26
C5	8.00^{b}±1.83
C7	8.75^{b}±1.70
C14	11.50^{c}±1.29
Treatment	
T3	8.00^{b}±0.82
T5	13.00^{c}±1.83
T7	13.25^{c}±1.22
T14	16.75^{d}±1.71

^{a,b,c}Different superscript denotes significant difference with $P<0.05$.
SD: Standard deviation

difference between C5 groups and C7 groups. The same occurrence was found when comparing the number between treatment groups. However, while the highest number was found in C14 group for the control group, it has no significance different when being compared to the T5 and T7 groups of treatment group.

Neovascular graphic pattern results were shown in Figure 2. Although the growth pattern was similar between two groups, the number was higher in each of treatment groups when compared to the control groups. In both control and treatment groups, the increase of neovascular was found from day 3 to day 5, while it got stable from day 5 to day 7, before started to increase again on day 7 to day 14.

Discussion

Neovascular plays an important role in wound healing process. As the wound healing process occurs, neovascular distributes the nutrition required by the wound tissue and

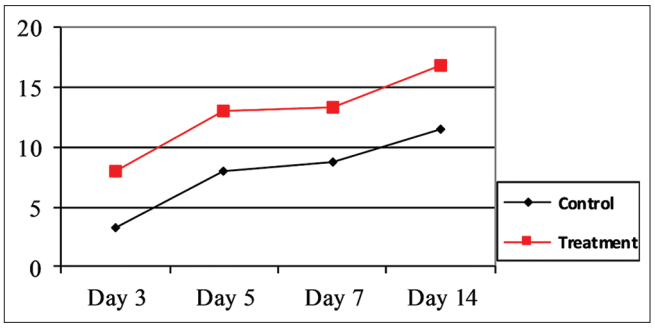

Figure 2: Neovascular growth pattern in both control and treatment groups

helps the regeneration of the loss of tissue caused by injury or trauma. The formation of neovascular is effected by few kind of cells and factors.

The results of this study showed that the neovascular growth pattern of both groups was similar although the number of neovascular found in treatment groups was greater compared to the control groups. This was caused by the active substances of *C. longa* Linn as several previous study has confirmed that curcuminoid has a role in the angiogenesis process.[11-13] Meanwhile, zinc oxide also can hasten the wound healing process; however, the role of zinc oxide in angiogenesis process still needs to be further investigated.[9]

Curcuminoid in the *C. longa* Linn. extract have proangiogenic properties by increasing the expression of VEGF, TGF-β dan FGF-2 which have an important role in angiogenic process, although the mechanism of curcuminoid itself is still unknown.[12] Besides the proangiogenic properties, curcuminoid also has antioxidation and anti-inflammation properties.

The anti-inflammation effect of curcuminoid was happened through several pathways. When the inflammation reaction occurs, curcuminoid would downregulate the cyclooxygenase-2 (COX-2) enzyme and the inducible nitric oxide synthase by inhibiting the activation of inactive NF-κB and inflammation κB kinase, the release of inhibitor κβ (Ikβα) from NF-κβ, also the dealkylation of arachidonic acid by downregulate COX-2 and lipoxygenase.[16,17]

The 3rd day of wound healing process is the inflammation phase. The significant difference neovascular number found between C3 and T3 was due to the excess inflammation reaction happened on day 3 in C3 group. The excess reaction of inflammation can inhibit the angiogenesis as a result of tissue hypoxia causing an acidic and anaerobic environment of wound tissue, which results in low number of neovascular as found in C3 groups.[12] While, for T3 group, the excess inflammation was suppressed to remain on the required amount of wound healing process due to the inhibition of NF-κB activation, which leads to the increase of prostanoid including prostaglandins and the

expression of VEGF, TGF-β, and FGF-2 as proangiogenic factors resulting in neovascularization.[16,18-20]

From the 3rd day to 5th day of the healing process, the inflammation phase still occurs but in the lower level. The 5th day of the healing process was characterized by the decrease of inflammatory cell activity until a required amount was left to regulate the pro-angiogenic factors and the increase of VEGF, TGF-β, and FGF-2 expression which leads to neovascular growth increase as a preparation of the proliferative phase.[21] Significantly higher neovascular number in T5 group compared to C5 groups was caused by the curcuminoid properties resulting in higher expression of pro-angiogenic factors.

The 5th day until the 7th day of the healing process was a transition from the inflammatory phase to the proliferative phase and the apoptosis of unnecessary inflammatory cell happens as the inflammation phase subsides, which leads to the decrease of proangiogenic factors production.[22] As a result, the neovascular growth becomes impeded.

The T7 group has a higher number of neovascular than the C7 group because the curcuminoid as an antioxidant agent has a ROS scavenging ability by increasing the expression of heme oxygenase-1.[12-14] Both curcumin activity as anti-inflammatory and antioxidant can reduce the inflammatory phase which will stimulate the beginning of healing phase and produce the neovascular growth can occur even in small amount.

From the 7th day until the 14th day, the proliferative phase occurs. The proliferative phase is characterized by granulation tissue formation, collagen deposition, fibroblast proliferation, and the apoptosis of unwanted cells. The granulation tissue formation in wound healing process is characterized by neovascular formation and fibroblast infiltration which facilitate the remodeling phase.[12] At the beginning of the proliferative phase, there was an elimination of unnecessary inflammatory cells resulting in a decrease in proangiogenic factor production, and this was confirmed by a slower rate of neovascular growth compared to the inflammation phase although there was still an increase in both C14 and T14 compared to their respective precedence group. T14 has a higher neovascular number compared to C14 as a result of the curcumin content in wound dressing combination increase the expression of proangiogenic factors resulting in an increase of neovascular numbers.[12] The limitation of 14 days in this study not enough to evaluate the neovascular in wound healing, a longer observation time is needed. However, based on the significant result obtained from this study and as the angiogenesis is a distinctive feature of wound healing and wound repair, the combination of zinc oxide and *C*. longa extract as an alternative wound dressing could be considered.[23]

Conclusion

Wound dressing combination of zinc oxide and C. longa extract can increase the number of neovascular formed in the wound healing process, while the optimum application duration is until the day 14 as the curcuminoid composition in the wound dressing still can enhance the number of neovascular necessary for the wound healing process.

Financial support and sponsorship

This study was financially supported by the Ministry of Research, Technology and Higher Education of Indonesia.

Conflicts of interest

There are no conflicts of interest.

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