This study aimed to investigate whether combined supplementation with vitamin C and vitamin E was able to modify the superoxide dismutase (SOD) and malondialdehyde (MDA) levels in the ovary of rats exposed to rhodamine B. Twenty-five female Wistar albino rats were divided into five groups (n = 5 each), including control (untreated group); rhodamine B group; rhodamine B group which received vitamin C (0.2 mg) + vitamin E (0.04 IU/g body weight); rhodamine B group which received vitamin C (0.4 mg) + vitamin E (0.04 IU/g body weight); and the rhodamine B group which received vitamin C (0.8 mg) + vitamin E (0.04 IU/g body weight). Analysis of MDA levels as a marker of lipid peroxidation was done spectrophotometrically. Analysis of SOD levels was done by enzyme-linked immunosorbent assay technically. Endometrial histology was analyzed in hematoxylin eosin staining. This increase in ovarian MDA was significantly (p < 0.05) attenuated by the two highest dose treatments of combined vitamin C and vitamin E. Rhodamine B significantly decreased SOD levels compared
to the untreated group. This decrease in ovarian SOD level was significantly attenuated by the second and third doses of the combined vitamin C and vitamin E. The vascular number and gland density were significantly lower in the rhodamine B group compared to the untreated control group ($p > 0.05$). All doses also significantly prevented rhodamine B-induced decrease in the vascular number and gland density. In conclusion, the protective effect of combined vitamin C and vitamin E against ovarian and endometrial toxicity in rats receiving oral rhodamine B is due to inhibition of the lipid peroxidation, modulation of SOD levels, and the endometrium repairing effect.

Introduction

In the reproductive system, reactive oxygen compounds play a role in physiological processes such as oocyte maturation. Excessive reactive oxygen compounds without adequate antioxidant defenses will trigger oxidative stress. Ovarian oxidative stress will lead to damage the structure of the oocyte and granulosa cells in the follicle. Reactive oxygen species can affect the quantity and quality of the ovaries and so have an impact on the capacity of the ovary. Women with impaired capacity will experience failure and high stimulation pregnancy failure. The endometrium is a complex tissue that lines the inside of the endometrial cavity. It is morphologically divided into functional and basal layers. The functional layer forms two thirds of the endometrial thickness and it presents different compartments, including the luminal epithelium, the glandular epithelium, stroma, and the vascular compartments.

One of the chemical compounds that can trigger oxidative stress is rhodamine B. This compound is a synthetic dye of green or red purple crystals. Utilization of rhodamine B dye includes paper, textile dyes, dyes of histology specimens, and cosmetics. When exposed to light, rhodamine B can form reactive oxygen compounds. Reaction formation of reactive oxygen compounds is divided into two types. The first reaction will increase the energy of rhodamine B and transfer to biomolecules to form reactive oxygen compounds. The second reaction is the reaction of the energy transfer to molecular oxygen to form singlet oxygen.

Various studies have shown that exposure to rhodamine B triggers oxidative stress on ovarian follicles and a decrease in the number of primary, secondary, and Graafian follicles. To inhibit oxidative stress requires intake of antioxidants.

The α-tocopherol is an antioxidant in the lipid compartment to protect against lipid peroxidation, changing gene expression, modulation of cell signaling, and proliferation. This compound is found in significant amounts in the ovaries and follicular fluid. Combined supplementation of vitamin C and vitamin E is the best choice for antioxidant treatment. As a result of continuous oxidative stress, there is an increase in the concentration of ascorbate radical that shows as a peak followed by a steady decline. After disappearance of the ascorbate radical, the tocopheroxyl radical appears. This study aimed to investigate whether combined supplementation of vitamin C and vitamin E was able to modify the superoxide dismutase (SOD) and malondialdehyde (MDA) levels in the ovary of rats exposed to rhodamine B. In addition, the effects on endometrial histology were also explored.

Material and Methods

Animals

Twenty-five female Wistar albino rats, aged 8–12 weeks, weighing 160–250 g were used for the present investigation. The animals were divided into five groups ($n = 5$ each), including control (untreated group); rhodamine B group; rhodamine B group receiving vitamin C (0.2 mg) + vitamin E (0.04 IU/g body weight); rhodamine B group receiving vitamin C (0.4 mg) + vitamin E (0.04 IU/g body weight); and the rhodamine B group receiving vitamin C (0.8 mg) + vitamin E (0.04 IU/g body weight). They were housed in a clean wire cage and maintained under standard laboratory conditions (temperature $25 \pm 2^\circ C$ with dark/light cycle 12/12 hours). They were fed a standard pellet diet and received water ad libitum. The animals were acclimatized to laboratory conditions for 1 week prior to the experiment.

Tissue sampling

At the end of the treatment, the animals in all groups were anesthetized. The ovary and endometrium were collected, weighed, and later rinsed with physiological saline. All samples were stored at $–80^\circ C$ until analyzed.

Rhodamine B

Rhodamine B was dissolved with double-distilled water and administered orally using a probe. The duration of administration of rhodamine B in the treatment group refers to the previous study related to subchronic toxicity tests of rhodamine B administered for 36 days.

Vitamin C and vitamin E

Vitamin C was dissolved with aqua dest 0.5 mL, but vitamin E was dissolved with sesame oil 0.5 mL. All of these
substances were given orally by gavage to rats at 10:00 AM every day for 36 days.

**MDA analysis**

The Bioxytech MDA-586 spectrophotometric assay for MDA assay kit (Catalog No: 21044) was purchased from Oxis International, Inc. (Foster City, CA, USA). The analysis was done according to detailed procedures in the kit.

**SOD analysis**

The EnzyChrom SOD assay kit (Catalog No: ESOD100) was purchased from BioAssay Systems (Hayward, CA, USA). The analysis was done according to detailed procedures in the kit.

**Hematoxylin eosin staining**

The histopathological profile of endometrial tissues was analyzed using hematoxylin eosin staining according to the previous study.16

**Ethics**

This research was approved by the Research Ethics Committee, Faculty of Medicine, University of Brawijaya, Malang, Indonesia.

**Statistical analysis**

Data are presented as mean ± standard deviation (SD) and differences between groups were analyzed using the Student t test and one-way analysis of variance (ANOVA) with SPSS 15.0 statistical package (SPSS Inc., Chicago, IL, USA). The post hoc test was used if the ANOVA was significant. A p value < 0.05 was considered statistically significant.

**Results**

Table 1 presents the MDA levels in the ovarium from each experimental group. The level of MDA was significantly higher in the rhodamine B group compared to the untreated control group (p < 0.05). Only the two highest doses significantly prevented rhodamine B-induced increase in ovarian MDA level. There was no significant difference between the effects of these two highest doses.

Table 2 presents the SOD levels in the ovarium from each experimental group. The SOD levels were significantly lower in the rhodamine B group compared to the untreated group. These decreased levels of SOD in the rhodamine B group were significantly elevated by both second and third dose administration of combined vitamin C and vitamin E. Indeed, administration of the highest dose to the rhodamine B group increased SOD levels to those comparable to the untreated group.

Table 3 presents the endometrium histology levels in each experimental group. The stromal density was significantly higher in the rhodamine B group compared to the untreated control group (p > 0.05). The vascular number and gland density were significantly lower in the rhodamine B group compared to the untreated control group (p > 0.05), but the difference in epithelial thickness was insignificant. All doses significantly prevented rhodamine B-induced increase in stromal density and epithelial thickness level. In addition, all doses also significantly prevented rhodamine B-induced decrease in the vascular number and gland density. There was no significant difference between the effects of these doses.

**Discussion**

In the present study, we observed a significant increase in ovarian MDA levels in rats exposed to rhodamine B. Ovarian MDA is a decomposition product of peroxidized polyunsaturated fatty acids that acts as a marker for detection of reactive oxygen species reactivity toward lipid peroxidation.17,18 This altered lipid profile is accompanied with elevations in reactive oxygen species formation and reduction of antioxidant activities, including SOD and Glutathione peroxidase (GSH-Px).19 This study also found that the SOD level was significantly reduced in the rhodamine B group compared with the control group (p < 0.05). Our finding indicated that rhodamine B treatment increases superoxide radical formation. This free radical will spontaneously dismutate by SOD to form hydrogen peroxide. Subsequently, hydrogen peroxide is involved in the Fenton reaction to form hydroxyl radicals. Hydroxyl radicals are an initiator for lipid peroxidation in the ovarium. Our data are consistent with previous studies that rhodamine B can significantly increase ovarian MDA levels compared with levels in the control female rats (p < 0.05).11

Vitamin E, α-tocopherol, is the most important antioxidant in the lipid phase of cells. Vitamin E acts to protect cells against the effects of rhodamine B-induced free radicals, which are potentially damaging byproducts of the

| Table 1 | Level of ovarium malondialdehyde in each experimental group. |
|------------------------|------------------------|------------------------|------------------------|------------------------|
| **Level** | **Control** | **RB** | **RB + vitamin E (0.04 IU/g body weight)** | **RB + vitamin E (0.04 IU/g body weight)** |
| **MDA (μM)** | 0.20 ± 0.10 | 0.42 ± 0.08* | 0.35 ± 0.10 | 0.25 ± 0.15** |
| **Vitamin C (0.2 mg)** | **Vitamin C (0.4 mg)** | **Vitamin C (0.8 mg)** |

Note: values are presented as mean ± standard deviation.

* p < 0.05; in comparison with the control group.

** p < 0.05; in comparison with the RB group.

IU = international units; MDA = malondialdehyde; RB = rhodamine B.
In conclusion, the protective effect of combined vitamin C and vitamin E against ovarian and endometrial toxicity in rats receiving orally administered rhodamine B is due to inhibition of the lipid peroxidation and modulation of SOD levels, and the endometrium repairing effect.

**Conflicts of interest**

The authors declare that there are no conflicts of interest regarding the publication of this article.

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