Topical administration of Curcuma longa L. extract gel increases M2 macrophage polarization and collagen density in skin excision

Afiat Berbudi1,2*, Hendi Anshori1, Andromeda Andromeda2, Alexander Kwarteng4, Almahitta Cintami Putri5, Nur Atik6
1Department of Biomedical Sciences, Parasitology Division, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia.
2Department of Parasitology, Faculty of Medicine, Universitas Pasundan, Bandung, Indonesia.
3General Surgery Residency Program, Department of Surgery, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia.
4Department of Biochemistry and Biotechnology, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.
5Department of Plastic Reconstructive and Aesthetic Surgery, Department of Surgery, Faculty of Medicine, Universitas Padjadjaran / Dr. Hasan Sadikin General Hospital, Bandung, Indonesia.
6Department of Biomedical Sciences, Cell Biology Division, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia.

ABSTRACT
While M1 macrophages initiate wound healing by inducing the inflammation phase, M2 macrophages are crucial in the proliferation phase by producing growth factors that accelerate new tissue formation. Turmeric (Curcuma longa L.) is known to accelerate wound closure; however, mechanisms underlying the wound healing properties of C. longa L. remain to be documented. Therefore, this study investigated the mechanisms by which C. longa L. accelerates wound healing. Here, an experimental study was conducted using 30 male Swiss Webster mice. Curcuma longa L. extract was applied on the wound bed tissue starting on day 3 after wound induction. The Arginase-1 (Arg-1) and inducible nitric oxide synthase (iNOS) (M1 and M2 markers) gene expressions from wound skin tissue were analyzed, while collagenesis during tissue repair was observed by histopathology analysis of excised skin on day 6 after wound induction. Upregulation of Arg-1 in the treated group was observed on day 6 after wound induction compared to the positive control (p = 0.0394) and the negative control groups (p = 0.0313). In addition, the Arg-1/iNOS ratio revealed a significant increase of M2 polarization in the C. longa L.-treated wound compared to negative controls (p < 0.0001), but not significant when compared with positive controls (p = 0.0535). Similarly, collagen density was significantly higher in the C. longa L.-treated wound than the negative control (p = 0.0418) and the positive control (p < 0.001). These results suggest that administration of C. longa L. extract gel improved wound tissue repair on skin excision by inducing M2 polarization on wound tissue during healing.

INTRODUCTION
Acute and chronic wounds do not only cause pain but also significantly impose an economic burden (Guest et al., 2017; Järbrink et al., 2017). As a result, productivity, business returns, and tax revenue are greatly affected (Nussbaum et al., 2018; Sen et al., 2009). Given that the rate of accidents is very high in Indonesia, research into wound care has become a great priority (Jusuf et al., 2017; Karina et al., 2019; Tarawan et al., 2017).

Tissue repair in wound healing consists of three consecutive phases including inflammatory, proliferation, and maturation phases. Macrophages play an important role in all the phases of tissue repair (Shedoeva et al., 2019). Following activation, macrophages are polarized into two types, i.e., classically activated macrophages (M1) and alternatively activated macrophages (M2) (Yuliani et al., 2018). Inhibition of macrophage activity has been reported to be associated with prolonged wound healing time (Shedoeva et al., 2019). Similarly, as described among diabetic patients, persistent M1 macrophages in wound tissues and inhibition of M2 macrophage polarization in wound tissues make it difficult for chronic wounds to heal (Shah et al., 2017). Prolonged wound healing results in prolonged treatment and eventually increases the treatment cost. In view of
this, a new breakthrough in wound care is required to produce effective, efficient, and inexpensive treatment options.

Medicinal plants for wound healing are not only cheap and affordable but also safe to use. These plants induce healing and regeneration of lost tissues by various mechanisms (Gao et al., 2015). According to the 2014 Horticultural Data, Curcuma longa, commonly known as turmeric rhizome, is the second highest biopharmaceutical plant in Indonesia (Jacob et al., 2007).

Furthermore, turmeric rhizome plants are widely used as an additive to food and also as a medicine for treating tissue inflammation problems (Chen et al., 2015). The turmeric rhizome contains curcumin, which modulates inflammatory responses through downregulation of cyclooxygenase-2 activity, lipoxigenase, and inducible nitric oxide synthase (iNOS) enzymes (Riquelme et al., 2013). In addition, in a macrophage cell line (Raw264.7), curcumin has been shown to induce polarization of M2 macrophages (Krzyszczyk et al., 2018).

Since curcumin in turmeric rhizome has been identified to modulate the inflammatory response and promote the polarization of M2 macrophages in vitro, investigating the effect of topical administration of turmeric rhizome extract gel on wound healing is warranted.

MATERIALS AND METHODS

Animal model

Thirty (30) Swiss Webster male mice, each weighing 22–26 g and about 8–10 weeks old, were obtained from the Faculty of Biology, Bandung Institute of Technology. Previously, these mice had been adapted for 1 week. The mice were divided into three groups and each group consisted of 10 mice. For wound induction, two circular skin excisions were performed on the back of each mouse using a biopsy punch with a diameter of 6 mm. After excision, the wound was cleaned with physiological NaCl, dried with sterile gauze, and covered with a transparent dressing; then, they were covered with dry gauze and plastered. Treatment of the wound was carried out on the 3rd day after the injury. In the positive control group, the open wounds were treated with tulle (polymyxin B sulfate + neomycin sulfate + bacitracin zinc). In the negative control group, the open wounds were given blank gel. In the last group, the open wounds of the mice were given turmeric (C. longa) rhizome extract gel. All mice were killed by cervical dislocation technique on the 6th day after the injury for excision and collection of the wounded skin tissue. For histopathology observation, the skin tissues collected were fixed for microscopic observation. One part of the skin tissue was taken and stored in liquid nitrogen immediately for Arginase-1 (Arg-1) and iNOS (M2 markers) gene expressions analysis using quantitative Polymerase Chain Reaction (qPCR).

Turmeric rhizome extract gel

Turmeric rhizome (C. longa)-based extract gel (3%) was provided by the Pharmacy Laboratory, Faculty of Pharmacy, Universitas Padjadjaran.

Histopathology (Collagen) observation

Collagenesis was observed by histopathology analysis of excised skin on day 6 after wound induction. Collagen was observed through Mason’s trichrome staining as zones of purple strands. In this study, collagen density was measured by taking an average of three collagen fibers that appeared intact on the two edges and center of the wound using a micrometer under a light microscope with a magnification of 10x and 40x objective lens. Scoring 0–4 indicates more solid collagen.

RNA extraction

The collected skin tissues were transferred into a homogenizer tube containing ceramic beads and 1 ml of Qiazol was added (Qiagen, Germany). Tissue lysis was carried out using Precellys homogenizer (6,000 rpm). The lysate was then transferred into 1.5 ml of microcentrifuge tube and 100 μl of chloroform was added gently. The tube was shaken for 10 seconds and incubated for 10 minutes at room temperature. The centrifugation was then carried out at 12,000 × g for 12 minutes at 4°C. After three phases were formed, 350 μl of the clear top layer consisting of the ribonucleic acid (RNA) was transferred into new tubes and washed with one volume of 70% ethanol. Afterward, RNA precipitate was transferred into a minicolumn tube, which consisted of RB column and 2 ml collection tube (Geneaid RNA Extraction kit, Taiwan) and centrifuged for 1 minute with 14,000 × g at 4°C. The next step was performed according to the manufacturer’s (Geneaid, Taiwan) instructions. The extracted RNA was quantified using a spectrophotometer and the concentration of RNA was measured using absorbance at 260 and 280 nm.

Gene expression analysis

The Arg-1 and iNOS (M1 and M2 markers) gene expressions from wound skin tissues were analyzed using the quantitative real-time PCR method. At least 4.2 μg/ml from the total RNA was reverse-transcribed to be complementary deoxyribonucleic acid (cDNA) using ReverTra Ace® qPCR RT Master Mix (Toyobo, Japan), according to the manufacturer’s instruction. PCR was then carried out using Thunderbird® SYBR® qPCR Mix (Toyobo, Japan) kit, according to the manufacturer’s instruction. Real-time PCR was performed using Rotorgene Q cycler (Qiagen, Germany). The gene expressions assessed were Arginase-1 (Arg-1) as a marker for M2 macrophage and iNOS as M1’s marker. The gene expressions were analyzed using the following formula: 2^{-ΔΔCt}, in which ΔΔCt = (Ct gene of interest−Ct reference gene) treatment group – (Ct gene of interest−Ct reference gene) control group.

The degree of gene expression was normalized with housekeeping gene β-actin as a reference gene and the result presented as a fold change to the control group. The primer sequences (mouse) are presented in Table 1. Quantitative real-time PCR was carried out at 95°C for 10 minutes, followed by at least 40 cycles at 95°C for 20 seconds and 60°C for 1 minutes.

Ethical statement

This study was approved by the Research Ethics Committee, Universitas Padjadjaran (705/Un6.C.10/PN/2017). All efforts were made to minimize the suffering of animals during the experimental procedures.

Statistical analysis

The Shapiro–Wilk test was used for the normality analysis and the Levene test was used for the homogeneity
analysis. Analysis of the mean difference between the two groups based on the results of this normality test used independent t-test for variables with normal distribution or the analysis of variance (ANOVA) test, followed by Tukey’s multiple comparison test to compare more than two groups. Meanwhile, for variables with abnormal distribution, the Mann–Whitney U test was used for comparison between two groups. The analysis was performed with statistical software GraphPad Prism 8.

RESULTS

Histopathology

Representative pictures of microscopic skin tissue among positive control, negative control, and C. longa treatment groups are shown in Figure 1. The expression of collagen was highest in the C. longa treatment group compared to the positive (p < 0.0001) and negative control (p = 0.0418) groups. However, there was no statistical difference between the positive and negative control groups (Fig. 2).

Gene expression marker analysis for M1 and M2

Gene expression analysis on Arg-1 showed a significant increase in the C. longa treatment compared to the positive control (p = 0.0394) and negative control (p = 0.0313) (Fig. 3). Statistical analysis of the iNOS gene expression showed a decreasing trend in the C. longa treatment compared to the positive control and the negative control, but it was not significant (Fig. 4).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5′–3′)</th>
<th>Reverse (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse Arginase-1</td>
<td>CCTATGTGTCATTTGGGTGGA</td>
<td>CAGGAGAAAGGACACAGGTTG</td>
</tr>
<tr>
<td>Mouse iNOS</td>
<td>CCAAGGCCCTACCTACCTCC</td>
<td>CTCGAGGCGTGACACAAAGG</td>
</tr>
<tr>
<td>Mouse β-actin</td>
<td>AGAGGGAATCGTGCGTGAC</td>
<td>CAATAGTGATGACCTGGCGGT</td>
</tr>
</tbody>
</table>

Figure 1. Microscopic appearance of skin on the 6th day after injury; group I was positive control, group II was negative control, and group III was treated by 3% C. longa extract gel. The histopathological observation shows more collagen in the tissues that were treated with 3% C. longa extract gel.

Figure 2. Comparison graph of collagen density in positive controls (Cont +), negative controls (Cont −), and C. longa (CL). Data are presented as median ± interquartile range. Statistical analysis between two groups was tested using Mann–Whitney U test; *p < 0.05; ****p < 0.0001.
Comparison of Arg-1 and iNOS gene expression ratios (Fig. 5) showed a significantly higher ratio in C. longa compared to negative controls ($p < 0.0001$), but it was not significant when compared with positive controls ($p = 0.0535$).

**DISCUSSION**

In this study, we observed that C. longa has the ability to increase wound healing properties through the upregulation of Arg-1 gene in M2 polarized macrophages. The highest collagen fibers were formed in tissues that were treated with C. longa compared with the other groups. The higher amount of collagen fibers in mice of the C. longa-treated group could be due to the presence of beta-diketone and polyphenolic functional groups which act as strong antioxidants (Sha et al., 2019; Shedoeva et al., 2019). As a result, lipid peroxidation decreases but vascularity and collagen synthesis, as well as cross-linking of collagen significantly improve (Yuliani et al., 2018). Curcuma longa has anti-inflammatory properties and, therefore, topical application of C. longa gel extracts may have negative effects on the inflammatory phase in wound healing (Shah et al., 2017; Xue et al., 2018). To avoid this occurrence, the gel extract of C. longa was applied to the wounds of the mice on the 3rd day after injury. This study showed that Arg-1 significantly increased in the mice treated with C. longa compared to the positive controls and negative controls, and therefore, coupled with increased collagen density, wound healing was accelerated in the C. longa-treated group. These findings

---

**Figure 3.** Comparisons of Arginase-1 gene expression in positive controls (Cont +), negative controls (Cont −), and C. longa (CL). Graph is presented as median with interquartile range. Statistical analysis between two groups was tested using Mann–Whitney U test; *$p < 0.05$.

**Figure 4.** Comparison of iNOS gene expression in positive controls (Cont +), negative controls (Cont −), and C. longa (CL). Graph is presented as mean with SD. Statistical analyses were tested using ANOVA, followed by Tukey’s multiple comparison test.
corroborate with several studies, wherein curcumin increased IL-4 and IL-13 proteins and messenger RNA (mRNA) expression which induced M2 polarization by upregulation of M2 markers (Arg-1, macrophage mannose receptor (MMR) gene, and Peroxisome proliferator-activated receptor gamma (PPAR-γ)) in Raw264.7 macrophages treated with C. longa (Gao et al., 2015; Jacob et al., 2007). According to other report, the inhibition of arginase activity significantly delayed wound healing time, and therefore, C. longa gel extracts can accelerate wound closure (Campbell et al., 2013). Generally, iNOS is induced by M1 macrophages and its expression is often low in human blood monocyte-derived macrophages (Ley, 2017; Riquelme et al., 2013). In this study, iNOS expression decreased in mice treated with C. longa compared with the negative controls and the positive controls. This may suggest that the local inflammatory processes and the resultant oxidative stress during injury may be reduced by topical application of C. longa gel extracts (Graf et al., 2017), thereby improving and hastening the healing of wounds (Choudhary et al., 2018). This finding concords with studies conducted by Ibrahim et al. (2018) and Mohanty et al. (2017), where C. longa gel extracts decreased the expression of iNOS and improved tissue repair, as well as the healing process.

Even though iNOS protein expression can hamper wound healing, it is a very essential enzyme that produces nitric oxide, a proinflammatory molecule that inactivates and destroys infectious microbes and as a result protects humans from diverse infectious diseases (Bogdan et al., 2000; Ellis et al., 2018). Since iNOS also helps the host, it is imperative to treat wounds with medications that balance the Arg-1 and iNOS ratio that will ensure that the benefits of both proteins are maintained but with decreased toxicity of iNOS. In this study, we revealed that the ratio of Arg-1 to iNOS was significantly higher in the C. longa treatment group than the negative controls; nevertheless, there was no substantial difference between C. longa treatment group and the positive controls. A high ratio of Arg-1 to iNOS activity improves the healing of wounds (Campbell et al., 2013), and by the findings of our study, it can be suggested that C. longa gel extracts will promote tissue repair and wound healing.

CONCLUSION

Based on this study, topical application of the turmeric rhizome (C. longa) gel extract accelerates the proliferation phase in the acute wound healing process through the induction of M2 macrophages and increasing collagen density in the wound tissue. However, further research is needed to explore more about the benefits of turmeric rhizome (C. longa) gel extract on the maturation/remodeling phase of the wound healing process.

ACKNOWLEDGMENTS

The authors thank the Directorate for Research and Community Service, Universitas Padjadjaran, for their financial support through Internal Research Grant for Fundamental Research.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

FUNDING

This work was supported and funded by the Internal Research Grant for Fundamental Research of Universitas Padjadjaran (No. 855/UN6.3.1/PL/2017).

REFERENCES


Yuliani S, Mustofa, Partadiredja G. Turmeric (Curcuma longa L.) extract may prevent the deterioration of spatial memory and the deficit of estimated total number of hippocampal pyramidal cells of trimethyltin-exposed rats. Drug Chem Toxicol, 2018; 41(1):62–71.

How to cite this article: