ORIGINAL ARTICLE

Red algae extract suppresses caspase-3 gene expression and induces catalase antioxidant enzyme in testicles of rats induced by boric acid

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ABSTRACT

Aim To determine the effect of red algae extract on the gene expression of catalase and caspase-3 in testicules of rats induced by boric acid (BA).

Methods This is experimental research with post-test control group design. Twenty four healthy male Wistar rats were divided into four treatment groups: a healthy group, negative control group, two treatment groups with red algae extract 400mg/kgBW/day (T1) and red algae extract 800mg/kgBW/day (T2). Each group was treated with BA 500mg/kgBW/day for 14 days, whereas the healthy group did not receive BA. In the treatment groups T1 and T2 were given red algae extract for 14 days. On day 15 all treatment groups were terminated and catalase and caspase-3 gene expression were analysed using qRT-PCR.

Results In the healthy group, the expression of the catalase gene was 1.39±0.67 and the expression of the caspase-3 gene was 1.06±0.17. In the negative control group, there was a significant decrease in catalase gene expression, 0.68±0.27 ($p<0.05$), and a significant increase in caspase-3 gene expression, 5.71±2.47 ($p<0.05$). Treatment groups T1 and T2 showed a significant increase in catalase gene expression, 2.67±0.69; and 2.85±0.64, respectively ($p<0.05$) and caspase-3, 3.96±1,16 and 1.89±0.84, respectively, compared to the control group.

Conclusion: The administration of red algae extract had a significant effect on increasing the expression of the catalase gene and decreasing the expression of the caspase-3 gene. This suggests that red algae extract has the potential to be developed as a protective agent against exposure to the effects of BA.

Key words: boric acid, caspase-3, catalase, red algae extract, testicular

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INTRODUCTION

Boric acid (BA) and inorganic borates are abundant in nature and widely used in industry, agriculture, cosmetics, and a variety of smaller applications (1). Orally administered BA is readily and completely absorbed in rats, rabbits and humans, as well as other animal species. In animals and humans, absorbed BA appears to be rapidly distributed throughout the body water via passive diffusion (1). A previous study reported that a high-dose BA exposure produces testicular lesions in adult rats characterized by inhibited spermiation that may progress to non-recoverable atrophy (2). Another study reported that BA at 1000 to 2000 ppm induces testicular atrophy leading to testicular apoptosis (3). The World Health Organisation (WHO) estimates that around 50 - 80 million married couples (1 in 7 couples) have infertility problems, and each year around 2 million infertile couples appear. The incidence of infertility in Indonesia increases annually by 10-15% (4). The use of borax as a food additive causes male infertility up to 40%.

A previous study stated that BA at doses of 250 mg/kgBW/day significantly induces reactive oxygen species (ROS) levels, decreased serum arginase activity, sperm quality and DNA content in sperm. Increasing the dose of borax acid to 500 mg/kgBW/day causes testicular atrophy, damage to spermatogenesis, failure of sperm formation (5). High levels of ROS inhibited the expression of antioxidant enzyme such as catalase, glutathione peroxidase (GPx), superoxide dismutase (SOD) (6). In addition, BA exposure induces intrinsic and extrinsic apoptosis through increases in intracellular free radicals and then converts adenosine triphosphate (ATP) and Ca^{2+} into the mitochondrial membrane wall to produce pro-apoptotic factors, activating caspases through the release of mitochondrial cytochromes to the cytosol including caspase-3 (7). The body can normally produce antioxidant enzymes such as catalase and endogenous antioxidant enzymes (8). Increasing ROS levels above the threshold due to BA exposure causes the antioxidant enzymes produced to be unable to stabilise ROS, thus causing oxidative stress and requiring additional antioxidant compounds from outside the body (9–11). In this case the body needed an external antioxidant to stabilizing high levels of ROS.

Previous studies have shown that red algae extract (*Eucheuma cottoni*) are a source of flavonoids, which may have an antioxidant activity (12,13). Red algae have a mechanism of action in preventing inflammation, and prevent the occurrence of ROS by providing ions and inhibiting the formation of ROS directly by ROS scavengers or indirectly by increasing antioxidant levels such as SOD, catalase, and GPx (14). The flavonoid content in red algae can protect lipid peroxidase by reducing hydrogen ions in hydrogen peroxidase (H_2O_2) into an active hydroxyl radical form (OH-). The redox potential of flavonoids (FI-OH) can reduce the formation of free radicals (15). However, no evidence supporting a favourable role of red algae extract in the regulation of catalase and caspase-3 gene expression in the BA exposure.

The aim of this study was to evaluate the effect of red algae extract in the regulation of catalase and caspase-3 gene expression in the BA exposure.

MATERIALS AND METHODS

Material and study design

This study was conducted in the Stem Cell and Cancer Research Laboratory, Indonesia, from December 2022 until February 2023. The experiment was carried out according to the internationally valid guidelines and the Faculty of Medicine Sultan Agung Islamic University, institutional animal Ethics Committee under the No. 296/VIII/2022/Komisi Bioetik.

Methods

Animals. Adult male Wistar rats (60 to 70 days old, 200–250 g) were obtained from Semarang local breeding laboratories, Central Java, Indonesia. The animals were acclimated for 7 days to the Integrated Biomedical Laboratory Sultan Agung Islamic University, Indonesia. Animals were housed five per polycarbonate cage with 12:12 h light/dark cycle, 50%±2% humidity, and an ambient temperature of 25±1.

Extraction of red algae extract. Red algae were collected from Jepara in Central Java Indonesia in October 2022 (Latitude 6.5805 and Longitude 110.6790). For biological studies, the plant was dried in a renewal air oven and circulated at 40°C until it was completely dehydrated. A biologist at the Ecology and Biosystematics Laboratory, Faculty Science and Mathematics, Diponegoro University, Semarang, Indonesia confirmed the identification of the plant. Red algae were rinsed with tap water followed by distilled water to remove the dirt on the surface. The dried red algae were blended into small pieces and sieved with a mesh size of 120 mesh. 500 g of red algae were extracted in a maceration apparatus with 5 L 70% ethanol for 24 h. When filtrated, it was then evaporated under the rotary vacuum evaporator (IKA), and the crude extract was kept in refrigerator at 4 (16,17). Red algae extract was dissolved in carboxymethyl cellulose (CMC) suspension for oral administration. The formulations were stored at 4 until further analysis (17,18).

BA exposure. Twenty four rats were computer-randomized by body weight and assigned to the healthy group (n=6/group), negative control (n=6/group), and 2 treatment groups (T1, T2) (n=6/treatment group). After further 7-day acclimation, the negative group and the treatment group were fed BA doses 500mg/kgBW/day for 14 days. The treatment groups received red algae extract doses 400mg/kgBW/days for 14 days (T1) and 800mg/kgBW/days for 14 days (T2) under oral administration. On day 15 after the treatment, all rats were terminated, and testicular tissue was isolated for further analysis.

Catalase and caspase-3 gene expression by qRT-PCR. Total RNA from rat testicular tissue was extracted with TRIzol (Invitrogen, Shanghai, China) according to the manufacturer's protocol. Briefly, first-stranded cDNA was synthesized with 1g of total RNA using Super-Script II (Invitrogen, Massachusetts, USA). SYBR No ROX Green I dye (SMOBIO Technology Inc, Hsinchu, Taiwan) was used for reverse-transcription in a real-time PCR instrument (PCR max Eco 48), and mRNA levels of the catalase and caspase-3 genes were measured using the respective primers (Table 1). The thermocycler conditions were as follows: initial step at 95 °C for 10 minutes, followed by 50 cycles at 95°C for 15 seconds, and 60°C for 1 minute. The gene expression was recorded as the cycles threshold (Ct). Data were obtained using Eco Software v5.0 (Illumina Inc, San Diego, CA, USA). All reactions were performed in triplicate, and data analysis used the $2^{-\Delta\Delta}$ Ct method (Livak method) (19,20).

Table 1. Primer sequences for catalase, caspase-3 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes

Gene	Primer sequence 5' → 3'
Catalase	Forward Catalase 5'- CTTGGAACATTGTACCCGCT-3' Reverse Catalase 5'- GTCCAGAAGAGCCTGAATGC-3'
Caspase-3	Forward Caspase-3 5'- GTGGGACTGAAGATGACA-3' Reverse Caspase-3 5'- ACCCGAGTAAGAATGTG-3'
GAPDH	Forward GAPDH 5'- GTCTCCTCTGACTTCAACAGCG-3' Reverse GAPDH 5'- ACCACCCTGTTGCTGTAGCCAA-3'

Statistical analysis. All data are presented as mean±standard deviation (SD). The normal distribution and the homogeneity of the data were analysed under Shapiro-Wilk and Levene test, respectively ($p>0.05$ described as normal distribution and homogen). Thus, the significance of the difference between means of the negative control and T1/T2 treated rats were analysed using one way analysis of variance followed by post hoc least significant difference; the level of significance was set at $p<0.05$.

RESULTS

The red algae extract in this study was obtained from Jepara waters and extracted by the maceration method using ethanol solvent and produced an extract yield of 67 grams (13.4%) from 500 grams of dry algae. The results of phytochemical screening of red algae extracts show that red algae extracts are positive for alkaloid, saponin, tannin, flavonoid, steroid and terpenoid compounds. The flavonoid content of red algae extract was 338.54±0.33 QE/g extract.

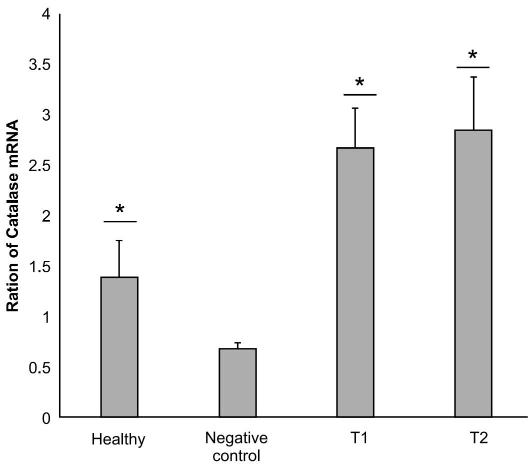

Figure 1. The effect of red algae extract on catalase gene expression on rats model induced by boric acid (BA) in the healthy (did not receive any treatment), T1 (received red algae extract of 400mg/kgBW/days for 14 days) and T2 (received red algae extract of 400mg/kgBW/days for 14 days) groups
Data are presented as fold change in gene expression relative to BA unexposed group; *p< 0.05

The results of catalase and caspase-3 gene expression were obtained in rat models induced by BA 500mg/kgBB/day for 14 days and treated with red algae extract. In the healthy group, the catalase gene expression ratio was 1.39±0.67, in the control group 0.68±0.27, in the T1 group 2.67±0.69, and in the T2 group it was 2.85±0.64. Catalase gene expression ratio increased in a dose-dependent manner (Figure 1).

In this study we also evaluated the effect of red algae extract on caspase-3 gene expression. Administration of red algae extract decreases caspase-3 gene expression induced by BA, in T1 group causes a decrease of 3.96±1.16 and in T2 of 1.89±0.84 compared to the negative control group, which causes an increase in caspase-3 expression by 5.71±2.47. In the healthy rat group, there was a normal caspase-3 gene expression of 1.06±0.17 (Figure 2). This is due to the low content of flavonoid compounds in T1 and T2 that can inhibit oxidative stress so as to reduce the expression of caspase-3 gene that causes apoptosis.

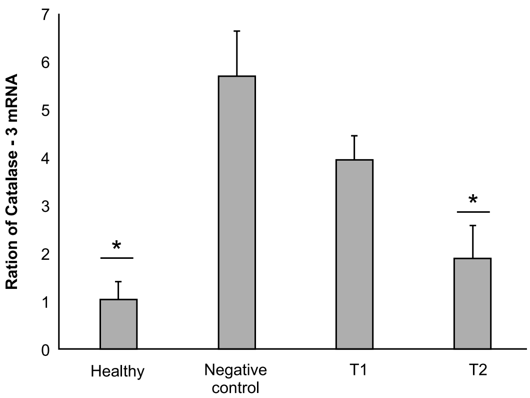

Figure 2. The effect of red algae extract on caspase-3 gene expression on rats model induced by boric acid (BA) in the healthy (did not receive any treatment), T1 (received red algae extract of 400mg/kgBW/days for 14 days) and T2 (received red algae extract of 400mg/kgBW/days for 14 days) groups
Data are presented as fold change in gene expression relative to BA unexposed group; *p< 0.05

DISCUSSION

Previous studies reported that antioxidant compounds such as β-carotene have free radical capture properties from ROS related to their ability to form stable radicals. Flavonoids can also effectively capture free radicals by forming semiquinone radicals that will bind with free radicals to form stable quinone structures (21).

In our study, red algae extract proved positive for alkaloid, saponin, flavonoid, steroid, and terpenoid compounds. Previous studies have confirmed that antioxidant compounds such as flavonoids are able to reduce and maintain the balance of ROS levels by increasing the formation of antioxidants (22–25). The regulation of the balance between ROS and antioxidant levels is crucial in several cellular signal transduction pathways including the regulation of differentiation, proliferation, migration, survival, and apoptosis (26). The results of this study indicate that red algae extract can induce catalase gene expression significantly compared to the control group with an effective dose of 800mg/kgBW. This is because at a dose of 800mg/kgBW they significantly decreased caspase-3 expression approaching the expression of the caspase-3 gene in healthy conditions. At a dose of 800mg/kgBW there was also a significant increase in catalase gene expression compared to a dose of 400mg/kgBW. Flavonoid compounds such as quercetin will form hydrogen bonds with Ser212 through the 3'-OH group causing inhibition of protein kinase kinase (MEK1) activity. Quercetin also inhibits phosphoinositide 3-kinase (PI3K) activation and activates protein kinase (MAPK) to induce antioxidant enzyme expression (27). A previous study also reported that the ability of flavonoid compounds as antioxidants was proven to reduce oxidative stress conditions by increasing the catalase enzyme (18,28).

This study also evaluated the effect of red algae extract on caspase-3 gene expression due to BA exposure. Previous studies reported that flavonoid quercetin increased the expression of Nrf2, which induces the production of various antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase (29,30). This suppresses ROS and increases the release of the antiapoptotic protein survivin, which inhibits the expression of caspase-3 (31,32). The C-6 structure on flavonoids inhibits the expression of the NF-kB signalling pathway (33). This structure, the expression of caspase-3 is also inhibited and inhibits apoptosis. The ring B structure on flavonoids also acts as a capture for hydroxyl free radicals, so that ROS generated by boric acid exposure can be suppressed (34). Downregulation of ROS inhibited the caspase-3 activation pathway (35,36).

The limitation of this study was not measuring ROS levels and the effect of red algae extract on other apoptotic pathways so that the molecular mechanism of action could not be found clearly.

In conclusion, this study found that the 800mg/kgBW dose of red algae extract is the most effective dose to have antioxidant activity and inhibit the gene expression of pro apoptotic factor caspase-3 due to BA exposure. This suggests that red algae extract has the potential to be developed as a protective agent against exposure to the effects of BA.

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TRANSPARENCY DECLARATION

Conflict of interest: None to declare.

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