Original Article

# New insight into the role of a combination of zinc oxide and turmeric rhizome liquid extract in osteogenic marker expression

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## Abstract

**Aim:** This research was aimed to determine the potential for treating osteogenesis with a combination of zinc oxide and turmeric (ZOT) rhizome liquid extract.

**Setting and Design:** *In vivo*, post test-control group design.

**Material and Methods:** The mandibular incisors of Wistar rats were extracted and left untreated or received an application of zinc oxideeugenol (ZOE) 10% or ZOT rhizome liquid extract at various concentrations (10%, 20%, and 40%). The mandible was then subjected to immunohistochemical analysis to detect RUNX2 and alkaline phosphatase (ALP) activity.

**Statistical Analysis Used:** One-way ANOVA and Tukey HSD using SPSS software.

**Results:** All groups demonstrated increasing RUNX2 and ALP activity. ZOT 40% showed the highest activity in all groups on day 3 and day 7, although there were no significant differences with ZOE 10%.

**Conclusion:** A combination of ZOT rhizome liquid extract can induce the osteogenic process in postextraction sockets. The results highlight the need for further investigation of the potential osteogenesis of curcumin in humans.

**Keywords:** Medicine, osteogenesis, tooth extraction, turmeric, zinc oxide

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## INTRODUCTION

Turmeric (Curcuma domestica val) a herb frequently used by Indonesians, Thais, and Indians both as an ingredient in cooking and traditional medicine. Curcumin, an active ingredient of turmeric, is clinically proven to have low toxicity and promote several biological activities, including antimicrobial, anti-fungal, anti-inflammatory, and antioxidant, possess anti-carcinogenic properties and act as an antidiabetic.[1]

As an anti-inflammatory agent, curcumin is able to modify NF-κB signaling, inflammatory cytokines such as

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interleukin and phospholipase A2; inhibit cyclooxygenase-2, myeloperoxidase, and lipoxygenase activity in addition to inducible nitric oxide synthase.[2-4] Curcumin also plays a role in modulating various signaling molecules such as pro-inflammatory cytokines, apoptotic proteins, C-reactive protein, prostaglandin E2, prostate-specific antigens, adhesion molecules, phosphorylase kinases, transforming growth factor-β, triglycerides, ET-1, creatinine, HO-1, aspartate aminotransferase, and alanine aminotransferase in humans.[5]

In recent decades, zinc oxide-eugenol (ZOE) has been renowned for its therapeutic effect in periodontal dressing which, postsurgery, can protect wounds from mechanical trauma and promote wound stability during the healing process.[6] Periodontal dressing made from ZOE has the benefit of producing both antibacterial and anaesthetic effects, the latter of which reduce pain. On the other hand, eugenol is known to cause allergies, cytotoxicity (at both high and low doses), and increase tissue inflammation and soft-tissue necrosis with the result that wounds do not heal readily.[7]

Zinc oxide has become valued for its potent antimicrobial properties, considered safe by the US Food and Drug Administration, which are often employed as food additives. It is also frequently applied in the development of dentistry implants and scaffold as well as bone regeneration. Scaffold reinforced with zinc oxide can support superior attachment and proliferation of 2.5 wt% content. Moreover, zinc oxide can promote both osteoinduction and osteoconduction, while also demonstrating effective mechanical and biological properties.[8] Periodontal dressings made from zinc oxide can reduce inflammatory responses after 28 days in the tooth sockets of rats, while simultaneously promoting new bone formation.[9]

Curcumin present in the turmeric extract can promote osteoblast differentiation in human adipose-derived mesenchymal stem cells irrespective of any oxidative injuries.[10] A combination of turmeric rhizome liquid extract and zinc oxide is effective as an anti-inflammatory agent in reducing the expression of MAC 387, cycloocygenase-2, and COX-2 receptors in the healing process of incisions in the vertebralis thoracis region of rodents.[11,12] The effect of zinc oxide-turmeric (ZOT) rhizome liquid extract on the process of osteogenesis is still unclear but is thought to encourage osteoblast formation. To the best of our knowledge, this represents the first study examining the effects of curcumin on the promotion of bone formation *in vivo*. Three and seven days were selected as the day of execution of this study. Alkaline phosphatase (ALP) messenger ribonucleic acid expression in MG-63 cells in the presence of 10 μM curcumin at 7 days was not significantly different to 21 days.[13]

In accordance with the data referred to above, it is believed that the multiple roles of curcumin present in turmeric can synergize with zinc oxide resulting in bone formation within the extraction socket. This combination of material was needed in terms of socket preservation to maintain jaw bone volume after tooth extraction procedure.[14] At present, the potential for osteogenic formation resulting from a combination of ZOT rhizomes liquid extract remains unknown. The purpose of this study was to determine the expression of osteogenic in a combination of ZOT rhizome liquid extract in postextraction tooth sockets.

## MATERIALS AND METHODS

### Ethical approval

This laboratory-based research was conducted in accordance with the ethics governing experimental use of animal subjects as approved by the Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga, No. 250/HRECC.FODM/IX/2018.

### Materials

#### *Preparation of turmeric rhizome liquid extracts*

Ten-month-old fresh turmeric rhizome (Curcuma domestica Val.) was obtained from suppliers in Malang, Indonesia, harvested and minced into 4 mm thick slices, before being dehydrated through exposure to direct sunlight for several days. The dried turmeric rhizome was subsequently macerated with 96% ethanol. Five hundred grams of turmeric powder was hydrated with 500 mL 96% ethanol and mixed in a jar until dissolved with 3 liters of ethanol. The jar was agitated at 60 rpm in a digital shaker (SHO-2D, Wisd, Witeg Labortechnik GmbH, Wertheim, Germany) for a period of 24 h and the liquid extract subsequently filtered through a cloth. This procedure was repeated twice, with material produced by the first and the second filtering being mixed and evaporated at 60°C in a rotary evaporator (Buchi® Rotavapor® R-215, Merck KGaA, Darmstadt, Germany) for 6 h. Once the ethanol solvent had become fully separated, it was soaked in a water bath for 2 h at 37°C.[11]

### Preparation of zinc oxide and turmeric rhizome liquid extract in gel form

Gel preparation was carried out by weighing 1 gram of sodium CMC powder pharmaceutical grade (Ashland, Alizay, France), mixing it with 100 ml aquadest and stirring until it became homogeneous. Ninety nine percentage zinc oxide was purchased from Merck (EMSURE®, Merck KGaA, Darmstadt, Germany). The combination of ZOT rhizome liquid extract produced three concentrations,

namely 10%, 20%, and 40%. For the manufacture of gels with a material concentration of 10%, a gel weighing 18 g was mixed with 1 g of zinc oxide powder and 1 g of turmeric rhizome liquid extract, before being agitated in a mortar until homogeneous. For the manufacture of gels with a material concentration of 20%, a gel weighing 16 g mixed with 2 g of zinc oxide powder and 2 g of turmeric rhizome liquid extract, prior to being stirred in a mortar until homogeneous. For the manufacture of gels with a material concentration of 40%, a gel weighing 12 g was mixed with 4 g of zinc oxide powder and 4 gof turmeric rhizome liquid extract, before being blended in a mortar until homogeneous. The gel was then put into a 1 cc syringe to facilitate postextraction application of the material into the socket.

### Preparation of zinc oxide and eugenol in gel form

The process for producing (ZOE) gel is similar to the preparation of ZOT rhizome liquid extract gel. To produce ZOE gel at a concentration of 10%, gel weighing 18 g was combined with 1 g of zinc oxide powder and 1 g of eugenol, the resulting mixture being stirred together in a mortar until homogeneous. The gel was then inserted into a 1 cc syringe to facilitate postextraction application of the material into the socket.

### Experimental animals

Fifty male, albino, Wistar rats (170–320 g in weight) were obtained from Wistar Farm, Malang, Indonesia and acclimatized for 1 week prior to the conducting of the experiment. These participants were subjected to a 12-h light/12-h dark cycle and fed standard pellets with water *ad libitum*. The behavior and environmental situations of the participants were observed both before and during the study.

After the adaptation period, each subject was injected intramuscularly with an appropriate dose (0.1 ml/kg) of a cocktail of ketamine (KEPRO, ZA, Denmark), xylazine (Interchemie werken, Venray, Holland) and acepromazine (Castran, Venray, Holland). The mandibular left incisor of each subject was extracted,[15] and the socket subsequently administered with a gel containing ZOT rhizome combination extract of either 10%, 20% or 40%, ZOE gel, or no gel and closed with 5–0 nylon suture (Aylee, Busan, Korea). The sockets were dissected at the intervals of 3 and 7 days after extraction and fixated in 10% neutral buffer formalin for 48 h. All experiments were performed in compliance with the relevant institutional guidelines.

### Decalcification, embedding, and sectioning

The sockets were processed by the administering of 10% ethylenediaminetetraacetic acid (EDTA) (Titriplex® III, Merck KGaA, Darmstadt, Germany) at the room temperature which was replaced every second day. The 10% EDTA was obtained by dissolving 60 g of EDTA in 600 ml of distilled water. After decalcification had been completed, the samples were processed and embedded in molten paraffin wax (60°C) (Paraplast, Surgipath, Leica, Nussloch, Germany). Paraffin blocks of 3 μm thickness were cut with a rotary microtome (Accu-Cut SRM, Sakura Seiki Co. Ltd., Japan), flattened in a 40°C-water bath (Memmert, Germany), placed on a poly-L-lysine slide and heated to 30°C–35°C on a hot plate for 24 h in order to remove the paraffin.

### HE staining

Slides were immersed in a solution of xylol, then put in a solution of 96% ethanol twice, 80% ethanol, and 70% ethanol respectively, each for 2 minutes.[16] The slides were washed with water for 10 min, then put in Mayer's Hematoxylin solution for 15 min, rewashed with water until clean. After that, the slides were put in 1% eosin solution for 30 s, and then dehydrated by putting it in 80% ethanol solution and 96% ethanol specifically for three times each for 2 min. For final step, the slides were immersed in xylol,[1] xylol,[2] and xylol[3] each for 5 min and then mounted with an EZ mount and covered with a glass cover.

### Immunohistochemical staining

Immunohistochemical examination was performed in accordance with the avidin-biotin complex. The slides were incubated with 3% hydrogen peroxide ($H_2O_2$) for 30 min to inhibit endogenous peroxidase activity and washed with distilled water for 10 min. In order to block nonspecific binding of antibodies, the slides were incubated in normal goat serum. The sections were subsequently incubated with a specific monoclonal antibody RUNX2 (Santa Cruz Biotechnology™, RUNX2 27-K sc-101145) and ALP (Santa Cruz Biotechnology™, BALP sc-271431), diluted in fetal bovine serum at a ratio of 1:100 for 1 h at the room temperature and washed. The sections were incubated with biotinylated universal secondary antibodies (D-BioSys, The Hague, Netherlands) before being re-washed in PBS pH 7.2. The sections were subsequently incubated with Strep-Avidin HRP, conjugated (D-BioSys, The Hague, Netherlands) and re-washed. A diaminobenzidine substrate kit (Histofine-Nichirei Bioscience, Tokyo, Japan) was used to detect peroxidase. After being washed in tap water for 10 min and dehydrated, the nuclei were stained with hematoxylin and the sections mounted with Entellan (Merck KGaA, Darmstadt, Germany).

### Imaging

The immunohistochemistry slides were examined under a light microscope (Nikon Eclips E 100, Japan) at ×400 to assess and calculate the expressed antibodies and structure. The area observed was the apical third of the socket, as much as 20 fields.[17] Observation at ×1000 was performed to confirm the presence of a specific structure or antibody in brown color and photographs were taken with a Sony ILCE α6,000 camera (Sony, Tokyo, Japan).

### Statistical analysis

Statistical analysis was performed using a Statistical Package for the Social Sciences software (SPSS) 24.0 edition (SPSS™, Chicago, United States), and the results expressed as mean ± standard deviation. The data obtained were tested statistically with $P < 0.05$ using both a Shapiro–Wilk test and Levene's test. Group differences were identified using a one-way analysis of variance test followed by a *post-hoc* Tukey test.

## RESULTS

Increasing RUNX2 activity was observed in the postextraction sockets of all treatment groups between day 3 and day 7. The highest level of RUNX2 was found in the ZOT 40% group on day 7 ($P < 0.05$, compared to negative control group on day 3 and day 7), while the lowest was that in the negative control group on day 3. The group that demonstrated the largest difference in RUNX2 between day 3 and day 7 was the ZOT 10% [Figure 1]. The expression of RUNX2 resulting from immunohistochemical staining observed under a light microscope was marked with red arrows, as shown in Figure 2.

There was increasing ALP activity in the postextraction sockets of all treatment groups between day 3 and day 7. The highest level of ALP occurred in the ZOT group 40% on day 7 ($P < 0.05$, compared to negative control group on day 3 and day 7), while the lowest was that of the negative control group on day 3. The group experiencing the largest difference in the number of ALPs was the ZOT group of 40% [Figure 1]. The expression of ALP from immunohistochemical staining observed under a light microscope and marked with red arrows is shown in Figure 2.

The osteoblast expression also showed similar significant trend as expressed by RUNX2 and ALP. When osteoblast showed higher number, the osteoclast showed lower number and vice versa. The histological figure is shown in Figure 3.

In all osteogenic expressions, the most significant differences were found in the ZOT 40% group compared to all other groups, although there were no significant differences with 10% ZOE group.

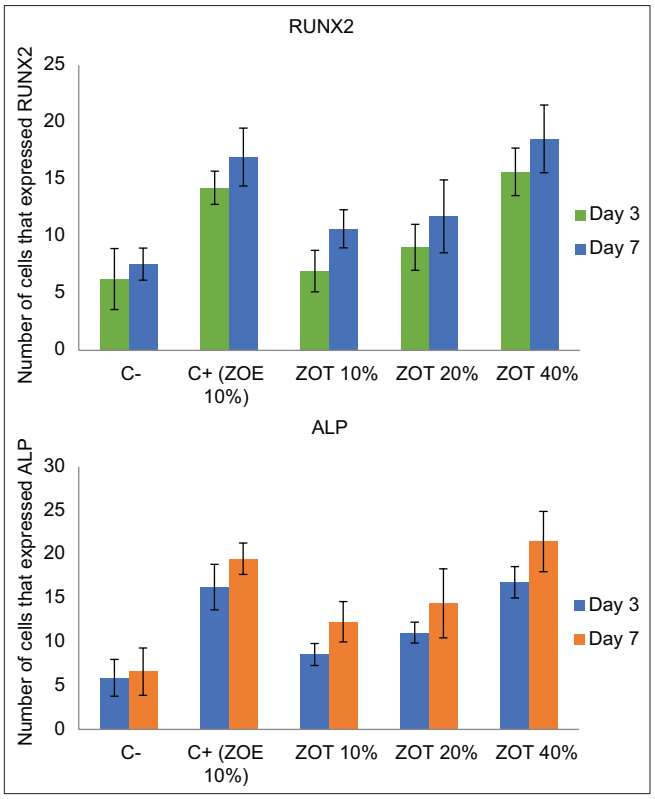


**Figure 1:** Comparison of mean RUNX2 and alkaline phosphatase expression in the control and treatment groups on day 3 and day 7. C–: negative control, without treatment. C+: Zinc oxide-eugenol 10% application, zinc oxide-turmeric 10%: zinc oxide-turmeric rhizome liquid extract 10% application, zinc oxide-turmeric 20%: zinc oxide-turmeric rhizome liquid extract 20% application, zinc oxide-turmeric 40%: zinc oxide-turmeric rhizome liquid extract 40% application

## DISCUSSION

Turmeric is a plant often used as a cooking spice and as a medicinal plant. Curcumin, an active component in turmeric rhizome, has been shown to have a pharmacokinetic, efficacious, and harmless effect during the treatment for more than a quarter of a century. Therefore, turmeric is worthy of further research to identify its other potential health benefits. Zinc oxide was selected because of its antibacterial properties and its being recognized as safe by the US Food and Drug Administration. Zinc oxide is used as a food additive because zinc is an essential micronutrient element often applied in the development of implants and scaffold in the fields of orthopedics, dentistry, and bone regeneration.[18] Research conducted by Indrastie *et al.*(2015) and Jessica *et al.*(2016) indicates that the combination of these two ingredients has antibacterial properties effective against *Streptococcus mutans* and *Porphyromonas gingivalis*.[19,20]

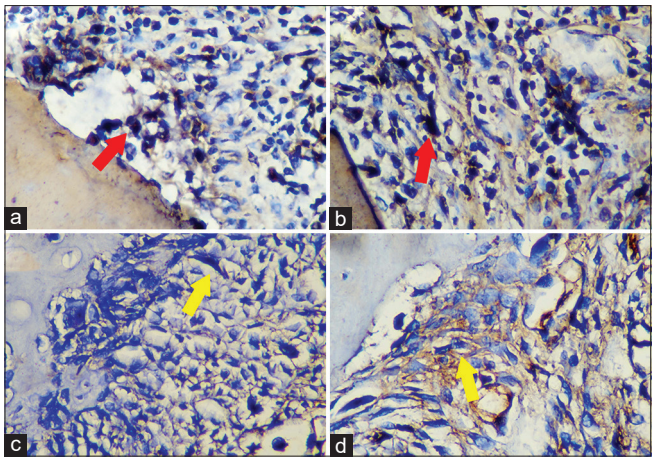


**Figure 2:** RUNX2 (a, b) and alkaline phosphatase (c, d) expression in postextraction sockets on day 7 observed through a light microscope at ×400 magnification, as indicated by the red and yellow arrow. (a, c) Negative control, extraction without application. (b, d) Extraction followed by the application of 40% zinc oxide-turmeric gel

Toxicity tests on human gingival fibroblasts show that a combination of these ingredients at certain doses does not prove noxious.[21]

According to previous *in silico* study, a combination of ZOT rhizome extract produces greater anti-inflammatory activity than ZOE. This result is consistent by *in vivo* study which finds that the expression of tumor necrosis factor α in ZOT group is lower than ZOE group on day 3 and day 7. This suggests that the combination of ZOT rhizome extract shows higher anti-inflammation effect and is more effective than ZOE.[11,12]

This study used ethanol as a turmeric rhizome extract solvent because ethanol can dissolve curcumin more effectively than other solvents, thereby containing more of the substance.[22] Ethanol is also a solvent frequently employed to extract curcumin. In this study, the content of curcumin found was 0.26%, while in other research, it reached as high as 0.58%–3.14%.[23] The difference in the curcumin content of turmeric is probably due to a combination of nutritional factors, acidity in the soil, and gene diversity.[23-25] Turmeric plants which have not undergone hybridization and introgression possess a higher curcumin content.[26] In this study, pure strain tracing was not carried out, with the result that homogeneity of the turmeric plants employed was unknown.

The treatment group material was produced in the form of a gel to facilitate the application of material to the interior of the postextraction socket. The 1% gel preparation was chosen because the material can be easily inserted into the syringe and demonstrates sufficient flow to reach the

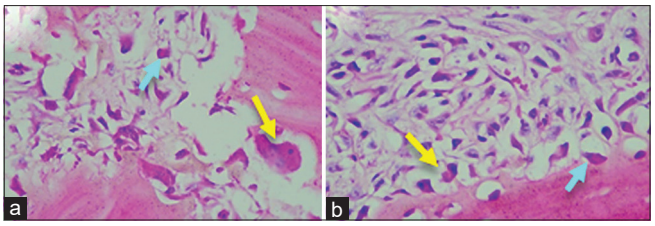


**Figure 3:** Expression of osteoblasts and osteoclasts with HE staining in postextraction sockets on day 7 observed through a light microscope at ×400 magnification, as indicated by the yellow and blue arrow. The yellow arrows showed osteoclasts and the blue arrows showed osteoblasts. (a) Negative control, extraction without application. (b) Extraction followed by the application of 40% zinc oxide-turmeric gel

apical of the alveolar socket. The mandibular left incisor was chosen as the subject for the treatment because of its anatomical shape and ease with which it can be removed. Each of these factors has a greater potential to reduce the risk of fracture during extraction compared to that with molars. Extraction of the incisors also occurs in a more flexible workspace than is the case with molars.

According to a study by Son *et al*., the mechanism that might occur involved curcumin inducing the differentiation of osteoblasts through activation of RUNX2 expression and through stress on the endoplasmic reticulum through the ATF6 gene in osteoblasts. This occurred because curcumin plays a similar role to BMP2 by inducing differentiation of osteoblasts through ATF6 expression in osteoblast cells and then increasing osteocalcin (OC) expression by binding directly to TGACGT sequences in OC promoter genes.[27] Mesenchymal stem cells in the early stages form preosteoblasts, proliferate close to the bone surface and secrete ALP, the initial marker of osteogenesis.[28] This is in line with the research conducted by Moran *et al*.(2012) which states that there is an increase in ALP expression in human osteoblast-like cells stimulated by 7 days of postculture curcumin.[13] Runx2 and Osterix, which are transcription factors for osteogenic differentiation, are induced by the infusion of $Zn^{2+}$ derived from zinc oxide. Runx2 and Osterix regulate ZIP1 expression with the result that it induces $Zn^{2+}$ influx, contributing to the positive charge supply in the zinc-Runx2/Osterix-ZIP1 loop regulation during osteogenic differentiation, thereby accelerating the process of osteogenesis.[29] Therefore, the combination of zinc oxide and liquid turmeric rhizome extract has the potential to form new bone through the expression of several osteogenesis markers so that the hypothesis can be accepted.

Despite all the results that have been mentioned earlier, there are some limitations. The concentration of ZOT used in this study was 10%, 20%, and 40%. Zinc is known to

have the potential to support the osteogenesis process in a dose-dependent manner,[30] as does turmeric, through its active ingredients (curcumin) which can support osteoblast differentiation in human adipose-derived mesenchymal stem cells at certain doses.[31] The appropriate dose needs to be identified to maximize the therapeutic benefits and minimize possible negative side effects. In addition, the process of bone formation is a time-consuming process. Osteogenesis markers in this study are the markers of osteogenesis in the early stages, so longer observations are needed to determine the effectiveness of the combination of these two ingredients in terms of bone regeneration.

## CONCLUSION

The levels of RUNX2 and ALP osteogenic expression produced by the combination material of ZOT rhizome liquid extract gel lead to no significant difference when compared to ZOE gel. By considering the positive and negative aspects of the two ingredients, it can be concluded that ZOT gel can be recommended as an alternative postextraction socket dressing to increase osteoblast differentiation. Further studies of drug delivery and concentration are needed to maximize the efficacy of this material.

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Nil.

### Conflicts of interest

There are no conflicts of interest.

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