Purple Roselle Fortified Yogurt Supplementation Prevents Dioxin-Induced Oxidative Testicular Impairment in Rats

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Abstract

Dioxins are highly toxic and hurt multiple organs and systems, including the reproductive organs. This study determined the supplementation of purple roselle (*Hibiscus sabdariffa* var. altissima) extract into yogurt to prevent testicular deterioration induced by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The fortification of purple roselle extract into yogurt (RY) was used as a supplement because bioactive components are known to be efficacious in preventing cell damage. Five groups of male Wistar rats were group as follows: 1) negative control (C), 2) TCDD (T), 3) TCDD and 0.5% RY (P_1), 4) TCDD and 1% RY (P_2), 5) TCDD and 1.5% RY (P_3). All treatments were carried out orally for 12 days. Testicular Malondialdehyde (MDA) level and histopathological changes were described quantitatively by one-way ANOVA. The study's results showed that treatment groups P_1 (0.5% RY) and P_2 (1% RY) had significantly ($P<0,05$) lower MDA levels than the T group, while group P_3 had no significant decrease in MDA level. It proved that supplementation of purple roselle yogurt reduced the formation of MDA in testicular tissue. Histopathology changes show that all groups exposed to TCDD have depleted spermatogenic, Sertoli, and Leydig cells compared to the C group. Suggests that dioxin exposure can have adverse effects on male reproductive organs. This research concludes that supplementing yogurt fortified with purple roselle extract can prevent increased testicular MDA levels. The decrease in testicular cells due to TCDD toxicity was inevitable. However, antioxidant activity from roselle yogurt supplementation can prevent further damage in rat testicular cells.

Keywords: Histopathology, MDA, Purple Roselle, Testis, TCDD, Yogurt.

INTRODUCTION

Unintentionally, combustion processes and manufacturing operations release environmental pollutants that are dangerous to human health. Chlorinated organic chemicals known as dioxins and their congeners have detrimental effects, especially 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). This persistent organic substance is capable of accumulating in the food chain. Exposure to dioxins in humans and animals can be through the environment, food, drinking water, and air pollution [1]. The TCDD compound is a toxic anthropogenic substance and, in low concentrations, can be dangerous to the health of living creatures. Toxicity caused by TCDD can affect the male reproductive system. This compound causes a reduction in testicular size, atrophy, decreased sperm count, increased abnormal sperm count, impaired spermatogenesis, and increased malondialdehyde (MDA) levels [2,3].

Dioxin compounds activate the aryl hydrocarbon receptor (AhR), followed by membrane and organelle damage. In addition, TCDD induces oxidative stress, forming reactive oxygen species (ROS), which causes cell proliferation, impaired growth, and apoptosis [4]. The TCDD compound that enters the body has a different half-life depending on the species, organ, and dose administered. The half-life in rats is estimated to be 12 to 31 days [5]. Cell damage due to oxidative stress causes the natural antioxidant system in the body to work harder, so it is necessary to increase the intake of exogenous antioxidants to prevent damage to the body's cellular components [6]. Yogurt contains bioactive components generated during fermentation that act as antioxidants by reaction with free radicals [7].

The addition of natural ingredients in yogurt is intended to increase antioxidant activity. Roselle flower (*Hibiscus sabdariffa* L) contains potential phytochemical compounds, including phenols, alkaloids, tannins, flavonoids, saponins, organic acids, and anthocyanins that have activity

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as antioxidants [8]. In previous studies, fortification of purple roselle extract into yogurt had preventive action against cardiotoxicity and renal toxicity and has gastroprotective activity [9–11]. This research explored the potential combination of plant ingredients, purple roselle, and dairy products such as yogurt to prevent testicular cell damage caused by TCDD exposure.

MATERIAL AND METHOD

Materials

Yogurt starter (Yógourmet, LYO-SAN. INC 500 Aéroparc, C. P. 598, Lachute, QC. Canada, J8H 4G4) containing the bacteria *L. bulgaricus*, *S. thermophilus*, and *L. acidophilus*, TCDD (2,3,7,8-TCDD Sigma 48599). Purple roselle flowers with plant species determination report from herbal laboratory Materia Medica, Batu, Indonesia, identified as *Hibiscus sabdariffa* var. altissima. Corn oil was use as solvent due to lipophylic characteristic of TCDD.

Purple roselle flower extraction

Dried roselle calyxes were pulverized into flour and sieved through a 60-mesh colander. The flour was dissolved using a 20 g per 100 mL water ratio. The solvent had been pasteurized at 63-65°C for 30 minutes. After 30 minutes, the liquid and sediment were separated [12]. The liquid was separated into another bottle for the following process.

Yogurt fortified with purple roselle extract

A total of 100 mL raw cow milk was pasteurized for 15 seconds at 72°C, allowed to reach 45°C, and inoculated with a 3% yogurt starter. The mix was incubated for 2-3 hours at 45°C until the consistency thickened with pH 4.5-5. 100 mL of the yogurt was homogenized using a blender, followed by adding 0.5%, 1.0%, and 1.5% purple roselle extract, and blended to homogenize the mixture [13].

Animals and study design

The Institutional Animal Care and Use and the Research Ethics Committee of Brawijaya University approved this experimental study (doc. number 1123-KEP-UB). White rats (*Rattus norvegicus*) Wistar strain, male, 6-8 weeks old, weighing 150-200 g, obtained from the Animal Laboratory of Biosains, Universitas Brawijaya, Malang. A total of 25 rats were divided into five groups. The experimental animals underwent acclimatization for approximately seven days. Food and water were provided ad libitum during the study. The control group (C) was only given corn oil; four groups (T, P_1, P_2, and P_3) were given TCDD 200 $ng.kg^{-1}$ body weight. Pure TCDD dissolved in corn oil. Oral administration of 1 mL each was carried out using a gastric probe daily at 10 am. Roselle yogurt (RY) treatment was given to group P_1 (0.5% RY), P_2 (1% RY), and P_3 (1.5% RY) at 1 pm, 1 mL for 12 days. The animals were then euthanized for tissue collection.

Measurement of Testicular Malondialdehyde Levels

Malondialdehyde level was measured using the thiobarbituric acid method [14]. A total of 0.1 grams of testicular tissue was crushed and added with 1 mL of distilled water. The homogenization process was continued by centrifuging at 1000 rpm for 10 minutes. A spectrophotometer set to 532 nm was then used to measure the absorbance of the resultant supernatant.

Histopathology Analysis

Organs were collected and placed in a 10% formaldehyde solution as a fixative. The organ had been cut to size 1 x 1 x 1 cm and put in a tissue cassette. Next, the dehydration process was done by immersing it in a series of alcohols starting from 70%, 80%, 90%, absolute alcohol I, and absolute alcohol II for two hours at each concentration. Then, xylene is used to clear the tissue to remove alcohol. The following process was embedded in paraffin and then blocked, so the tissue was printed in a paraffin block and stored in a cooler. Paraffin blocks were cut with a microtome to a thickness of 4-5 microns. The cutting results were placed on a glass object for hematoxylin-eosin (HE) staining.

The histopathology parameters observed include calculating the number of cells in the spermatogenesis stages (spermatogonia, spermatocytes, and spermatids), Sertoli cells, and Leydig cells. Counting spermatogenic cells was done by taking five seminiferous tubules and counting them using Image Raster software (ImageJ).

Data Analysis

Changes in MDA levels and histopathological features were analyzed using a one-way analysis of variance method with a confidence level of 95%. Then, it followed up with the honestly significant difference test.

RESULT AND DISCUSSION

Based on the results of the testicular MDA levels in white rats (Table 1), all treatment groups given purple roselle yogurt showed decreased testicular MDA levels. Statistical analysis showed that there were significant variations ($p<0.05$)

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between treatment groups P_1 and P_2 compared to the positive control group (T). Meanwhile, group P_3 was not significantly different from group T. These results mean that preventive yogurt supplementation fortified with purple roselle extract (RY) can prevent increased MDA levels in the testicular organs of rats exposed to TCDD. Based on the concentration of purple roselle extract, the P_2 group, which was given 1% purple roselle extract in yogurt, has the lowest MDA levels.

This study showed that administering purple roselle extract yogurt had a role in preventing the formation of malondialdehyde testicular due to dioxin intoxication. The TCDD exposure increases ROS generation and lipid peroxidation. It decreases the activity of cellular antioxidant enzymes, creating an imbalance in the prooxidant and antioxidant system and leading to oxidative stress [2]. Consequently, the concurrent rise in ROS production in the TCDD-treated group may have contributed to the increase in testicular MDA in the current study.

Table 1. Average testicular MDA levels of white rats in various treatments

Treatment groups	MDA level ($ng.mL^{-1}$) average ± SD
C (corn oil)	274.66 ± 37.712^{ab}
T (TCDD 200 $ng.kg^{-1}$ BW)	508.88 ± 137.379^{b}
P_1 (TCDD and 0.5% RY)	244.11 ± 55.284^{a}
P_2 (TCDD and 1% RY)	210.22 ± 28.284^{a}
P_3 (TCDD and 1.5% RY)	317.99 ± 92.629^{ab}

Note: superscript notations mean significant differences ($p<0.05$). TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin, BW= Body Weight, and RY= Roselle Yogurt.

This difference in the MDA level in RY supplementation groups proved that yogurt and roselle extract had antioxidant effects. Antioxidant-rich food can reduce the harmful effects of oxidative stress. Bioactive peptides derived from milk protein α-lactalbumin, β-lactoglobulin, and α-casein have antioxidant activity by scavenging free radicals, preventing liposome oxidation, and inhibiting lipid oxidation [15]. Previous studies indicated that adding purple extract has increased antioxidant activity IC_{50}, consistent with yogurt's added concentration [11]. Adding purple roselle extract, which contains bioactive compounds such as anthocyanins and tocopherols, provides a synergistic effect in increasing antioxidant activity and preventing the excessive production of free radicals, thus preventing further damage to the cells [16].

The milk fermentation process by lactic acid bacteria hydrolyzed milk proteins, carbohydrates, and lipids into secondary forms. These molecules, such as free amino acids and peptides, have antioxidant activity to capture free radicals [17]. The addition of purple roselle extract plays an essential role in yogurt production. As shown by De Moura *et al.* [18], the natural compositions of roselle that are relevant for pharmacological use are organic acids, anthocyanins, polysaccharides, and flavonoids. Therefore, these antioxidant compounds from purple roselle can be a functional additive for yogurt.

In the histopathological findings of the testicle, the T group (Fig. 2B) that was given TCDD showed vacuolization in testicular cells, which results in degenerative changes that decrease the number of germ cells. In the basal lamina, the release of germ cells was seen due to the adverse effect of TCDD in the cells of seminiferous tubules. This situation showed that TCDD affects the development of spermatogenic cells from the early to late stages. Dioxins and other environmental pollutants are known to cause reproductive toxicity by upsetting the balance between prooxidants and antioxidants, which may lead to oxidative stress [2].

Table 2. shows the observed testicular cells from all groups. The mean number of spermatogenic, Sertoli, and Leydig cells in the positive control group (T) decreased due to exposure to TCDD compared to the negative control group (C). The dioxin compound may cause testicular morphological lesions. It was demonstrated that seminiferous tubules, which only contain Sertoli cells and very few germ cells, were severely damaged and necrosed. Therefore, it implies that aberrant spermatogenesis was brought on by the toxicity of TCDD in the testicles.

Treatment groups P_1, P_2, and P_3 were not significantly different in the number of testicular cells from the positive control group. This result suggests that exposure to TCDD in the testicles caused abnormal spermatogenesis [2]. The TCDD interferes with endocrine and reprotoxic activities by binding to Ahr and blocking the ARNT complex, disrupting steroidogenesis. TCDD also inhibits Sertoli cell proliferation. The hypothalamic-pituitary-thyroid (HPT) axis is also disturbed due to exposure to TCDD, which causes a decrease in spermatogenesis, Leydig cell number, and testosterone [19].

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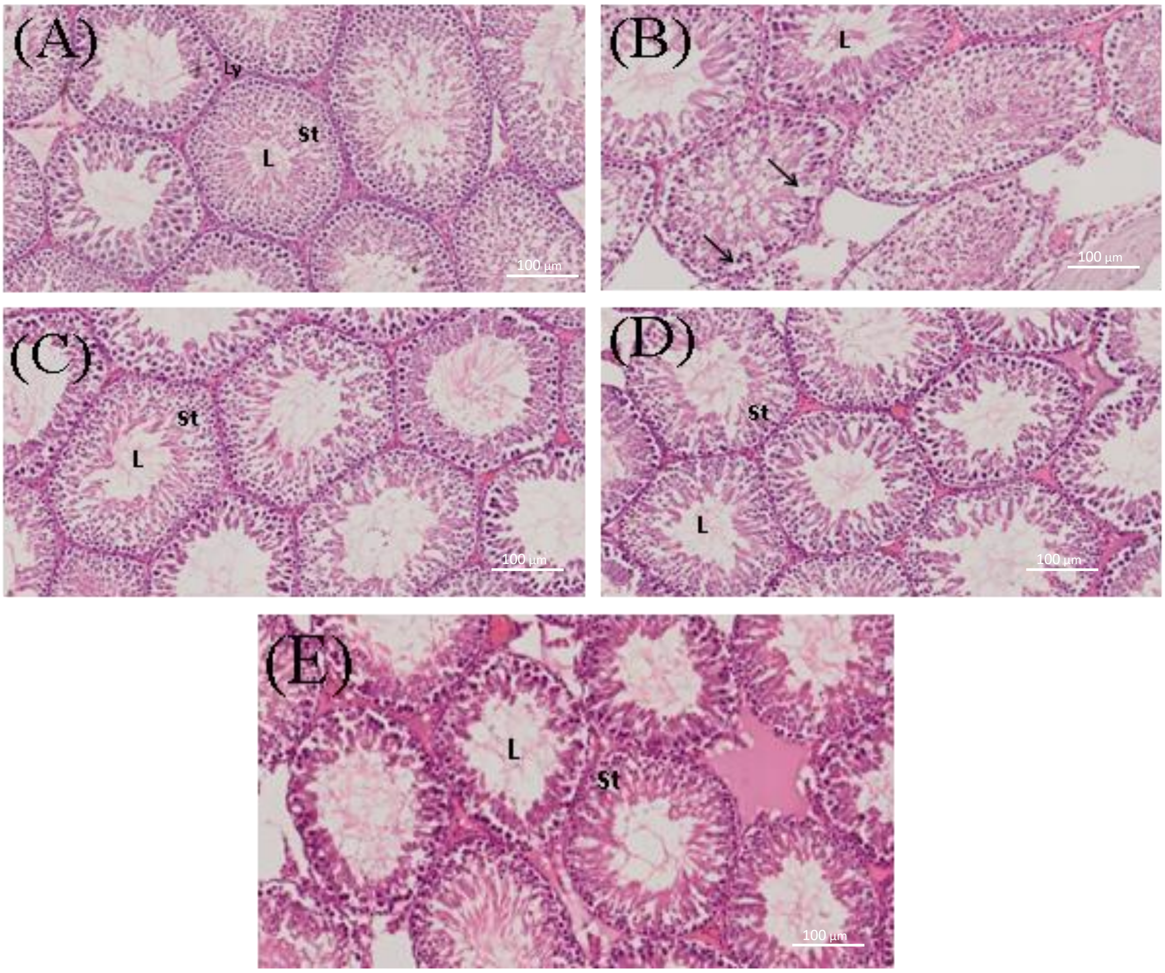

Figure 2. Histopathology of rat testicular section. Hematoxylin and eosin (H&E) stained, Magnification 100X; (A) C group – normal control rat showed normal histological structure of seminiferous tubule with normal spermatological cells, (B) T group (TCDD) - Seminiferous tubules possess irregular basal lamina and are separated from each other, wide gaps between neighboring cells and enlargement of the intercellular spaces. Interstitial tissues showed vacuolation, In treatment groups TCDD+RY (C) P_1 group, (D) P_2 group, (E) P_3 group, the normal tubules were seen. Abbreviation: seminiferous tubules (St), Leydig cells (Ly), lumen (L), vacuole (→).

Exposure to TCDD causes histopathological changes in the seminiferous epithelium, which causes a decrease in Sertoli cells and germ cells, thereby disrupting the stages of spermatogenesis. Histopathological changes in testes exposed to TCDD are characterized by changes in the seminiferous tubules' shape, spermatogonia's degradation, and a decrease in the number of spermatids [19,20]. Increased testicular sensitivity due to TCDD exposure via CYP1A1-induced oxidative stress can exacerbate inflammation and apoptosis, decreasing the number of Sertoli cells, spermatocytes, and spermatids. Additionally, TCDD stimulates Leydig cell apoptosis via Ahr activation [21,22].

The TCDD compound affects Sertoli cell junctions and apoptosis, disrupting the blood testicular barrier (BTB) structure and function, resulting in male reproductive dysfunction. Toxic compounds can cross BTB Sertoli cells by downregulating efflux drug transporters. Downregulation of some drug transporters can trigger the entry of xenobiotics and undesirable compounds into the testes through the BTB [23].

The decrease in the average spermatogenic, Sertoli, and Leydig cells is possible because antioxidant activity was not optimal in stabilizing free radicals. Antioxidants react with free radicals through different mechanisms such as hydrogen atom transfer, single electron transfer mechanisms, or a combination [24]. Supplementation of yogurt with purple roselle extract acted as an exogenous antioxidant to prevent the decline of spermatogenic cells caused by exposure to TCDD.

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Table 2. The average number of spermatogenic, Sertoli, and Leydig cells in each treatment

Treatment groups	Observed testicular cells				
	Spermatogonia	Spermatocyt	Spermatid	Sertoli	Leydig
C (corn oil)	64.28 ± 6.12^{b}	132.72 ± 36.43^{b}	115.52 ± 20.90^{a}	26.84 ± 2.35^{b}	22,56±4,16^{b}
T (TCDD 200 ng.kg^{-1} BW)	47.24 ± 7.90^{a}	56.64 ± 12.49^{a}	79.68 ± 21.95^{a}	14.44 ± 4.10^{a}	13,56±3,35^{ab}
P_1 (TCDD and RY 0.5%)	46.50 ± 7.10^{a}	102.95 ± 24.31^{ab}	113.50 ± 113.50^{a}	15.35 ± 3.60^{a}	15,60±5,35^{ab}
P_2 (TCDD dan RY 1%)	47.80 ± 9.61^{ab}	169.40 ± 42.14^{b}	125.00 ± 17,53^{a}	16.30 ± 0.14^{a}	13,80±1,13^{ab}
P_3 (TCDD dan RY 1.5%)	47.28 ± 8.19^{ab}	114.96 ± 36.23^{ab}	83.92 ± 19.40^{a}	12.48 ± 1.96^{a}	11,08±3,73^{a}

Note: superscript notations mean significant differences ($p<0.05$). TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin, BW = Body Weight, and RY= Roselle Yogurt.

Yogurt contains bioactive peptides formed from the hydrolysis of milk proteins during fermentation caused by the proteolytic activity of bacteria. Peptides bind to specific receptors on target cells and induce biological responses such as increasing antioxidant activity. Lactic acid bacteria can increase the structural profile of seminiferous tubules and spermatogenesis. Another positive effect of probiotic microbes is increasing testosterone levels and Leydig cells [25,26]. Purple roselle has anthocyanin compounds that donate hydrogen or electrons to free radicals and delocalize them in their aromatic structure. In addition, anthocyanins are responsible for increasing the growth of lactic acid bacteria so that antioxidant activity increases [8,27].

CONCLUSION

This research concludes that supplementing purple roselle-fortified yogurt can prevent increased testicular MDA levels in rats exposed to TCDD. Fortification at 0.5% and 1% RY concentrations significantly lowered testicular MDA levels. The calculation of the average spermatogenic cells, Sertoli cells, and Leydig cells demonstrated that TCDD toxicity would cause a decrease in testicular cells. However, antioxidant activity from roselle yogurt supplementation can stop additional damage to rat testicular cells.

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