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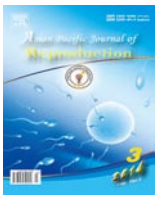

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Rhodamine B increases hypothalamic cell apoptosis and disrupts hormonal balance in rats

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ABSTRACT

Objective: To investigate whether orally exposure to rhodamine B could be changes the expression of Bax, Bcl-2 of the hypothalamic, and also levels of Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) in female rats. **Methods:** Twenty eight virgin female Wistar rats were divided into four groups, including control group, group exposed to dose of 4.5, 9 and 18 milligram/200 gram body weight (mg/200 g BW) of rhodamine B daily for 36 days. The hypothalamic expressions of Bax and Bcl-2 were examined immunohistochemically. The levels of serum FSH and LH were determined by the enzyme-linked immunosorbent assay (ELISA) technique. **Results:** The level of Bax was significantly higher in the rhodamine B treatment group compred to control group ($P<0.05$). Out of the 4.5, 9, and 18 mg/200 g BW doses of rhodamine B treatment, only the two highest doses significantly decreased the Bcl-2 levels compared to the control group ($P<0.05$). The serum FSH and LH levels were significantly lower in all dose´s rhodamine B treatment groups compared with the control ($P<0.05$). **Conclusion:** In conclusion, rhodamine B increases hypothalamic cell apoptosis and disrupts hormonal balance in rats.

1. Introduction

Synthetic colorations are generally used in numerous manufacturing industries such as paper printing, textile dyeing, cosmetics and pharmaceuticals. About 15%-20% of the total world production of dyes is lost during the coloring processes[1, 2]. Rhodamine B is widely used in industrial purposes and capable of cause irritation to the skin, eyes, gastrointestinal tract as well as respiratory tract [3]. It may cause carcinogenic and teratogenic effects[4]. Besides, exposure to toxic chemicals may be very harmful to the reproductive system, including the hypothalamic, pituitary, ovaries and reproductive tract. Disruption in one of these organs may ultimately manifest as disruption of steroid hormones, which can lead to ovarian failure. As a consequence, the destruction of the oocyte eventually disrupts endocrine balance, causing a decrease in estrogen and progesterone, and Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) elevations[5].

The process of cell death can occur in several ways, such as necrosis, apoptosis (programmed cell death type-1) and autophagic (programmed cell death type-2). When there is necrosis, a cell will have a cytoplasmic swelling, organelle structure disorganization, ruptured cell membrane and karyolysis in the cell nucleus[6]. On the contrary, different things will happen in cells undergoing apoptosis. Apoptotic pathway is induced by intracellular program regulation, where dead cells activate enzymes to degrade DNA in the nucleus and cytoplasm protein in the cell itself[7]. Apoptosis is regulated by various proteins, including the Bcl-2 (B-cell lymphoma-2) protein family. This family of proteins consists of both pro-apoptotic (*e.g.* Bax (Bcl-2 antagonist X), Bak, Bad, Bag and Bcl-xs) and anti-apoptotic (*e.g.* Bcl-2, Bcl-xL, Mcl-1) proteins. The ratio of Bcl-2/Bax heterodimeric to Bax/Bax homodimers determines whether or not a cell will undergo apoptosis, where excess Bax will promote cell death [8-9]. The anti-apoptotic Bcl-2 family of proteins (especially Bcl-2, Bcl-xL, Mcl-1) are frequently overexpressed in cancer cells, including solid tumors. Therefore, they prevent death

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in corresponding cells and increase resistance to traditional treatments[10].

The objective of the study is to investigate the effect of orally Rhodamine B on expression of Bax and Bcl-2 of the hypothalamus. In addition, in order to know the effects on hormone production, the levels of serum FSH and LH also determined.

2. Material and methods

2.1. Animal

Twenty eight virgin female, 8-10 weeks aged; 167-251 gram of Wistar rats were divided into four groups, including control group (without being exposed to Rhodamine B), exposed to Rhodamine B (4.5 mg/200 BW), exposed to Rhodamine B (nine mg/200 g BW), and exposed to Rhodamine B (18 mg/200 g BW). All experimental procedures were compliant with the Medical Faculty Brawijaya University Committee Guidelines on the Use of Live Animals in Research, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Rhodamine B treatment

Rhodamine B (Sigma, SigmaAldrich, Singapore) was dissolved with double distilled water (one mg/mL) and administered orally using gavage. We chose an oral route caused by illegally food coloring use. The duration of administration of rhodamine B in the treatment group according to the previous study which applied sub chronically toxicity tests of rhodamine B for 36 days[11].

2.3. Euthanasia

Cycle determination was started at the prior of group randomization. The cycle of each female rat was determined by observation of vaginal smears, which were taken using a plastic tip. Saline was placed on the vaginal opening, aspirated, and then placed on a microscopic slide. After the sample had dried, it was stained with hematoxylin-eosin. When the dye was removed, the slide was washed in de-ionized water and examined under a binocular microscope. The slide specimens were compared and matched according to Freeman[12]. Animals in the diestrus phase were used. The remainder of the animals continued to have their estrous cycles checked daily, being start euthanasia when in diestrus. After 36 days of Rhodamine-B treatment, euthanasia was performed in proestrus phase.

2.4. Analysis of Bax and BcL-2

The expressions of Bax and Bcl-2 were examined according to previous protocol[13]. Immunohistochemically was performed on the hypothalamus region. Hypothalamus regions consisting of supraoptic zone, tuberal, arcuatus periventricular, and paraventricular, which associated with FSH and LH secretion.

Brain tissue was excises, cleaned with ice-cold normal saline and were prepared for immunohistochemistry evaluation. Brain sections paraffin-coated slides (Surgipath Paraplast, Leica Microsystem, Europe) were prepared by Tissue Tex Processor. Twenty eight slides (seven slides per group) for Bax analysis. Analysis of Bcl-2 was also performed on twenty eight slides (seven slides per each group). After heating, brain sections were deparaffinized in xylol (Merck, USA) and rehydrated in a graded alcohol (Merck, USA). Sodium citrate buffer of concentration 10 mM was heated until boiling in a microwave for antigen retrieval. Immunohistochemistry staining was applied following the manual of the company (DakoCytomation, USA). Succinctly, 3% hydrogen peroxide in 0.5% methanol was used to block the endogenous peroxidase for 5 min followed by washing the tissue sections carefully using wash buffer and then incubated with Bcl-2-associated × protein (Bax) (1:100), and antiapoptotic protein Bcl-2 (1:100) (Santa Cruz Biotechnology Inc., California, USA) biotinylated primary antibodies for 120 minutes. After incubation, tissue sections were carefully washed with washing buffer and conserved in the buffer bath. After adding streptavidin-HRP, sections were kept for 40 minutes incubated and then washed. Diaminobenzidine substrate chromogen was applied to the sections and reincubated for over 20 min followed by careful washes and hematoxylin counterstaining for 5 seconds. Dot slide Olympus Camera ×C10 (OLYMPUS, Japan) was used to examine the immunostaining analysis. (Magnification ×400). All regio of the brain was stained but only hypothalamus will be analyzed. Sections were scored semiquantitativley as follows: (0) negative; (1) weakly positive; (2) moderately positive; (3) strongly positive ; and (4) very strongly positively were determined according to the immunodetection of stain intensity and amounts of positive cells by two pathologists.

2.5. Analysis of serum FSH and LH levels

Levels of FSH and LH were determined in the serum of rats in proestrus phase. The enzyme-linked immunosorbent assay (ELISA) method was applied. FSH in serum was measured immunoenzymetric using an ELISA method (Abnova Rat FSH Elisa Kit, Catalog Number 2535). LH in serum was measured immunoenzymetric using an ELISA method (USCN Rat FSH Elisa Kit, Wuhan, PRC, Catalog Number CEA441RA). All procedure was done according to kit instruction.

2.6. Ethics

This research has been approved by research ethics committee Faculty of Medicine University of Brawijaya, Malang, Indonesia.

2.7. Statistical analysis

Data are presented as mean ± SD and differences between groups were analyzed using 1-way ANOVA with SPSS 19.0 statistical package. Post Hoc test was used if the ANOVA was significant. $P<0.05$ was considered statistically significant.

3. Results

Table 1 showed the hypothalamic Bax and Bcl-2 levels in each group. The level of Bax was significantly higher in the rhodamine B treatment group compared to control group (P<0.05). Out of the 4.5, 9, and 18 mg/200 g BW doses of rhodamine B treatment, only the two highest doses significantly decreased the Bcl-2 levels compared to the control group (P<0.05). There was no significant difference of Bcl-2 levels between first dose compared to control group or between two higher doses (P>0.05).

The levels of serum FSH and LH levels in all groups are presented in the Table 2. The serum FSH and LH levels were significantly lower in all dose's rhodamine B treatment groups compared with the control (P<0.05).

Table 1
Level of Bax and Bcl-2 in hypothalamic of exposed groups and control rats.

Level (unit)	Rhodamine B treatment groups			
	Control	4.5 mg/200 g BW	9 mg/200 g BW	18 mg/200 g BW
Bax	1.86±1.22	5.00±0.82[a]	8.46±2.04[ab]	16.43±2.76[abc]
Bcl-2	6.71±1.38	5.00±1.83	3.86±2.27[a]	2.71±1.38[ab]

Note: values are presented as mean ± SD; [a]P<0.05 in comparison with control group; [b]P<0.05 in comparison with first dose treatment groups; [c]P<0.05 in comparison with second dose treatment groups.

Table 2
Level of serum FSH and LH in exposed groups and control rats.

Level (μg/mL)	Rhodamine B treatment groups			
	Control	4.5 mg/200 g BW	9 mg/200 g BW	18 mg/200 g BW
FSH	19.19 ± 1.45	17.11 ± 0.63[a]	15.75 ± 0.70[ab]	13.41 ± 0.69[abc]
LH	18.97 ± 0.55	16.49 ± 0.80[a]	15.05 ± 0.37ab	12.66 ± 1.63[abc]

Note: values are presented as mean ± SD; [a]P<0.05; in comparison with control group; [b]P<0.05; in comparison with first dose exposed groups; [c]P<0.05; in comparison with second dose exposed groups.

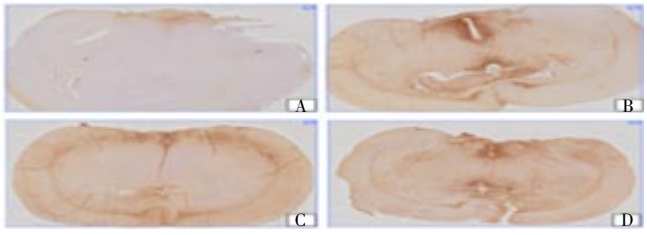

Figure 1. Immunohistochemical of hypothalamic Bax expression after Rhodamine B treatment.
Representative microscopic picture of brain stained imunnohistochemically with antibody against Bax at non treatment group (A), 4.5 mg/g 200 BW rhodamine B treatment group (B), 9 mg/g 200 BW rhodamine B treatment group (C), and 18 mg/g 200 BW rhodamine B treatment group (D). (Magnification ×40).

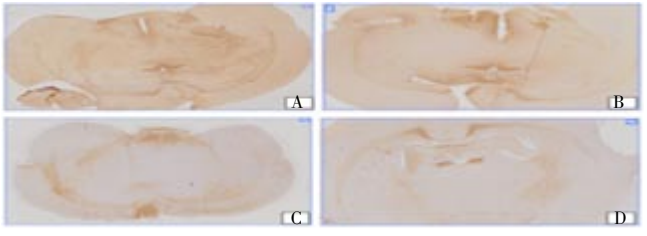

Figure 2. Immunohistochemical of hypothalamic Bcl-2 expression after Rhodamine B treatment.
Representative microscopic picture of brain stained imunnohistochemically with antibody against Bax at non treatment group (A), 4.5 mg/g 200 BW rhodamine B treatment group (B), 9 mg/g 200 BW rhodamine B treatment group (C), and 18 mg/g 200 BW rhodamine B treatment group (D). (Magnification ×40).

4. Discussion

In the present study, the pro-apoptotic marker (Bax expression) was significantly higher in the doses of rhodamine B-treatment group compared to the control group. This finding indicated that hypothalamus is sensitive to orally rhodamine B route. Rhodamine able to penetrate into the cells and accumulate in the mitochondria then disrupt the respiratory chain reactions[14]. Mitochondria plays important roles in cellular pathways; such as cellular energy metabolism, apoptosis regulation, cell redox signaling, as well as reactive oxygen species production[15-19]. The leak of the mitochondrial respiratory chain may increase reactive oxygen species level then turn the apoptosis pathway. The mitochondrial pathway has frequently been implicated in neuronal apoptosis, along with the pro-apoptotic Bax protein, a major component of this pathway[20]. Besides, compromised mitochondrial membrane integrity includes rearrangement of proapoptotic Bax and antiapoptotic Bcl-2 molecules in its membrane. Bax is a soluble protein present predominantly in the cytosol, whereby during the induction of apoptosis; it shifts to mitochondrial membranes. Bcl-2 is present in mitochondria and functions as a repressor of apoptosis[21-24]. In the present study, only the two highest doses significantly decreased the Bcl-2 levels compared to the control group. We speculated that rhodamine stimulates down regulated of Bcl-2 protein, which need further studied. In cancer cells, extracellular regulated kinase (ERKs) inhibitor downregulated the expression of Bcl-2[25].

Gonadotropin-releasing hormone (GnRH) neurons will increase luteinizing hormone secretion as the response of estradiol level[26]. Administration of rhodamine B in female rats significantly reduces FSH and LH levels compared to the control. Previous studies revealed that norepinephrine concentration at the hypothalamic level exhibits a circadian variation that correlates with circulating LH and FSH levels[27]. We hypothesized that norepinephrine may be affected by rhodamin B to decrease LH and FSH levels. Besides, rhodamine B may be affecting the basophilic cells of the adenohypophysis where FSH and LH are synthesized then turn a primary hyposecretion of FSH and LH.

In conclusion, rhodamine B increases hypothalamic cell apoptosis and disrupts hormonal balance in rats. Therefore, the illegal use of rhodamine B for food coloring need more awareness.

Conflict of interest statement

The author(s) declare(s) that there are no conflict of interests regarding the publication of this article.

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