# The Role of TLR2, NF-κB, TNFα as an Inflammation Markers of Wound Dressing Combination of Zinc Oxide With Turmeric Liquid Extract

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**Abstract**

The aim of this study was to examine the TLR2, NF-κB, TNFα expression as an inflammation marker to evaluate the anti-inflammation properties of combination zinc oxide and turmeric rhizome liquid extract as wound dressing. Twenty three male Wistar rats, aged 3 months, weighing 175-275 grams, adapted one week before, were devided into 3 groups. Group A without any intervention as reference for normal data (n=3), Group B (excision at 3rd day), Group C (excision at 7th day). Group B and C contains 10 animals and has two subgroups: control (excision, n=5) and experimental group (excision with dressing application, n=5).The tissues around wound excision were removed and used to analyze histopathologically and immunohistochemistry. ANOVA was used for the statistic and regression was used for path analysis.

The results showed that combination of zinc oxide and turmeric rhizome liquid extract as a dressing, were found to inhibit the inflammation, as it indicates by lowering the TLR2 at the 7th day, blocking the NF-κB expression, lowering the TNFα expression. Path analysis found a strong relationship between dressing combination zinc oxide and turmeric rhizome liquid extract to TLR2 expression (B= -0,772), NF-κB (B=0,712), TNFα (B=0,722).

In conclusion, these data indicate that zinc oxide with turmeric rhizome liquid extract have beneficial effects to accelerating the inflammation through lowering TLR2, NF-κB and TNFα expressions.

***Experimental article (J Int Dent Med Res 2016; 9: (3), pp. 173-177)***

**Keywords:** Wound dressing, zinc oxide, turmeric liquid extract, inflammation effect.

**Received date:** 29 September 2016 **Accept date:** 07 November 2016

## Introduction

Wounds in the oral cavity have the characteristics that can heal itself, but some situations require isolated injury of the oral environment, indications ranging from a tooth extraction, flap surgery (wound excision) and coverage of sutured on the wound edges[1]. Wounds can be isolated from the surrounding environment, generally using a wound dressing. Using the dressing, it can minimize post-operative infection, bleeding and facilitates healing by protecting the wound surface to trauma during mastication as well as provide protection against pain, which caused by the wound when it contact with food and tongue during mastication. Since there are many bacteria in the oral cavity, it would be better if the dressing has the anti-bacteria, instead anti-inflammation too. Two types of dressing materials that are widely used were zinc oxide eugenol and zinc oxide non-eugenol. Eugenol from zinc oxide eugenol dressing and rosin from zinc oxide non-eugenol can cause allergies[2]. This negative effects causes to find a replacement for eugenol / rosin which one that has minimal side effects. Turmeric (*Curcuma domestica* Val) is a famous herbal plant in Indonesia, which had known for its anti- inflammation and anti-bacteria properties. Turmeric extracts macerated with ethanol, has a number of biological activities including anti-bacterial, anti-cancer and

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inhibits topoisomerase, as well as anti-oxidant and anti-inflammatory. The active ingredient of turmeric rhizome liquid extract is curcuminoid, a flavonoid compound consisting of curcumin, demethoxycurcumin, and bisdemethoxycurcumin[3].

Curcuminoid of turmeric extract is a highly pleiotropic molecule, able to interact with a variety of molecular targets involved in inflammation. Toll-like receptor (TLR) is part of pattern recognition receptors (PRR) that build the body's first line of defense. In addition to the invasion of pathogenic bacteria, TLR is also activated by endogenous ligands in a sterile inflammation. Signal danger-associated molecular patterns (DAMP) regardless of necrotic cells or stress, which triggers an inflammatory response after injury or trauma. Expression or secretion of endogenous ligands, such as heat shock protein 70 (HSP 70), will directly stimulate TLR2 signaling in response to tissue damage, causing an inflammatory response[4,5].

Curcumin in high doses, given at heparin veins isolated from a healthy human in vitro studies, can reduce TLR2[6].

Curcumin has anti-inflammatory and anti-bacterial properties[7,8]. Zinc also has anti-bacterial properties, particularly against gram-positive bacteria[9]. Zinc oxide can reduce the bacterial adhesions, maintain the integrity of the membrane caused by enterotoxigenic Escherichia coli, and reduces the expression of inflammatory cytokines[10].

Anti-inflammatory and anti-bacterial curcumin from turmeric and also zinc oxide, lead researcher wishes to mix zinc oxide with turmeric rhizome liquid extract. This combination is expected to be used as a wound dressing. So far no proper investigation has evaluated the effect of wound dressing combination of zinc oxide and turmeric rhizome liquid extract and our hypothesis was that its usage may accelerate the inflammation stages of wound healing process. Thus, the purpose of this in vivo study was to evaluate wound dressing combination of zinc oxide and turmeric rhizome liquid extract effectiveness in inflammation phase of wound healing.

## Materials and methods

Animals. Twelve weeks old, the Wistar strain of Rattus norvegicus, each Wistar is 175–275 g in weight (Wistar Farm-Malang) adapted one week before, were randomly allotted into one of three group. Group A without any intervention as reference for normal data (n=3), Group B (excision at 3rd day), Group C (excision at 7th day). Group B and C contains 10 animals and has two subgroups: control (excision, n=5) and experimental group (excision with dressing application, n=5). Twenty-three healthy rats were utilized in this study, were fed a standard chow and tap water ad libitum. The experimental procedure was approved by the Ethical Clearance of Health Research Committee Faculty of Dental Medicine - Universitas Airlangga, Indonesia.

Materials. Dressing material: Combination of zinc oxide and turmeric rhizome liquid extract (1:1), were mix with stainless steel spatula on a mixing pad. Turmeric rhizome liquid extract which macerated with ethanol which purchased from Health Department of East Java Province (Materia Medica-Batu). The extract was analyzed with Thin Layer Chromatography (TLC) - Densitometry in Testing Service Unit in Faculty of Pharmacy, Universitas Airlangga, contain 32,34% curcuminoid. Zinc oxide 99,8% purchased from Merck-Germany, catalog 1.08849.0500, batch K43371349.

Surgical protocol: Previously we followed reported wound excision model[11] with modification. After the induction of general anesthesia with intraperitoneal ketamine (1ml/100g body weight), dorsal regions were shaved and cleaned with ethanol 70 %. The full-thickness excision wound 6x6mm was made with a surgical blade No. 15 (Swann Morton-England, Lot. 5201411), cleaned with a physiological saline solution (0,9 NaCL).

Wound management. The control excision wound (B1, C1) was left undressed, covered by hypo allergenic tape (Hypafix Germany, Lot. 44120230). The experiment wound (B2, C2) was dressed with the application of combination of zinc oxide with turmeric rhizome liquid extract (0,3g:0,3g) and covered by hypo allergenic tape. The rats were euthanized with an overdose of anesthesia at 3 and 7 days after wounding. The granulation tissue formed on

the injury was excised leaving a 5 mm margin of normal skin for histopathologically assessment and immunohistochemistry. The wound tissue sample was collected at each time point: zero (A, n=3), 3 days (B1, B2, n=5 each sub groups) and 7 days (C1, C2, n=5 each sub groups).

Preparation of histological specimens. Histopathologic evaluation: The wound tissues were embedded in the paraffin blocks after they had been fixed in 10% neutral buffered formalin. Sections of 4 µm were obtained, deparaffinized and stained with routine Haematoxylin-Eosin (HE). HE stain was used to counting the number of macrofag. Immuno-histochemical staining.

Paraffin blocks are section at 4 µm thickness. Immunohistochemical reactions were performed according to the avidin-biotin complex (ABC) followed by previous experiment[12] with minor modification.

The procedure involves the following steps:(1) endogenous peroxidase activity was inhibited by 3 % $H_2O_2$ in methanol for 30 min;2 the sections were washed in distilled water for 10 min;3 non-specific binding of antibodies was blocked by incubation with normal goat serum (NGS) (Santa Cruz Biotechnology), phosphate buffered saline (PBS), diluted 1:4;4 the sections were incubated with specific polyclonal/monoclonal antibody (TLR2 (H-175):sc-10739; NF-κB p65 (F-6):sc-8008; TNFα (54B83):sc-52746, all from Santa Cruz Biotechnology) diluted 1:100 in fetal bovine serum (FBS) for 1 h at room temperature;5 the sections were washed in PBS pH 7,2;6 the sections were incubated with biotinylated anti-mouse IgG (Santa Cruz Biotechnology Kit);7 the sections were washed in PBS pH 7,2;8 the sections were incubated with ABC (Santa Cruz Biotechnology Kit);9 the sections were washed in PBS ;10 peroxidase was detected with diamino benzidine (DAB) substrate kit (Santa Cruz Biotechnology);11 the sections were washed in tap water for 10 min and then dehydrated;12 the nuclei were stained with hematoxylin, and13 the sections were mounted with entelan (Merck). All dilutions and thorough washes between steps were performed using PBS unless otherwise specified. All steps were carried out at room temperature unless specified.

Statistical analysis. The results were expressed as mean ± standard deviations (SD). One-way analysis of variance (ANOVA) followed by LSD test was applied to assess the statistical significance of the differences between the study groups at $p<0.05$ (IBM SPSS Statistics 21).

## Results

Data were presented in Tabel 1. It was estimated at a mean from 20 microscopic fields of 1000x magnification[13]. Sub groups with a wound dressing application of zinc oxide and turmeric rhizome liquid extract, have a higher number of macrofag, lower in TLR2, NF-κB p65, and TNFα. Macrofag demonstrated increased in sub group (B2,C2) with dressing combination zinc oxide with turmeric rhizome liquid extract application.

| | | Macrofag (HE) | TLR2 | NF-κB p65 | TNFα |
|---|---|---|---|---|---|
| | Normal | 6.33 $^{a}$ ± 2.082 | 2.33$^{a}$±1.528 | 3.33$^{a}$±0.577 | 6.67$^{ab}$±2.082 |
| Day 3 | Control | 3.00 $^{a}$ ± 1.581 | 10.60$^{c}$±2.702 | 14.60$^{c}$±1.517 | 12.40$^{de}$±2.074 |
| | Dressing | 11.00 $^{b}$ ± 3.082 | 6.60$^{b}$±1.949 | 3.60$^{a}$±1.140 | 8.20$^{bc}$±1.924 |
| Day 7 | Control | 3.40 $^{a}$ ± 1.140 | 14.00$^{d}$±2.000 | 19.80$^{d}$±2.775 | 13.60$^{e}$±1.817 |
| | Dressing | 15.20 $^{b}$ ± 2.387 | 4.80$^{ab}$±1.924 | 3.40$^{a}$±1.517 | 5.20$^{a}$±.1.483 |

Note : * significantα=0,05
$^{a,b,c,d,e}$*same superscript* was no significant differences

**Table 1.** Mean and SD of each group.

**Evaluation of immunohistochemical staining.** The TLR2 expression was stronger increased in the macrofag of sub group without dressing (B1, C1), and decreased in the 7$^{th}$ day sub group with dressing (C2), showed at the brown expression cell with nucleus. The NF-κB p65 expression positively increased in sub group without dressing (B1,C1), showed the brown color cell with nuclei at the group. The TNFα expression increased almost the same with another pro- inflammatory expression.

**Path analysis.** Path analysis showing that dressing combination of zinc oxide with turmeric rhizome liquid extract, have a strong negative relation with TLR2 (B= -0.772; p=0.05), TLR2 have a strong positive relation with NF-κB p65 (B=0.712; p=0.02), NF-κB p65 have a strong positive relation with TNFα (B=0.722; p=0.03).

## Discussion

The wound-healing process consists of four highly integrated and overlapping phases: hemostasis, inflammation, proliferation, and tissue remodeling. These phases and their biophysiological functions must occur in the proper sequence, at a specific time, and continue for a specific duration at an optimal intensity. There are many factors that can affect wound healing which interferes with one or more phases

of this process, thus causing improper or impaired tissue repair[14]. None of these phases corresponds to a precisely defined period of time and all phases overlap. In this experiment, we focus in inflammation phase.

Macrophages at the site of wound repair consist of two populations. The first is the 'resident' tissue macrophage that is present in tissues at all the times. Normal skin contains resident macrophages at a low density of approximately 1–2 per $mm^2$. The other major population is newly recruited from hematogenous precursor cells, called monocytes. Once newly recruited monocytes migrate through the vessel wall, they release enzymes that fragment extracellular matrix (ECM) proteins, which creates space for monocytes to migrate into the wound bed. Subsequently, in reaction to the micro-environment, monocytes differentiate into macrophages. Macrophage numbers increase during the phase of inflammation, peak during the phase of granulation tissue formation about the 5$^{th}$ day and decline during the maturation phase[15]. In this study, the number macrofag in sub group with dressing application was higher, in day 3$^{rd}$ and day 7$^{th}$, and the control sub group still below the normal data.

The data generally support our initial hypothesis that the use of zinc oxide and turmeric rhizome liquid extract combination may accelerate the inflammation stages of wound healing process. First, the number of macrofag increased positively with the dressing. Second, TLR2 as a pattern recognition receptors slightly increased at 3$^{rd}$ day, because TLR activation will be expressed the antigen presenting cell (APC) to initiate an adaptive immune response[16]. Optimal TLR2 signaling itself is required to activate our innate immune system, but an excessive amount of TLR2 signaling and expression and over production cytokines lead to inflammatory disease[6]. In this experiment, expression TLR2 decreased at 7$^{th}$ day. Third, this dressing can inhibit the expression NF-κB p65 and lower the expression of TNFα, so the next phase of healing will be done faster, although they are overlapping.

NF-κB proteins are a family of transcription factors and they are central importance in inflammation and immunity. The mammalian NF-κB proteins consist of five different related family members that bind as homodimers or heterodimers to 10-base pair κB sites. All of these family members have a Rel-homology (RHD) domain that is essential for DNA binding and dimerization. The three Rel members of the family, RelA (also known as p65), RelB, and cRel, have a C-terminal transcription activation domain (TAD) that serves to positively regulate gene expression. Although there are many ways of activating NF-κB, two main signaling pathways have been described that lead to the activation of NF-κB target genes. These are referred to as the canonical (or classical) and noncanonical (or alternative) pathways. The canonical NF-κB pathway is activated mostly by the stimulation of pro-inflammatory receptors, such as the TNF Receptor superfamily (TRAFs), the Toll-Like receptor family (TLRs), and by cytokine receptors for the interleukins[17] the same as in this experiment. In the control group (B1, C1), showed significantly increased expression of TNFα compared to the normal state (A). This is due to NF-κB would release pro-inflammatory cytokines, including TNFα, marks the day 7$^{th}$ is still in the inflammatory phase. The combination of zinc oxide turmeric rhizome liquid extract showed expression of TNFα lower than the control group and decreased again at day 7$^{th}$, indicating an inflammatory process has subsided and transformation into the proliferative phase, in accordance with the results of the expression of NF-κB p65. This proves dressing zinc oxide turmeric rhizome liquid extract, effectively counteracting oxidants and free radicals that regardless of HSP 70, as a result of trauma excision. These results are consistent with studies that showed increased phagocytic activity of macrophages in experimental animals that were given curcumin. The action of curcumin on macrophages explained by an increase in the oxide-free catching capacity in non-inflammatory conditions[18]. In addition, the effects of curcumin topical use in healing wounds in the inflammatory phase can inhibit the transcriptional activity of NF-κB, thus reducing the production of cytokines TNFα and IL-1, further reduce or accelerate the inflammatory process[7].

The path analysis showed that dressing combination of zinc oxide and turmeric rhizome liquid extract had a strong negative relation with TLR2, strong positive relation to NF-κB p65 and TNFα, so this dressing can inhibit the expression of pro-inflammatory cytokine. NF-κB p65 and TNFα is a pro-inflammatory marker. Although the dressing can inhibit the NF-κB p65

expressions, the expression of NF-κB is would not end, because NF-κB p65 bind to NF-κB p50 as heterodimer, binding to DNA and dimerization[17], and it would follow the next step of healing, although there are overlapping.

## Conclusions

The results presented here indicating that wound dressing combination of zinc oxide and turmeric rhizome liquid extract was effective in lowering TLR2, NF-κB and TNFα expressions.

## Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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