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Supplementation of curcuma domestica extract reduces cox-2 and inos expression on raw 264.7 cells

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Abstract. The expression of COX-2 and iNOS have an important role in inflammation. Turmeric (Curcuma domestica) contains active compounds such as curcumin, which can inhibit the expression of the enzyme so that the risk of chronic inflammation and cancer can be reduced. The aim of this study was to determine the anti-inflammatory effects of turmeric extract concentrations on the expression of COX-2 and iNOS in RAW 264.7 cells induced by lipopolysaccharide (LPS). This study was an experimental post-test only with a control group design. The RAW 264.7 cultured cells were divided into six groups (G) on a 24-well plate; GA (control group without LPS), GB (control group with LPS), GC (control group without primary antibody), GD (62,5µg/mL extract concentration), GE (125µg/mL extract concentration), and GF (250µg/mL extract concentration). After 18 hours of treatment, all the cells were fixed with ethanol and stored and tested with immunocytochemistry. Results show the expressions of COX-2 and iNOS in GA (10,51±5,15; 12,51±3.10), GB (82,29±1,49; 82,70±1,67), GC (29,01±5,19; 16,33±1,61), GD (32,19±5,36; 58,58±10,31), GE (24,29±5,88; 52,55±9,03), and GF (40,42±3,15; 29,24±7,84). The test results of data analysis by one-way ANOVA (COX-2) and Kruskal-Wallis (iNOS) showed significant differences ($p < 0.05$) along with Post Hoc test Tamhanes (COX-2) and Mann-Whitney (iNOS), which also showed significant differences ($p < 0.05$). The most substantial differences shown by the mean of the treatment group turmeric extract concentration were 125 µg/mL (COX-2) and 250µg/mL (iNOS). These results suggest that turmeric extracts can reduce the effects of inflammation on RAW 264.7 cells induced by lipopolysaccharide

1. Introduction

Inflammation is a protection mechanism mediated by proinflammatory cytokines such as tumor necrosis factor (TNF) -α, interleukin (IL) -1β, IL-6, interferon (IFN) -γ, and inflammatory mediators such as bradykinin, leukotrienes, prostaglandins (PG), and nitric oxide (NO) [1]. The excessive production of cytokines and inflammatory mediators can cause prolonged inflammation and trigger various chronic diseases [2]. Chronic inflammation leads to the damage of deoxyribonucleic acid (DNA), which precedes cancer [3].

Inflammation, the result of the pathobiological effect of cancer, is still a problem in cancer management. Currently, corticosteroid and non-steroid have been an inflammation drug relief used by many health centers. Both steroid drugs and Non-Steroid Anti-Inflammation Drugs (NSAID) have adverse effects such as gastrointestinal hemorrhage, cardiovascular disease, osteoporosis, and both can destroy kidney's function [4]. Herb medicine, Turmeric (*Curcuma domestica),* is reported to have an anti-inflammation and chemopreventive agent. The curcumin compound works through the inhibition of cyclooxygenase-2 (COX-2) and inducible Nitric Oxide-Synthase (iNOS) production in cancer cells [5]. This study tried to show the effect of the ethanol extract of turmeric (*Curcuma domestica)* on the expression of COX-2 and iNOS in RAW 264.7 cells induced by LPS.

2. Materials and methods

2.1. Ethical clearance

An ethical clearance certificate was obtained from The Committee of Research Ethics in the Medical Faculty of Jenderal Soedirman University (UNSOED), Purwokerto, Indonesia.

2.2. Materials

Materials used in this study included the 95% ethanol extract of turmeric; murine cells of RAW 264.7 macrophage cell line; lipopolysaccharide (Sigma-Aldrich, Singapore); primer antibodies to the target protein COX-2 and iNOS (Sigma, Singapore); and secondary antibodies with biotin-labeled protein and Avidin-peroxidase enzymes (Sigma-Aldrich, Singapore).

2.3. Cell Culture

RAW 264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, New York, USA), the buffer contains 10% fetal bovine serum and 1% penicillin/streptomycin. Cells then were cultured in Biosafety Cabinet II (Esco, Horsham, PA, USA) using 5% CO2 at 37° C. Cells were harvested after 80% becoming.

2.4. Extract Administration

Control groups were divided into three categories; Group A (cells without LPS), Group B (cells with LPS), and Group C (cells with absent of LPS and primary antibody). Treatment groups were divided into 3 categories; Group D (LPS-induced cells and given turmeric extract at concentration of 62.5 μg/mL), Group E (LPS-induced cells and given turmeric extract at concentration of 125 μg/mL), and Group F (LPS-induced cells and given turmeric extract at concentration of 250 μg/mL).

Coverslip was put into a 24 wells plate and filled with 1000 mL of cell suspension at a concentration of $5x10^4$ cells/mL in 1000 and then incubated for 24 hours. Turmeric extract was dissolved in DMSO and given media (20 μL) according to the prescribed concentration (62.5 μg/mL, 125 μg/mL, and 250 μg/mL). Treatment cells were incubated for 1 hour and were given LPS μg/mL (1 μL), and then incubated for 21 hours for COX-2 expressions and 18 hours for iNOS expressions.

2.5. Immunocytochemistry

Immunocytochemistry was using a protocol of Biocare Medical. In the first step, cells were fixed with a 300 μl methanol 100%, 90%, 70%, and hydrogen peroxide. Then cells were given Prediluted blocking serum (4 drops) and incubated for 15 minutes, primary monoclonal antibodies COX-2 and iNOS (4 drops) and incubated for 1 hour. A total of 500 μL of PBS was added and removed. The secondary antibody (4 drops) was incubated for 10 minutes and rinsed with 500 mL PBS.

Avidin-peroxidase complex reagent (4 drops) was incubated for 10 minutes, PBS (500 μL) was incubated for 5 minutes. DAB chromogen solution (4 drops) was incubated for 10 minutes. Following, 500 μL of distilled water was added and then thrown back. Mayer-Haematoxylin solution was incubated for 3 minutes. Again, 500 mL of distilled water was added and then thrown back. The cover slip was placed on an object glass slide, and mounting was done using 1-2 drops xylol, closed with a coverslip contact.

One researcher observed the expression of COX-2 and iNOS and one inter-observer at 4 x 400 magnification at random field of view using a light microscope Nikon Ni-U LED, taken using Optilab. Brown color indicates a positive expression of COX-2 and iNOS, while blue color indicates the expression of COX-2. In this study, iNOS was negative.

2.6. Data Analysis

Data from two observers was tested using the correlation of the Pearson test. One-way ANOVA with a 95% confidence level was done after the transformation Square for COX-2 data. Meanwhile, iNOS expression was done by Kruskal-Wallis, since the data was not homogenous. The Post Hoc test Tamhanes then followed the analysis for COX-2 and Mann-Whitney for iNOS.

3. Results

Turmeric extract was applicate into lipopolysaccharide-induced RAW 264.7 cells after 21 hours of incubation for COX-2 and 18 hours for iNOS. The expressions were then figured using the immunocytochemistry test. Cells that expressed COX-2 and iNOS (positive) were brown in the cytoplasm, while negative cells were blue (figure 1). The number of positive cells and the total number of cells were then calculated and compared. Data were obtained in the form of the proportion of COX-2 and iNOS against a total number of cells.

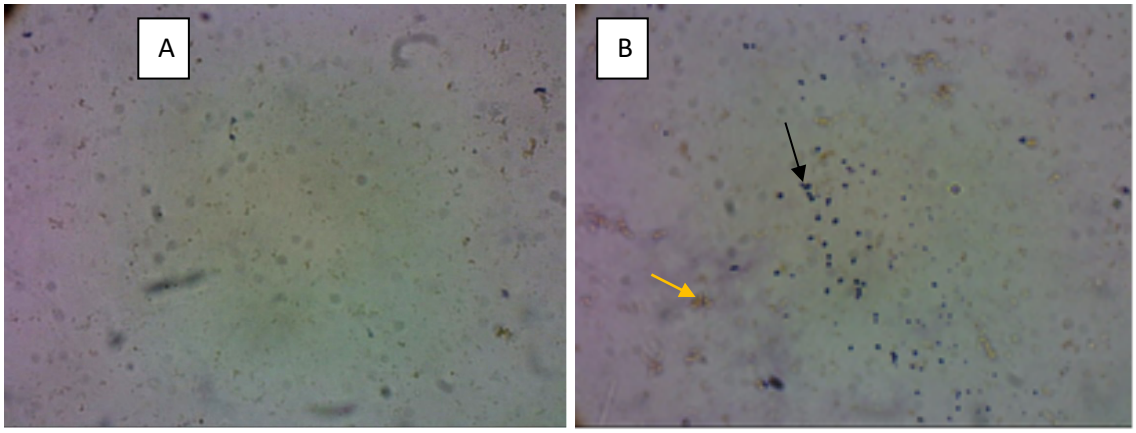

Figure 1. Microscopic Overview of Immunocytochemistry Test Result (Magnification of 400x). Positive cells were brown in the cytoplasm (orange arrow). Negative cells were blue in the cytoplasm (black arrow). (A) COX-2; (B) iNOS

Results of the expression of COX-2 and iNOS showed group B to be a group of LPS-induced RAW 264.7 cells which express COX-2 and iNOS the most and group A the least. All treatment groups D, E, and F showed a decrease compared to group B. In the expression of COX-2, group E (LPS with turmeric extract of 125 µg/ml) had the closest results of group A, as a control without LPS while on the expression of iNOS, group F (LPS at 250µg / mL extract) had the results that most approached group A (figure 2).

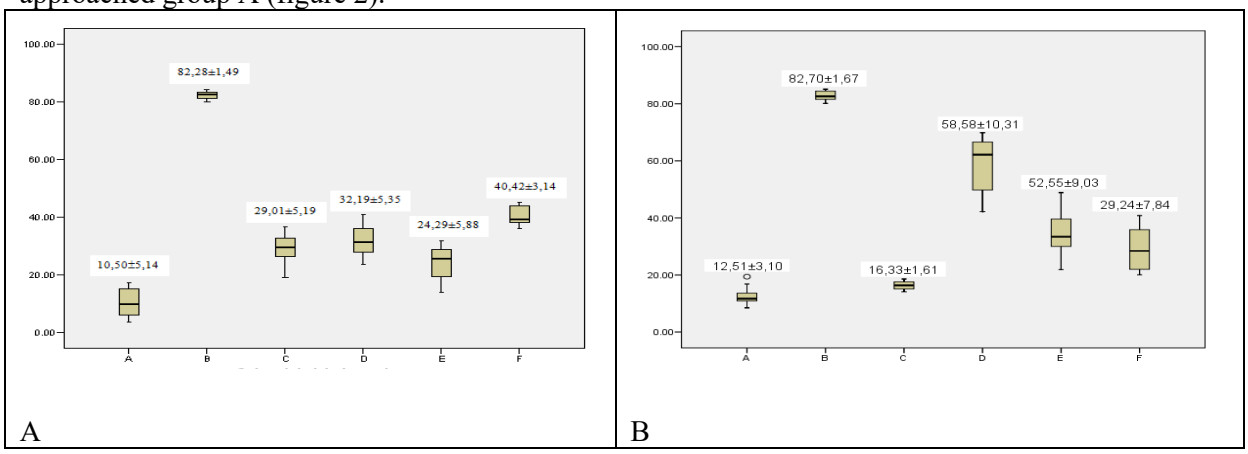

Figure 2. Average ($\bar{x}$) ± SD of cells proportion of COX-2 (A) and iNOS (B). A: cells without LPS as negative control cells; B: control cells with LPS; C: control cells without LPS and primary antibody; D: cells that given turmeric extract at a concentration of 62.5 µg/mL; E: cells that given turmeric extract at a concentration of 125 µg/mL; F: cells that given turmeric extract at a concentration of 250 µg/mL.

The Pearson correlation test compared data from two observers and obtained significant results with a very strong correlation ($p < 0.05$; $r = 0.96$). Normal data distribution of COX-2 expression was not homogeneous. Therefore, the Square transformation was done, and the results were obtained as

homogenous COX-2 expression data ($p > 0.05$), but not in iNOS ($p < 0.05$). One way ANOVA analysis was carried out for the COX-2 data and Kruskal-Wallis was used for the iNOS data.

In the one way ANOVA test for COX-2, data was obtained as significant results ($p < 0.05$). Further analysis with the Tamhanes Posthoc test did not pay attention to the assumption of variance. The treatment group using turmeric extract 125 μg / ml (test group E) was compared with the group with LPS (group B) and showed the mean difference was biggest compared to two control groups (test group A and C) and two groups of other treatments (group D and F). Overall, the test showed a significant result ($p < 0.05$) compared to group D and E as compared to group C.

A nonparametric test of Kruskal-Wallis was performed for iNOS data and showed statistically significant with $p = 0.000$ ($p < 0.001$). Based on the results of Mann-Whitney, it showed that the treatment group with a concentration of 250μg / mL (group F) showed the values most statistically significant compared to the three control groups (Group A, B, and C) and a group of other treatments (groups D and E) with $p = 0.000$ ($p<0,001$).

4. Discussion

Results of the analysis showed that the ethanol extract of turmeric has anti-inflammatory effects that were significant statistically compared to the control group. Turmeric has an active compound called curcumin that can be used as an anti-inflammatory agent. Some studies suggest that curcumin can suppress the expression of COX-2 and iNOS in LPS–induced RAW 264.7 macrophages cells. Previous data suggests that curcumin can inhibit COX-2 expression in RAW 264.7 cells induced by LPS of 1 μg/ml through the inhibition of NF-κB and modulation of COX-2a with a concentration of 5 and 10 μM but cytotoxic at a 25 μM with a 24 hour an incubation while researchers incubated for 21 hours after treatment [6]. The previous study also proved that curcumin could inhibit the expression of COX-2, LTB4, and the release of arachidonic acid in RAW 264.7 cells induced by 2 μg/mthe l LPS. Turmeric extract was performed 1 hour before administration of LPS then the cells were incubated for 18 hours with curcumin concentrations of 10, 25, and 50 μM. In contrast to these studies, the sufficient concentration in this study was 125 μg/ml extract of turmeric (10.17 μM curcumin) while at a concentration of 250 μg / ml (20.34 μM curcumin), a decrease in the effectiveness of turmeric extract was found. It may be related to the concentration of LPS used and a longer incubation time (21 hours) [7].

Another research group also found that the cells were not induced by treatment with LPS also express COX-2 at 14-hour incubation while the 24-hour incubation showed COX-2 was not expressed. This statement shows incubation time may affect the effectiveness of curcumin. A compound takes time to bind to the target; it also occurs in the compound curcumin. More extended incubation allows time for cells to absorb the compound curcumin on the extracellular so that a more extended incubation can improve effectiveness [8]. Also, curcuminoid compounds known to have a greater inhibitory effect on COX-2 peroxidase compared to COX-2. Ben and colleagues suggest that curcumin at concentrations of 5 μM, ten μM, and 20 μM can reduce NO production and iNOS expression in RAW 264.7 cells induced by LPS. At a concentration of 20 μM curcumin, significantly expressed iNOS mRNA levels can decrease by 55% and reduce levels of iNOS protein by 70% in RAW 264.7 cells induced by LPS [9]. The previous study reported that the curcumin inhibited LPS-mediated upregulation of iNOS, the protein of COX-2 and also NF-κB activation. Curcumin also has a potential effect compared to the metabolites, of which tetrahydrocurcuim has an abundant result. The present study demonstrated that curcumin and its major metabolites induce inhibition mechanism of inflammatory response via blocking NF-κB translocation to the nucleus [10]. The continuous contribution of IL-1β and the addition of curcumin decreased expression amount of iNOS protein, resulting in inhibition of NO production. Supplementation of Curcumin also affects in reducing mRNA production of TNF-α and IL-6. Data showed that Curcumin inhibited the essential signaling ways for iNOS induction, NF-κB activation and type I IL-1 receptor regulation. Transfection study told that curcumin inhibits iNOS mRNA expression at the promoter activation and mRNA stabilization steps. The delayed administration of this kind of substance will inhibit iNOS induction [11].

Curcumin inhibits the production of COX-2 and iNOS by suppressing the activation of the transcription factor nuclear factor-κB (NF-κB) through TLR4. At the time of RAW 264.7

macrophages cells induced by LPS, PRR that was TLR4 on cell membrane recognizes LPS structures. TLR4 accessory molecules, myeloid differentiation protein 2 (MD2) extracellular protein will bind to the lipid A component of LPS and the complex formed, which then interacts with TLR4. LPS binding iatrogenic the formation of associate degree m-shaped receptor multimer composed of 2 copies of the TLR4-MD-2-LPS complicated organized symmetrically. LPS interacts with an outsized hydrophobic pocket in MD-2 and directly bridges the two parts of the multimer. Most of super molecule chains of LPS are buried deep within the pocket, and therefore the remaining chain is exposed to the surface of MD-2, forming a hydrophobic interaction with the preserved phenylalanine of TLR4. The F126 loop of MD-2 undergoes localized structural modification and supports this core hydrophobic interface by creating hydrophilic interactions with TLR4. TLR4 signal significantly improve optimal rejuvenation of programed cell dead in the injured tissue throught protection and reproduction of the cells, as well as MyD88. TLR4 is more powerfull in the short-term effect, but chronic induction through TLR4 may lowering the threshold of cronical proccess [12].

COX-2 expression was also confident in the group without LPS given only secondary antibody. It has become one of the limitations of the study in controlling variables. The study could not determine which pathways play a role in the inhibition of COX-2 and iNOS as observed only expressing cells and not expressing COX-2 and iNOS without noticing their inhibition path. Additionally, the negative cells were not stained blue staining entirely after cells were observed to be a translucent color (negative) and brown (positive).

5. Conclusion

The study concluded that there were anti-inflammatory effects of turmeric (*Curcuma domestica*) extract in RAW 264.7 cells induced by lipopolysaccharide. Optimal concentration was achieved in 125µg/mL for COX-2, and 250µg/mL for iNOS. Supplementation of *Curcuma domestica* extract can reduce the inflammatory effects.

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Conflict of interest

Authors declare there are no conflicts of interest regarding submission of this manuscript.