Thymoquinone Increased Expression of CD4CD25Treg in Sprague-Dawley Rats Induced Dimethylbenzanthracene

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Abstract

**BACKGROUND:** The carcinogen dimethylbenzanthracene (DMBA) is immunotoxic. Thymoquinone, meanwhile, is known to have antioxidant and anti-inflammatory effects.

**AIM:** This study aims to determine the effect of thymoquinone and tamoxifen on the CD4CD25Treg count in Sprague-Dawley (SD) rats induced by DMBA.

**METHODS:** The 50 SD rats were divided into five groups. Group I (normal control) was given standard drinking and food. Group II was given thymoquinone, Group III was given tamoxifen, Group IV was given DMBA, and Group V was given solvent control. Thymoquinone, tamoxifen, and solvent control administration started 2 weeks before DMBA administration and continued during DMBA induction. In the 3rd week, except for the normal group, all groups were created to be induced with 10 × 20 mg/kg body weight of DMBA for 5 weeks. In the 21st week, surgery and data collection were performed. The hematology profile and CD4CD25Treg number were carried out employing a flow cytometer. The difference in the average number of CD4CD25Treg and blood cells between groups was analyzed with one-way analysis of variance

**RESULTS:** The results revealed that DMBA induction reduced the number of erythrocytes, HB levels, platelet counts, and leukocyte counts (p < 0.05). The administration of thymoquinone and tamoxifen reduced the hematopoiesis effect of DMBA. The thymoquinone and tamoxifen group had a higher number of CD4CD25Treg and leukocytes than the DMBA group (p < 0.05).

**CONCLUSION:** There was no difference in the average CD4CD25Treg, leukocyte count, lymphocyte count, and monocyte count between the thymoquinone and the tamoxifen groups (p > 0.05).

Introduction

The T lymphocytes are an integral part of the neoplasm-defying cellular immune system [1]. Approximately 20–30% of the peripheral blood leukocyte population is cluster of differentiation 4 (CD4) Th cells [2]. ThCD4 lymphocytes act as activators of specific immune responses against intracellular infections and neoplasms by regulating lymphocyte proliferation and differentiation and cytokine production. Active CD4 Th lymphocytes will secrete cytokines, including interleukin-12 (IL-12), IL-2, and interferon gamma (IFNγ) [1], [3]. About 5–10% of the CD4 T-cell population is CD4CD25 T cells that act as Tregulator (Treg) cells [4]. Cytomegalovirus infection is often followed by increasing the CD4CD25 population, which is thought to suppress the immune response [5]. Increased CD25 is associated with immunity tolerance [6]. A decrease in CD25 count is correlated with autoimmunity and chronic inflammation [7].

The compound 7,12-dimethylbenzanthracene (DMBA) is one of the main ingredients of cigarette smoke, kitchen smoke, and motor vehicle fumes, which are carcinogenic [8], [9]. Besides being genotoxic, a carcinogen 7,12-DMBA is also immunotoxic [10]. As a genotoxic carcinogen, DMBA can lead to breast cancer, skin cancer, blood cancer, oral cancer, and lung cancer [11]. DMBA is metabolized in the body by cytochrome and microsomal epoxide hydrolase enzymes to become the ultimate genotoxic and immunotoxic carcinogen [12]. Ultimate carcinogens of DMBA has been shown to suppress bone marrow activity, thereby inhibiting erythropoiesis and lymphocyte formation. The ultimate carcinogen DMBA is also toxic to splenocytes [10], [13], [14].

The active substances of black cumin seed oil (BCSO) are empirically used as defense enhancers [15]. In the laboratory, BCSO has been shown to have biological activity as an anti-carcinogenesis chemopreventive, immunomodulatory, anti-inflammatory, hepatoprotective, and nephroprotective.
[16], [17], [18], [19]. BCSO was shown to increase the CD4 lymphocyte count and IFN levels on the 3rd day of cytomegalovirus infection [20]. In addition to unsaturated fatty oils, thymoquinone is the main BCSO [21]. Based on the previous studies’ results, it was proven that cancer patients’ consumption of black cumin seeds could increase the number of T cells [22]. Administration of black cumin ethanol extract can protect the damage to splenocyte DNA due to oxidative stress due to Ro ray radiation [23]. It has been shown that the critical content of black cumin seed essential oil, thymoquinone, enhances p53 expression in T47D cells [24], [25]. Thymoquinone is also an antioxidant and anti-inflammatory [26]. Besides, the administration of black cumin seeds can increase the phagocytosis activity of peritoneal macrophages in Sprague-Dawley (SD) rats induced by DMBA [27]. Administration of BCSO, with the main content of thymoquinone, in test animals induced by the immunotoxic DMBA carcinogen, is thought to increase the expression of CD25 [5], [28]. The immunotoxic impact of DMBA on the number and function of BCSO-acquired Treg lymphocytes is unclear so far. This study aimed to determine the number of CD4CD25 after administering thymoquinone to SD rats induced by DMBA.

**Methods**

**Research materials and tools**

BCSO with a level of 2.72% thymoquinone developed in the Pharmacy Biology Laboratory of the Faculty of Pharmacy, Ahmad Dahlan University, thymoquinone (Sigma) and CD4CD25 antibody were the materials used in this research. The growth medium utilized for lymphocyte cell culture was RPMI-1640 (GIBCO) medium, containing: 10% fetal bovine serum; 1 mM sodium bicarbonate; 2 mM L-Glutamine; 100 µ penicillin; and 0.5 mg streptomycin, 70% alcohol, 95% ethanol, heparin (anticoagulant), sodium oxalate, and distilled water. Sorenson’s buffer (0.67 mM) was prepared from a mixture of 7.2 ml of solution A + 2.8 ml of solution B + 90 ml of aqua dest. Solution A: 9.5 g Na2HPO4 + aqua dest up to 1 l. Solution B: 9.07 g KH2PO4 + aqua dest up to 1 l. All obtained from the Biological Sciences Laboratory of UGM, Yogyakarta.

Fifty female white SD rats aged 3–4 weeks with a weight ranging from 100 to 140 g were used in this study. The test animal was obtained from the UGM Laboratory of Life Sciences. The care and treatment of animals were carried out as recommended by the testing ethics committee on test animals. Test animals were kept in iron cages measuring 50 cm × 30 cm × 20 cm individually, fed with 529 pellets, and given enough drinking [29].

Some of the equipment needed in this study were a CO2 incubator, centrifuge, microplate reader, inverted microscope, and little camera set for cell culture. The 10 cc injection syringe, laminar air flow hood, scale, commonly used glassware, hemocytometer, dropper pipette, glass object, blender, filter, operating tools, and an operating table were for in vivo study. Furthermore, the flow cytometer was for immunoassay examination.

**Research procedure**

**Preparation of test animals, treatment, and DMBA induction**

The ethics committee of Gadjah Mada University (no: 222/ KEC-LPPT/III/2015) has reviewed and accepted the research procedures and the research protocol. A pharmaceutical biologist at the Pharmaceutical Biology Lab, Faculty of Pharmacy, UGM, carried out the identification of black cumin seeds. The manufacture of the BCSO was conducted by steam distillation method, using a distillation. Every 50 g of powder were wrapped in filter paper, then put into a distillation. After that, 500 ml of water was added.

Fifty randomly selected SD rats aged 4–6 weeks were grouped into five groups of 10 each. As a regular party, drinking and standard food were given to Group I mice. Test animals in Group II received thymoquinone 50 mg/kg body weight (BW)/day orally before and during the induction of DMBA. Tamoxifen 20 mg/kg BW/day was given to Group III test animals 2 weeks before and 5 weeks during DMBA induction. Tested animals in Group IV were administered DMBA at a dose of 10 × 20 mg/kg, 2 ×/week, orally with a probe. Test animals were administered corn oil at a dose of 20 mg/kg BW/day orally in Group V as solvent control 2 weeks before and 5 weeks during DMBA induction. DMBA was given orally using a sonde to all groups of tested animals. DMBA in corn oil has been dissolved. A dosage of 20 mg/kg BW was given ten times, that is, twice/week for 5 weeks, beginning in the 3rd week of treatment. Test animals’ development during and after being treated and induced by DMBA was observed for clinical conditions and measured their body weight once a week.

**Hemogram examination of peripheral blood and count the type of leukocytes**

Blood was drawn from the orbital vein by trained personnel. A hemogram examination was carried out in the Lab. LPPT UGM. The blood cell count was checked with a hemoanalyzer machine.

**Flow cytometer test for CD4CD25Treg count**

Calculation of the number of T lymphocyte types CD4CD25 was done using a flow cytometer. Blood was
drawn from the vena orbitalis of the test animals’ eyes and then connected in a Vacutainer tube containing an anticoagulant. The examination of samples with a flow cytometer using the following procedure: (i) Specimens were piped up to 50 μL (ii) into the falcon tube. Add 10 p.L of FITC/CD Tritest Reagent CD per CP. It (iii) mix homogeneously with a vortex mixer and incubate in a dark space for 15 min at 20–25°C. III. (iv) With 450 p.L of distilled water, dilute 50 p.L of I Ox FACS lysing solution and then blend homogeneously. Oh. (v) The 450 μL diluted FACS reagent (ix) was applied to the sample after the incubation time was over. (vi) Mix homogeneously, then incubate in a dark room for 15 min, 20–25°C. Uh. (vii) The study was performed using the FACS flow cytometer after the incubation cycle was completed.

**Data analysis**

Analysis of the mean difference test between groups with one-way analysis of variance (ANOVA) was applied to the number of blood cells, the type of leukocyte count, and the number of Tregs. Blood cell counts and CD4CD25 Treg were calculated for the mean difference between groups and followed by ANOVA and Tukey.

**Results**

**Overview of test animals**

Blood samples were taken through the orbital sinuses of SD rats using a hematocrit tube. The blood drawn was collected at Eppendorf, which had previously been cleaned and given ethylenediaminetetraacetic acid (EDTA). The addition of EDTA aimed to prevent clotting or, in other words, to function as an anticoagulant. The results of the hemogram are presented in Table 1.

As presented in Tables 1 and 2, DMBA has been shown to reduce the number of erythrocytes, platelets, hemoglobin, hematocrit, and mean corpuscular hemoglobin (MCH) and MCH concentration levels. Apart from reducing the number of erythrocytes, hemoglobin, and platelets, DMBA induction has also been shown to reduce the number of leukocytes.

Based on this study’s results (Table 2), it was proven that DMBA suppressed all types of blood cells. The number of RBC, leukocyte, and platelets in the DMBA group was more than in the normal group (p < 0.05).

**The results of the leukocyte count test**

Table 3 describes the leukocyte count test results. In general, the results revealed that DMBA induction decreased the number of lymphocytes, decreased the number of monocytes, and increased the number of neutrophils and eosinophils (p < 0.05). There was no difference in the number of lymphocytes, monocytes, eosinophils, and basophils in the normal thymoquinone and tamoxifen groups (p > 0.05).

Based on Table 3, it is also known that DMBA induction did not affect the number of basophils. The basophil count in the DMBA group did not differ from the basophil count in the normal, thymoquinone, and tamoxifen groups.

**CD4CD25 expression test results with flow cytometry**

DMBA is a carcinogenic polycyclic aromatic hydrocarbon. Table 4 presents the results of evaluating the impact of BCSO administration on CD4CD25Treg in SD rats induced by DMBA.

DMBA induction was shown to decrease CD4CD25 counts, as shown in Table 4. In the DMBA group, the average CD4CD25 count was below the average CD4CD25 count in the normal and solvent groups (p < 0.05).

**Discussion**

The induction of DMBA has been shown to decrease the number of lymphocytes and monocytes and increase eosinophils and neutrophils. As compared
to the other groups, the DMBA group had the lowest number of lymphocytes and the highest number of neutrophils and eosinophils. Before and during DMBA induction, the administration of thymoquinone to SD rats could prevent a decrease in the number of lymphocytes and monocytes and reduce the number of neutrophils and eosinophils. This study’s result is consistent with the previous studies where thymoquinone is anti-inflammatory by reducing prostaglandin activity and the number of polymorphonuclear cells. Thymoquinone is an immunostimulant and anti-inflammation. In vitro, it is known that thymoquinone inhibited NFkB activation [23].

### Table 4: Mean values and percentages (± SD) of CD4CD25 SD rats induced by DMBA after administration of thymoquinone and tamoxifen for 14 days and during DMBA induction

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>n</th>
<th>Mean absolute number of CD4CD25 T lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>10</td>
<td>68.5 ± 1276</td>
</tr>
<tr>
<td>Thymoquinone 50 mg/kgb</td>
<td>10</td>
<td>97.5 ± 2169</td>
</tr>
<tr>
<td>Tamoxifen 20 mg/kgb</td>
<td>10</td>
<td>84.5 ± 1346</td>
</tr>
<tr>
<td>DMBA</td>
<td>10</td>
<td>18.6 ± 40.17</td>
</tr>
<tr>
<td>Solvent</td>
<td>10</td>
<td>109.3 ± 64.02</td>
</tr>
</tbody>
</table>

*p<0.05 for normal control; **p<0.05 for the DMBA group; DMBA: Dimethylbenzanthracene.

Thymoquinone increased the CD4CD25 count. Table 4 displays the findings of the CD4CD25 Treg expression test with flow cytometry. The results showed that the neutrophil count was inversely linked to the CD4CD25 count. Neutrophils are a cellular component of acute and exaggerated inflammatory responses. The increase in CD4CD25 count suppresses acute inflammatory reactions resulting in a decrease in the number of neutrophils. This study’s result follows previous studies that thymoquinone is potential as an anti-inflammation-immunotolerance agent [30], [31].

Based on the study results, it is known that thymoquinone is anti-inflammatory by reducing polymorphonuclear and eosinophil cells. Thymoquinone has also been shown to increase immune tolerance by increasing the number of CD4CD25Treg cells. Further research can be carried out to clarify the mechanism of activation of CD4CD25 Treg cells.

### Conclusion

Based on the results of the analysis, it could be concluded that DMBA reduced the percentage of CD25 expression in TCD4 lymphocytes. Meanwhile, thymoquinone increased CD25 expression on CD4 T cells.

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### References

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