Antioxidant activity of casein yogurt against dioxin toxicity in rats liver

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Abstract. Prolonged exposure to dioxin can lead to negative effects. Oxidative stress is caused by dioxin toxicity due to interference of the metabolism process including lipid metabolism. This current study was to address the role of casein yogurt in the prevention of oxidative stress caused by dioxin intoxication. Substances used were 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) diluted in corn oil and casein from goat milk yogurt. A total of 24 Wistar rats divided into 6 groups: control, placebo with 600 mg/kg BW of casein, TCDD group with 100 ng/kg BW of TCDD, T1 to T3 groups were given with TCDD and casein in different dose: 300, 600, and 900 mg/kg BW. The respective dose was given orally daily for 21 days. Blood serum was collected for cholesterol and triglyceride analysis. Liver samples were collected for superoxide dismutase (SOD) activity assay. Statistic analysis using one-way ANOVA and post hoc Tukey test. Results showed that casein yogurt increased SOD activity in TCDD intoxicated rats. A significant increase (P<0.05) was shown by the group with 600 and 900 mg/kg BW of casein. Serum lipid profile exhibited no significant change in serum cholesterol and triglyceride level. Casein yogurt is potential to prevent cellular damage caused by dioxin toxicity.

1. Introduction

Dioxin compounds are undegradable in the environment. This persistent contaminant can cause an extensive toxic effect on human health. Dioxins are generated unintentionally from the incineration process of chemical wastes containing with chorine, community waste, metal industry, combustion of leaded gas, bleaching of paper products, and from a synthesis of herbicides and pesticides [1,2]. The substance 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the most toxic congeners of dioxins [3]. Some negative effects of TCDD exposure such as wasting syndrome, chloracne and skin damage, immunotoxicity, hepatotoxicity, carcinogenicity, teratogenicity, reproductive failure and disorders, endocrine cessation, neurotoxicity, and other biochemical alterations are due to the oxidative stress [4,5]. Dioxin intoxication induces alterations in liver metabolism, ruin cholesterol and estrogen metabolism, raise the activity of liver enzymes, and decrease the activity of antioxidant enzymes [6,7].

Food products of animal origin can be contaminated by TCDD from the environment and contaminated feed. Contamination incidents of TCDD in milk, meat, commercial eggs, and other products has been reported in many countries [8-10]. In developing countries, including Indonesia, industrialization and weak law enforcement risking hazards caused by environmental contamination. Health risks associated with dioxin were high in the Southeast Asia region [11]. The lipophilic nature of these compound contributes to their ability to accumulate in fat tissues thus it can be degraded slowly by chemical and biological process [2,12]. Prolonged half-life and
continuous exposure of TCDD in humans can lead to clinical signs of toxicity which will appear in future years [13,14].

Long-period exposure to TCDD in humans and other mammals can alter blood lipid profile and antioxidant enzyme activity. A cohort study on workers exposed to dioxins compound showed elevation in serum cholesterol and triglyceride [15-17]. The risk of exposure to TCDD increases the malondialdehyde (MDA) level and decreasing antioxidant enzyme activity like superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), and glutathione peroxidase (GSH-Px) [18]. A recent study on white rats exposed to TCDD showed an increase in serum γ-glutamyl transferase as an early indication of oxidative stress [19].

Natural antioxidant derived from foods has been studied and believed to have a biological function to prevent adverse effect caused by free radicals and have a protective effect to lower the development of many chronic diseases [20]. Milk naturally contains proteins with varied biological functions. Goat milk is known to have more health benefits in total solids and nutrients than cow’s milk [21]. Protein in milk is in the form of an inactive component, whereas fermentation by lactic acid bacteria is one of the proteolysis methods to release bioactive peptides from the precursor proteins [22]. Casein is known as a precursor protein for bioactive peptides that have activity as antimicrobial, antioxidative, antiinflammatory, anticarcinogenic, and immunomodulatory process [23]. This study aimed to explore the role of casein yogurt which was previously known to have antioxidant activity, to prevent negative effects caused by TCDD. Parameters such as lipid profile and antioxidants enzyme superoxide dismutase (SOD) activity were analyzed.

2. Methods

2.1. Materials preparation

Dioxin chemical used in this study was 2,3,7,8-Tetrachlorodibenzo-p-dioxin (Supelco, Cat No: 48599) with > 99% purity. Yogurt starter used was Yógourmet (Lyo-SAN INC: 500 Aeroparc, C.P. 589, and Lachute, QC. Canada, J8H, 464) containing three strains of lactic acid bacteria: Lactobacillus bulgaricus, Streptococcus thermophilus, and Lactobacillus acidophilus. Solution of 2,2-diphenyl-1-picrylhydrazil (DPPH) 0.2 nM was used for antioxidant activity assay.

Preparation of goat milk yogurt based on the modified method described in Padaga et.al [24]. Raw and unpasteurized goat milk was obtained from a local supplier in Malang, Indonesia. Viable lactic acid bacteria were measured using total plate count method, nutrition value of goat milk yogurt was assessed using proximate assay and antioxidant activity was analyzed using DPPH in accordance with a modified method by Pinela et.al [25]. Casein was separated from the whole yogurt by cold centrifugation (12,000 rpm, 5°C) for 10 minutes followed by filtration. The casein yogurt was stored at -20°C until used.

2.2. Animals preparation

Male albino Wistar rats between 8-12 weeks old were obtained from Biosains Institute Brawijaya University with uniform body weight in range 150-160 grams. All animals were housed at an animal facility under a controlled laboratory environment. Room temperature was maintained at 24±2°C with 12 hours day/night cycles. General health was observed during acclimatization for 2 weeks prior to experiment. Drinking water and a standard diet were given without restraint throughout this study.

2.3. Experimental study method

The ethical clearance certificate was issued by the ethics committee of Brawijaya University. A total of 24 albino Wistar rats was randomly distributed into 6 uniform groups. The Control group was given no treatment. Placebo group was treated using casein yogurt at an oral dose of 600 mg/kg BW/day
diluted in distilled water, whereas TCDD group was treated at an oral dose of 100 ng/kg BW/day of TCDD diluted in corn oil. Treatment groups (T1-T3): T1 with 300 mg/kg BW of casein and 100 ng/kg BW of TCDD daily. T2, given with 600 mg/kg BW of casein and 100 ng/kg BW of TCDD daily. T3 was given with 900 mg/kg BW of casein and 100 ng/kg BW of TCDD daily. The volume of the casein solution and TCDD solution was 1 mL for each rat. The study was conducted for 21 days. On the day 22, all animals were euthanatized, followed by blood and liver tissue collection. Blood serum was analyzed for cholesterol and triglyceride assay. Liver tissue was collected for antioxidant enzyme superoxide dismutase (SOD) activity assay.

2.4. Biochemical and tissue analyses

Blood samples were collected, left for 60 min to clot, and centrifuged for 10 min at 5000 rpm to obtain clear sera. Serum cholesterol and serum triglyceride level were analyzed using Hitachi 902 automatic analyzer (Hitachi Co. Ltd, Tokyo, Japan).

The liver samples were placed in sodium chloride (NaCl) perfused with the same solution to remove the blood cells, wrapped in aluminum foils, then stored in a -80°C freezer for further experiment. The SOD activity was analyzed using the nitroblue tetrazolium (NBT) method described by Beauchamp and Fridovich [26]. According to this method, NBT was reduced to blue formazan by superoxide ion (O$_2^-$), the SOD enzyme from the sample inhibited the formation of blue formazan, thus the color change can be measured using a spectrophotometer at 560 nm wavelength.

2.5. Statistical Analyses

Data were presented as Mean ± SD. Differences between groups were assessed by one-way Analysis of Variance (ANOVA), changes among groups were determined by Post Hoc multiple comparison tests (honest significant different test; HSD) with significance level at P<0.05

3. Results and Discussion

The results on goat milk yogurt analysis were shown in table 1. Total viable lactic acid bacteria were $3.4 \times 10^7$ CFU/mL and the IC$_{50}$ value for casein yogurt resulted 2543,00 mg/mL.

Table 1. Analysis of yogurt and casein.

| Sample                  | Test           | Value         |
|-------------------------|----------------|---------------|
| Yogurt                  | Viable count   | $3.4 \times 10^7$ CFU/mL |
|                         | Protein        | 3.58%         |
| Casein from goat milk yogurt | Protein      | 4.85%         |
|                         | Fat            | 9.32%         |
|                         | Water          | 78.85%        |
|                         | Ash            | 0.73%         |
|                         | Carbohydrate   | 6.25%         |
|                         | Antioxidant IC50 | 2543,00 mg/mL |

3.1. Effect of casein on lipid profile

Results on serum triglyceride values and lipid profile analysis presented in table 2 show an increase in cholesterol and triglyceride level in groups given with TCDD compared to the control group, even though no significant difference between groups (P>0.05) by statistical analysis. Treatment groups given with Casein yogurt dose 600 mg/kg BW (T2) shows a 5.76% decreased compared to control, and the group given with Casein yogurt dose 900 mg/kg BW (T3) shows a decrease in cholesterol level by 8.12% compared to TCDD group. Triglyceride level shows lower
values in treatment groups given with Casein yogurt dose 300 mg/kg BW (T1) by 31.57%, dose 600 mg/kg BB (T2) by 55.10%, and dose 900 mg/kg BB (T3) by 44.51% compared to TCDD group.

**Table 2.** Effect of casein on serum cholesterol triglyceride level of dioxin intoxicated rats.

| Groups | Cholesterol level (mean ± SD) mg/dl | Triglyceride levels (mean ± SD) mg/dl |
|--------|-------------------------------------|--------------------------------------|
| Control | 90.5 ± 9.00                      | 59.5 ± 11.82                      |
| TCDD   | 95.5 ± 9.47                      | 127.5 ± 45.18                     |
| Placebo | 85.0 ± 7.16                      | 65.5 ± 22.58                      |
| T1     | 95.5 ± 16.03                     | 87.25 ± 40.12                     |
| T2     | 90.0 ± 9.93                      | 57.25 ± 20.32                     |
| T3     | 87.75 ± 5.38                     | 70.75 ± 42.11                     |

3.2. **Effect of Casein on Liver Superoxide dismutase Activity**

Antioxidant enzyme superoxide dismutase (SOD) assay from liver tissue showed increased activity in groups given with casein dose of 300, 600, and 900 mg/kg body weight (T1, T2, T3), compared to TCDD group as presented in table 3. ANOVA test showed significant (P<0.05). Different between groups and a significant decrease was shown in group T2 and T3.

**Table 3.** Effect of casein on liver SOD activity of dioxin intoxicated rats.

| Groups | SOD Activity (mean ± SD) U/mL | Decreased in SOD activity compared to control (%) | Increase in SOD activity compared to TCDD group (%) |
|--------|-------------------------------|-----------------------------------------------|-----------------------------------------------|
| Control | 5.6915 ± 1.076abc             | -                                             | -                                             |
| TCDD   | 3.6918 ± 0.146a               | 35.14                                         | -                                             |
| placebo | 4.7333 ± 1.003ab              | 16.84                                         | 28.21                                         |
| T1     | 4.8165 ± 0.667ab              | 15.37                                         | 30.47                                         |
| T2     | 5.6363 ± 0.539bc              | 0.97                                          | 52.67                                         |
| T3     | 6.4973 ± 0.468c               | -                                             | 75.99                                         |

Differences in superscript notation indicate a significant difference between groups at P<0.05

3.3 **Discussion**

The results for goat milk yogurt and casein yogurt analysis were following minimum total lactic acid viable count for probiotic products given by Indonesia National Standard (SNI, 2009). Ranadheera et al. [27] stated minimum total lactic acid bacteria on probiotics that can give health benefits was $10^5 - 10^6$ CFU/g. Casein from yogurt is able to scavenge free radicals, as well as certain strains of *L. acidophilus* do scavenge the free radicals alpha,alpha-diphenyl-beta-picrylhydrazyl (DPPH) [20,28,29]

Serum cholesterol levels showed normal values in the control and treatment groups. Cholesterol values increase at 5.52% on the TCDD group and the T1 group compared to the control group. The lowest cholesterol value was reached by the placebo group treated with casein. Serum triglyceride levels of all groups remain normal. The highest value was reached by the TCDD group, which increased 114.29% compared to the control group. Treatment groups showed
a slight decrease in triglyceride levels compared to the TCDD group, although the statistic test showed no significant differences between groups.

Dioxin exposure is one risk factor for many metabolic diseases. A study on incinerator workers shows an association between TCDD exposure and elevated serum cholesterol and triglyceride [15]. Groups given with 100 ng/kg BW of TCDD for 21 days showed elevation in cholesterol and triglyceride levels. TCDD is a persistent organic pollutant that can induce metabolic changes in the liver and caused a disturbance in cholesterol metabolism [6]. Exposure to the combination of persistent organic pollutants (POPs) inhibits the genes for several enzymes that play role in bile acid signalling, such as CYP7A1 which is the key enzyme involved in bile acid biosynthesis from cholesterol. TCDD exposure reduced CYP7A1 expression in a rat liver model. The down regulation of the genes involved in lipid metabolism may assign to increase cellular cholesterol and raise the risk of fatty liver disease [30].

Decreased serum cholesterol and triglyceride values on treatment groups given with casein showed that casein yogurt could prevent the elevation on lipid profile caused by dioxin intoxication. There are some statements about the consumption of fermented milk that can significantly reduce serum cholesterol [31]. In the small intestine, simultaneous precipitation of cholesterol with deconjugated bile salts occurs as the pH declines from lactic acid production by lactic acid bacteria [32], hence cholesterol is excreted faster from the body.

Dioxin (TCDD) exposure on Wistar rats decreased liver SOD activity significantly (P<0.05) compared to the control group. As depicted in table 3, SOD activity on the treatment group given with casein 600 mg/kg BW close to normal value on the control group. Changes in the activity of the antioxidant enzyme can be used as indirect measures of oxidative stress induced by specific compounds [33]. Superoxide dismutase enzyme is the first body defence against the negative effect of free radicals [34]. A decrease in SOD activity is evidence of oxidative stress caused by dioxin intoxication. According to Turkez et al. [7], TCDD elevated the production of reactive oxygen species (ROS) due to oxidative stress and hepatotoxicity. The increased lipid peroxidation (LPO) in various tissues resulted in the inhibition of antioxidant enzyme activity.

In this study, TCDD treatment at dose of 100 ng/kg BW on Wistar rats for 21 days decreased liver SOD activity by 35.15% compared to control. Excess production of free radicals induces protective enzymes such as SOD and other antioxidant enzymes become overwhelmed lead to destructive and lethal cellular effect by oxidizing lipid membranes, cellular proteins, DNA enzymes, followed by shutting down cellular respiration [34].

Casein yogurt treatments on dioxin intoxication rats showed a protective effect against oxidative stress caused by TCDD. According to Pihlanto [35], casein generated activity as a cation chelator which can inhibit lipoygenase-catalyzed lipid autoxidation, as well as antioxidant activity as a radical scavenger. The free radical scavenging activity has been reported derived from the peptic digest of casein. The peptide, Tyr-Phe-Tyr-Pro-Glu-Leu has the potent superoxide anion radical scavenging activity. In addition, the fermentation process by lactic acid bacteria releases bioactive peptides from precursor proteins, these peptides are comprised of five to eleven amino acids among proline, histidine, tyrosine, or tryptophan in the sequence that contribute to the antioxidant activity [22,36]. Based on the liver SOD activity, casein yogurt was proven to have a protective effect against TCDD intoxication with the best dose was 900 mg/kg BW.

4. Conclusion
Casein yogurt exhibited antioxidant activity that contributed to prevent cell damage and further adverse effect caused by intoxication of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD). Casein yogurt can be potential as a functional food and natural antioxidant from animal products. Further studies need to be carried out in order to explore bioactive peptides in casein yogurt and its application as alternative prevention against diseases caused by environmental toxic exposure such as dioxins and other persistent organic pollutants (POPs).
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