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Rhodamine B Triggers Ovarian Toxicity Through Oxidative Stress

Rodamin B, Oksidatif Stress Aracılığı ile Ovarian Toksisitesi

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ABSTRACT

Purpose: The purpose of this study was to analyze the effects of exposure to rhodamine B on ovarian oxidative stress, ovarian follicles, hormone 17β-estradiol and thickness of endometrium.

Materials and Methods: A total of 28 female rats were divided into four groups consisting of control; groups treated with rhodamine B at doses of 4.5; 9, and 18 milligram/200 gram body weight. Rhodamine B was administered orally for 36 days with the probe. Analysis of MDA level was done spectrophotometrically. Analysis of the number of ovarian follicles and thickness of endometrium was done histopathologically by hematoxylin eosin staining. Analysis of 17β-estradiol level was done by ELISA.

Results: Rhodamine B administered in different doses in female rats can increase ovarian MDA levels significantly than the control ($P < 0.05$). Administration of rhodamine B of the second and third doses in female rats can reduce the number of primary, secondary, and De Graaf follicles significantly compared to the control ($P < 0.05$). Administration of rhodamine B of the second and third doses in female rats can reduce 17β-estradiol level significantly compared to the control ($P < 0.05$). The administration of rhodamine B could reduce thickness of endometrium significantly compared to the control ($P < 0.05$).

Conclusion: It was concluded that administration of rhodamine B triggered ovarian toxicity through oxidative stress, a decrease in the number of follicles, and decreased level of 17β-estradiol which ultimately lowered the thickness of endometrium.

Key Words: Dye, toxicity, ovarium, endometrium, oxidative stress.

ÖZET

Amaç: Çalışmanın amacı, rodamin B'ye maruziyetin, ovarian oksidatif stress, ovarian folikül, 17β-Estradiol hormonu ve endomerium kalınlığı üzerindeki etkisini araştırmak.

Materyal Metod: Toplam 28 dişi sıçan kontrol grubu ile birlikte 4 gruba ayrılmıştır. Gruplar 4.5, 9 ve 18 miligram/200 gram vücut ağırlığı olacak şekilde rodamin B ile muamele edilmişir.Rodamin B, 36 gün boyunca probla birlikte oral olarak uygulanmıştır. MDA seviyelerinin analizi spektrofotometrik olarak yapılmıştır. Ovarian folikül sayısı ve endometrium kalınlığı analizleri histopatolojik olarak hematoksilin eozin boyama ile yapılmıştır. 17β-Estradiol seviyesi ELISA ile ölçülmüştür.

Bulgular: Dişi sıçanlara farklı dozlarda Rodamin B uygulanması, ovarian MDA seviyelerini kontrollere gore anlamlı şekilde arttırmıştır (P < 0.05). Dişi sıçanlarda rodamin B'nin 2. ve 3. dozları primer, sekonder ve De Graaf folikül sayısını kontrollerle karşılaştırıldığında anlamlı olarak azaltmıştır (P < 0.05). Dişi sıçanlarda rodamin B'nin 2. ve 3. dozlarının uygulanması 17β-estradiol seviyesini kontrollerle karşılaştırıldığında anlamlı olarak azaltmıştır (P < 0.05). Rodamin B uygulanması endometrium kalınlığını kontrollerle karşılaştırıldığında anlamlı olarak azaltmıştır (P < 0.05).

Sonuç: Rodamin B uygulamasının, oksidatif stress aracılığı ile ovarian toksisitesini tetiklediği, folikül sayısını azalttığı ve, 17β-Estradiol seviyesini azaltalarak endometriyum kalınlığını düşürdüğü sonucuna varılmıştır.

Anahtar Kelimeler: Boya, Toksisite, ovarian, endometriyum, oksidatif stres

INTRODUCTION

The development of methods of storage, fabrication, and food processing in the small or large scale industries leads to increased use of food dyes[1,2]. Rhodamine B is a synthetic dye in the form of green or reddish purple, odorless and bright red crystal powders. This substance fluoresces with a molecular weight of 479.02 g/mol. Besides used as a food dye, it is also used as paper dye, textile dye; histological, biotechnological and color cosmetic applications[3].

Rhodamine is a compound that can be catalyzed by light to form singlet oxygen ($^1O_2$). There are two types of such reactions. The energy of the compound will be enhanced by light and transferred to the biomolecules and may trigger the formation of free radicals or transfer of energy to the oxygen to form singlet oxygen. This reaction is influenced by the presence of the halogen group and the strength of the light[4-6]. Furthermore, various types of reactive oxygen compounds will trigger many other processes. Reactive oxygen compound in reproductive system in normal level has a role in regulating physiological process of oocyte maturation; however, in excessive level it can augment oxidative stress that may damage the molecules and the structures of the oocyte and granulose cells in the follicles[7].

An imbalance between prooxidant and antioxidants can cause some of reproductive diseases such as endometriosis, polycystic ovarian syndrome, and infertility[8]. Infertility can be caused by the capacity of the ovary. The capacity may include the quantity and quality of oocytes. Several factors that may serve as predictors include age, ovarian volume, number of antral follicles and hormonal markers such as estradiol. A woman with a reduced capacity of ovaries has a high rate of stimulation failure and a high rate of pregnancy failure[9]. Based on the above theory, no study has evaluated the effect of rhodamine B on the female reproductive system. Therefore, the purpose of this study was to analyze the effects of exposure to rhodamine B on ovarian oxidative stress, ovarian follicles, hormonal 17 β-estradiol and thickness of endometrium.

MATERIAL and METHODS

Animal

Twenty eight adult female Wistar rats weighing 150-200 g were obtained from the Laboratory Pharmacology Brawijaya University. The rats were kept in a room with a 12-h light/dark cycle at 22^{o}C and were provided access to food and water ad libitum. Female rats were randomly divided into the following four groups: the control group, the rhodamine B administration group at dose 4.5 mg/200 gBW; 9 mg/ 200 gBW; and 18 mg/ 200 gBW. 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.

Administration of Rhodamine B

Rhodamine B was dissolved with double distilled water and administered orally using 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[10].

Malondialdehyde Level

Malondialdehyde level in ovary was measured thiobarbituric acid method using TBARS Assay Kit (R&D system, Catalog Series KGE013, USA).

The Number of Follicles

The numbers of primary, secondary, tertiary and de Graaf follicles were calculated from the right ovary cut transversely and then preparation was made and stained histologically with HE and the follicles were calculated using Dotslide Olympus Camera XC 10. The entire cross-section was analyzed and further identified with magnification of 100 X.

Cycle Determination

Cycle determination was started at the end of the rhodmain-B treatment. 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[11]. Animals in the diestrus phase were used. The remainder of the animals continued to have their estrous cycles checked daily, being sacrificed always when in diestrus.

Dissection

Dissection was performed on week 8 of the treatment. Before the rats were killed, the vaginal swap was done to determine the estrous cycle in rats; rats that were on proestrus phase would be killed and if rats were not the proestrus phase, wait until the proestrus phase.

Measurement of Estradiol

Estradiol in serum was measured immunoenzymatically using an ELISA method (Cusabio, Catalog Series CSB-E05110r, China).

The Thickness of the Endometrium

The thickness of the endometrium was determined by calculating the average thickness of the endometrium with the highest and lowest sizes at each incision (totally 10 locations) for sample using Dot Slide camera.

Ethics

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

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.

RESULTS

Table 1 presents the ovarian MDA levels in the control group and exposure of rhodamine B to female rats. Rhodamine B administered in different doses in female rats can increase ovarian MDA levels significantly than the control ($P < 0.05$). There was no significant difference in the administration of the second dose of rhodamine B compared to the third dose ($P > 0.05$).

Administration of rhodamine B of the second and third doses in female rats can reduce the number of primary, secondary, and De Graaf follicles significantly compared to the control ($P < 0.05$). For primary follicles, there were no significant differences among the three doses of rhodamine B ($P> 0.05$) as shown in Table 2.

17β-estradiol levels in the control group and the groups treated with various doses of rhodamine B are showed in Table 3. Administration of rhodamine B of the second and third doses in female rats can reduce 17β-estradiol level significantly compared to the control ($P < 0.05$).

The administration of rhodamine B could reduce thickness of endometrium significantly compared to the control (P < 0.05). The first and second doses did not generate significant difference in the thickness of endometrium (P> 0.05) as seen in Table 4.

Table 1. Level of malondialdehyde in rhodamin B-administered groups and control rats

		Rhodamine B-administered groups		
Level	Control	4.5 mg/200 g BW	9 mg/200 g BW	18 mg/200 g BW
MDA (mg/mL)	2.21 ± 0.03	2.36 ± 0.05[a]	2.46 ± 0.08[a]	2.61 ± 0.23[abc]

Note: values are presented as mean ± SD; ap<0.05; in comparison with control group; bp<0.05; in comparison with first dose administered groups; cp<0.05; in comparison with second dose administered groups; mg/200 g BW: miligram/200 gram body weight.

Table 2. Number of follicle in rhodamin B-administered groups and control rats

		Rhodamine B-administered groups		
Follicle Number	Control	4.5 mg/200 g BW	9 mg/200 g BW	18 mg/200 g BW
De Graaf	4.00 ± 1.15	3.00 ± 1.15	1.71 ± 1.11[ab]	1.57 ± 0.98[ab]
Primary	3.86 ± 1.21	2.86 ± 1.07	2.29 ± 1.11[a]	1.57 ± 1.13[a]
Secondary	5.14 ± 1.35	5.00 ± 1.41	3.00 ± 0.82[ab]	1.29 ± 0.95[abc]

Note: values are presented as mean ± SD; ap<0.05; in comparison with control group; bp<0.05; in comparison with first dose administered groups; cp<0.05; in comparison with second dose administered groups; mg/200 g BW: miligram/200 gram body weight.

Table 3. Level of 17β-estradiol in rhodamin B-administered groups and control rats

		Rhodamine B-administered groups		
Level	Control	4.5 mg/200 g BW	9 mg/200 g BW	18 mg/200 g BW
17β-estradiol (ng/mL)	19.25 ± 1.21	17.94 ± 1.56	17.14 ± 0.87a	16.73 ± 1.04a

Note: values are presented as mean ± SD; ap<0.05; in comparison with control group; bp<0.05; in comparison with first dose administered groups; cp<0.05; in comparison with second dose administered groups; mg/200 g BW: miligram/200 gram body weight.

Table 4. Thickness of endometrial in in rhodamin B-administered groups and control rats

		Rhodamine B-administered groups		
Level	Control	4.5 mg/200 g BW	9 mg/200 g BW	18 mg/200 g BW
Thickness (μm)	951.82 ± 227.37	610.53 ± 137.88[a]	509.35 ± 115.89[a]	307.33 ± 123.12[abc]

Note: values are presented as mean ± SD; ap<0.05; in comparison with control group; bp<0.05; in comparison with first dose administered groups; cp<0.05; in comparison with second dose administered groups; mg/200 g BW: miligram/200 gram body weight.

DISCUSSION

In this research, the administration of rhodamine B in various doses in female rats can increase ovarian MDA levels significantly than the control (P < 0.05). There was no significant difference in the administration of the second dose of rhodamine B compared to the third dose (P > 0.05). This indicates that the metabolism of

rhodamine B triggers an imbalance between the production of reactive oxygen compounds and endogenous antioxidant defenses. Rhodamine B able to induces cytochrome P450 system and lower the antioxidant enzymes in the liver[12]. In addition to the derivative of organochlorines, rhodamine B also contains a quinone structure[13]. Quinones are electron and proton carriers playing a major role in aerobic metabolism of every cell and performing redox reactions in mitochondria, golgi apparatus, plasma membrane and endoplasmic reticulum. Quinones also enter the redox cycle with semiquinone radicals to form reactive oxygen compounds[14,15]. In addition, rhodamine can also penetrate into the cells and accumulate in the mitochondria to disrupt the respiratory chain[16].

Folliculogenesis occurs in the ovarian cortex with two preantral and antral phases. Preantral phase is characterized by the growth and differentiation of the oocyte. Antral phase is characterized by a rapid increase of the size of the follicles, up to approximately 25 mm. Folliculogenesis in the ovary is affected by xenobiotics. Xenobiotics has toxicological effects which may cause loss of oogonia, oocytes, somatic cells and even a significant decline in the number of follicles[17]. Administration of rhodamine B of the second and third doses in female rats can reduce the number of primary, secondary, and De Graaf follicles significantly compared to the control ($P < 0.05$). This proves that the rhodamine B can inhibit folliculogenesis and result in decreased number of follicles. Rhodamine B may be induces apoptosis of follicle. Rhodamine able to penetrate into the cells and accumulate in the mitochondria to disrupt the respiratory chain then increase reactive oxygen species level and apoptosis pathway[16].

Ideally, the follicle will serve to provide a microenvironment that is necessary for growth and maturation of oocytes and produces steroid hormone[18]. Steroid hormone such as estradiol is very essential for reproduction. This hormone is synthesized from androgens by aromatase, an enzyme found in the endoplasmic reticulum of granulose cells. This hormone will work on specific receptors that are expressed in granulose cells of preantral and antral follicles[19-22]. In this research, administration of rhodamine B of the second and third doses in female rats can reduce 17β-estradiol level significantly compared to the control ($P < 0.05$). This indicates that the rhodamine B triggers a decrease in the levels of 17β-estradiol due to a decrease in the number of follicles or may be caused by impaired synthesis of 17β-estradiol.

In addition to the above function, estradiol may scavenge reactive oxygen compounds through hydrogen donor from phenol-hydroxyl ring. Estradiol may inhibit lipid peroxidation in plasma and liver tissues[23,24]. The current study showed that a decrease in 17 β-estradiol level as a result of administration of rhodamine B was also accompanied by increased levels of MDA. Cellular oxidative damage due to a decrease in estradiol causes apoptosis and follicular atresia[25,26]. Lipid peroxidation occurs in the plasma membrane of luteal cells and may be associated with loss of gonadotropin receptors and decreased cAMP formation thereby reducing steroidogenic ability of the corpus luteum during involution[27].

During the reproductive period, endometrium is dynamic and will experience cycles of proliferation, differentiation, and decay. In premenopausal women, endometrium is proliferative or secretory, depending on the phase of the menstrual cycle[28]. The administration of rhodamine B in different doses could reduce thickness of endometrium significantly compared to the control ($P < 0.05$). The first and second doses did not generate significant difference in the thickness of endometrium ($P > 0.05$). This is caused by decreased levels of 17 β-estradiol due to a decrease in the number of follicles as consequence of the ovarian oxidative stress.

It was concluded that administration of rhodamine B triggered ovarian toxicity through oxidative stress, a decrease in the number of follicles, and decreased level of 17 β-estradiol which ultimately lowered the thickness of endometrium.

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DECLARATION of inTEREST

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

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