Role of MDA, SOD and GPx Expression on Protective Mechanism of Xanthone Against 2-Methoxyethanol-Decreased Number of Sertoli Cell in Mice

Ernawati[1], Reny I'tishom[2], Sri Agus Sudjarwo[3*]

[1]Program Study of Doctoral Degree in Medical Science, Faculty of Medicine, Airlangga University
[2]Department of Medical Biology, Faculty of Medicine, Airlangga University,
[3]Department of Pharmacology, Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia

[*]Corresponding author : Email : ags158@yahoo.com

Abstract

Background: 2-Methoxyethanol **(**2-ME) can increase Reactive Oxygen Species (ROS) production that cause damages to the male reproductive organs such as Sertoli cell. Xanthone is one of the natural antioxidant that can neutralize ROS. Objective: The purpose of this research was to determine the protective mechanism of xanthone on 2-ME-induced Sertoli cell oxidative damage. **Methods:** The research used 35 male mice divided into 5 groups: control group (mice were given daily with water purified by distillation); 2-ME group (mice were given daily with 2-ME 200 mg/kg BW orally once in a day for 35 days); and the treatment group (mice were given the xanthone 60 mg, 120 mg, and 240 mg/kg BW orally once in a day for 38 days, and on the 3th day, were given 2-ME 200 mg/kg BW one hour after the xanthone administration). After 38 days, the testis tissues were collected to evaluate the immunohistochemical of the expression of malondialdehyde (MDA), Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) in Sertoli cell. Testis tissue was also taken to histological evaluations of Sertoli cell number. **Results:** The immunohistochemical evaluation showed that 2-ME administration significantly decreased the expression of SOD, GPx, and increased the expression of MDA in Sertoli cell. The histopathological evaluations showed that 2-ME also significantly decreased Sertoli cell number. However, administration of xanthone significantly increased the expression of SOD, GPx, and decreased the expression of MDA on Sertoli cell in immunohistochemical evaluation. Xanthone significantly increased Sertoli cell number in histopathological evaluation. **Conclusion:** In conclusion, our results indicate that *xanthone* as antioxidant agent is able to increase Sertoli cell number in mice treated with 2-ME through decreased MDA expression, and increased both SOD and GPx expression

Keyword: Antioxidant, Xanthone, Sertoli cell, Oxidative stress

Introduction

The occurrence of infertility disorders in animals and humans can be caused by infection, trauma, smoking, alcohol, radiation, diabetes mellitus, drug use, heavy metal poisoning (As, Pb, Cd, Hg) and Ethylene Glycol Monomethyl Ether (2-Methoxyethanol) poisoning (Choy and Ellsworth, 2012; Sharma, 2017). 2-Methoxyethanol (2-ME) is a glycol ether compound found in various industrial products including paints, inks, varnishes, nail polishes, hydraulic fluids, plastic materials, aircraft fuels and the food industry (Johanson, 2000; Bagchi and Waxman, 2008). 2-ME enters the body of animals and humans through inhalation, peroral and topical, which then be oxidized by Alcohol dehydrogenase to methoxyaldehyde (MALD); and MALD is rapidly oxidized by aldehyde dehydrogenase to 2-methoxyacetic acid (2-MAA) which is a stable and very toxic metabolite (Dayan and Hales, 2014). Some researchers report that 2-ME and its metabolites, 2-MAA, can cause disturbances in the testes and spermatozoa so can occur infertility (Adedara and Farombi, 2010; Kumar and Singh, 2018).

Oxidative stress has a very important role in the mechanism of action of 2-ME in causing the decrease in quality and quantity of spermatozoa cells in the epididymis, and testicular damage (Adedara and Farombi, 2010; Kumar and Singh, 2018). Oxidative stress can occur due to an increase in Reactive Oxygen Species (ROS) which includes superoxide (O_2^-), hydroxyl radical (OH^-), hydrogen peroxides (H_2O_2) and decreased endogenous antioxidants including Superoxide Dismutase (SOD), Catalase and Glutathione Peroxidase (GPx). The enzyme catalase and glutathione peroxidase work by converting H_2O_2 to H_2O and O_2 while the enzyme superoxide dismutase (SOD) works by catalyzing the dismutation reaction of the superoxide anion radical to H_2O_2 (Pamplona and Costantini, 2011; Asadi et al., 2017). The imbalance between ROS and antioxidants will cause oxidation of lipid, protein, and DNA of spermatozoa cells, Leydig cells and Sertoli cells in the testes so occur oxidative damage in the cell membrane lipids, protein molecules, and DNA that can produce Malondialdehyde (MDA) (Sudjarwo et al., 2019; Del Rio et al., 2019).

Cell damage due to 2-ME exposure to spermatozoa and testes can be inhibited by administering antioxidants (Adedara and Farombi, 2014; Adewoyin et al., 2017). Antioxidants work by donating one electron to an oxidant compound so that the activity of the oxidant compound can be inhibited (Asadi et al., 2017). Exogenous antioxidants such as have been reported to be used to protect spermatozoa and testicular cell damage due to increased production of ROS (free radicals) during 2-ME exposure (Adewoyin et al., 2017; Kumar and Singh, 2018). Some researchers report that antioxidants derived from plants such as Tribulus terrestris, Withania somnifera, Mucuna pruriens; Garcinia kola, and Garcinia mangostana can be used as protectors for spermatozoa and testicular cell damage due to 2-ME exposure (Zheleva-dimitrova et al., 2012;Tania and winarni, 2015; Hayati et al., 2017; Kumar and Sing, 2018). Several studies have proven the pharmacological activity of xanthone which one of the active compounds contained in Garcinia mangostana as an antioxidant (Thonga et al., 2015). Xanthones are a natural chemical substance that is classified as polyphenolic compounds. Xanthones have an antioxidant effect because xanthones have a hydroxil group (OH^-) that effectively binds to free radicals in the body (Jiang *et al*., 2004; Panda et al., 2013; Ernawati et al., 2019). The xanthones have a very strong antioxidant effect, therefore is needed research to prove that xanthone can be used to

prevent damage to Sertoli and Leydig cells due to exposure to 2-ME.

Material and Methods

Experimental animals

Male BALB/c mice weighing approximately 25-30 g (2-2.5 months) were obtained from Gadjah Mada University, Yogyakarta, Indonesia for experimental purpose. They were housed in plastic cages in an air-conditioned room with a temperature maintained at 26 ± 2 ^{o}C and 12 h alternates light and dark cycles. The rats were given *ad libitum* with tap water and fed with standard commercial rat chow. This study was reviewed by the Ethical Clearance Committee for preclinical research, Faculty of Medicine, Airlangga University and obtained ethical clearance under No.183/FK/12/2019.

Experimental design

The research used 35 male mice divided into 5 groups: negative control (mice were given daily with water purified by distillation); positive control (mice were given daily with 2-ME 200 mg/kg BW orally once in a day for 35 days); and the treatment group (mice were given the xanthone 60 mg, 120 mg, and 240 mg/kg BW orally once in a day for 38 days, and on the 3th day, were given 2-ME 200 mg/kg BW one hour after the xanthone administration). After 38 days, the testis tissues were collected to evaluate the immunohistochemical of the expression of malondialdehyde (MDA), Superoxide Dismutase (SOD) and Glutathione Peroxidase (GPx) in Sertoli cell. Testis tissue was also taken to histological evaluations of Sertoli cell number.

Immunohistochemical examination

For immunohistochemical studies, a LSAB System HRP (Dako, Carpinteria, CA), antisuperoxide dismutase polyclonal antibody (Abcam International, USA), glutathione peroxidase monoclonal antibody (Abcam International, USA), and anti MDA monoclonal antibody (Abcam International, USA) were used. In brief, according to Juárez-Rebollar et al., 2015, the sections were deparaffinized, after hydrated with decreasing alcohol concentrations and washed three times for 3 min each time in 0.01 M phosphate-buffered saline (PBS, pH 7.4) for heat-induced epitope retrieval; the sections were boiled in citrate buffer (pH 6 or 9) in a microwave oven for . The sections were preincubated with 0.3% hydrogen peroxide in PBS and later incubated with SOD antibody (1 : 100), GPx (1 : 100), and MDA (1 : 100) by 90 min at room temperature. Slices were washed two times with PBS for 2 min followed by incubation with a secondary biotinylated antisera and then immersed in avidin–biotin peroxidase complex (LSAB System HRP, Dako, Carpinteria, CA) for 20 min at room temperature. The immune reaction resulted in the oxidation of the 3,3-diaminobenzidine by peroxidase (Liquid DAB, Dako, Carpinteria, CA) into an insoluble brown precipitate. Counterstaining with hematoxylin was performed after immunostaining.

Histopathological examination

The tissue of testis was fixed in a 10% neutral buffered formalin solution, embedded in paraffin and used for histopathological examination with hematoxylin and eosin (H&E) stain.

Statistical analysis

Data were presented as means ± standard deviation. Oneway ANOVA has carried post hoc test and the statistical comparisons among the groups were performed with an LSD test using a statistical package program SPSS version 17.0 (SPSS Inc, Chicago, USA).

Results

Table 1 and Figure 1 showed the results of xanthone in protective 2-ME-decreased Sertoli cell number. The administration of 2-ME on mice caused a significant decrease Sertoli cell number compared to the control group. The treatment xanthone increase Sertoli cell number in a dose-dependent manner**.**

Table 1: Protective effect of xanthone in against 2-ME-decreased Sertoli cell number

Group	Sertoli cell number (Mean ± SD)
Control group	22.04^{a}±2.47
2-ME group	13.07^{b}±2.36
Xanthone 60 mg/kg BW	12.46^{b}±2.07
Xanthone 120 mg/kg BW	13.56^{b}±2.36
Xanthone 240 mg/kg BW	17.20^{c}±2.03

^{a,b,c}Different superscript within each column differ significantly ($P < 0.05$)

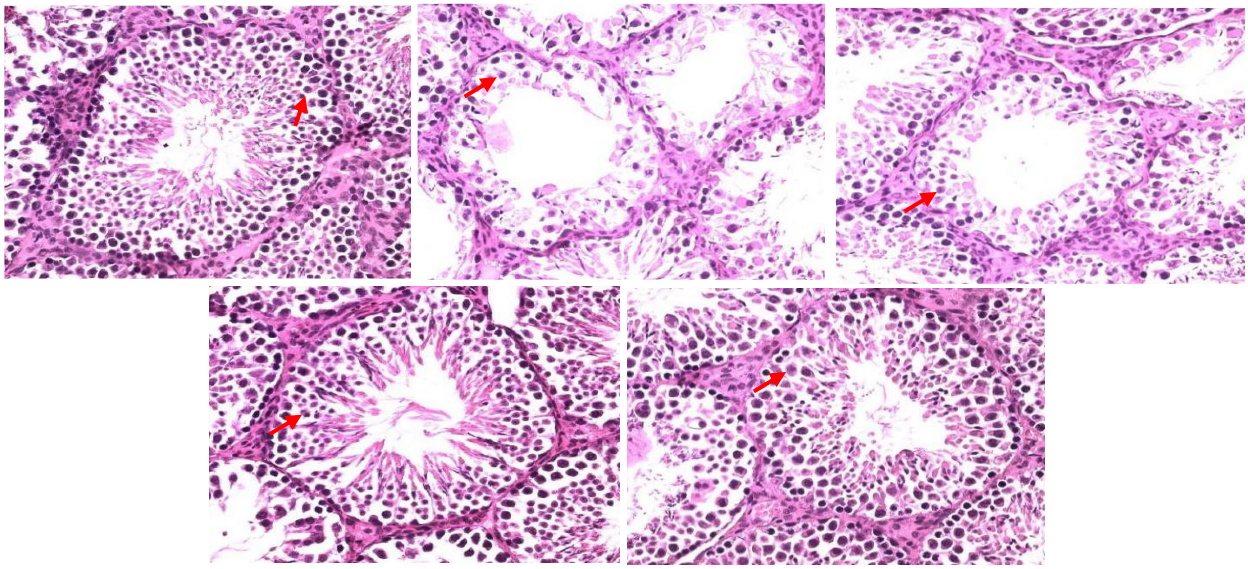

Figure 1: Histopathological study of xanthone in against 2-ME-decreased Sertoli cell number (indicated by red arrows). Control group (a); 2-methoxyethanol group (b); mice treated with xanthone 60 mg/kg BW; 120 mg/kg BW, and 240 mg/kg (c-e)

Table 2 and Figure 2 showed the results of xanthone on the expression of MDA in against 2-ME-decreased Sertoli cell number. The administration of 2-ME on mice caused a significant increase in the expression of MDA in Sertoli cell number compared to the control group. Dose dependent of xanthone decrease the expression of MDA in Sertoli cell number.

Table 2: Protective effect of xanthone on MDA expression in against 2-ME-decreased Sertoli cell number

Group	MDA in Sertoli (Mean ± SD)
Control group	$2.87^{a} \pm 0.28$
2-ME group	$4.79^{b} \pm 0.37$
Xanthone 60 mg/kg BW	$4.69^{b} \pm 0.36$
Xanthone 120 mg/kg BW	$4.56^{c} \pm 0.65$
Xanthone 240 mg/kg BW	$3.54^{c} \pm 0.49$

^{a,b,c}Different superscript within each column differ significantly ($P < 0.05$)

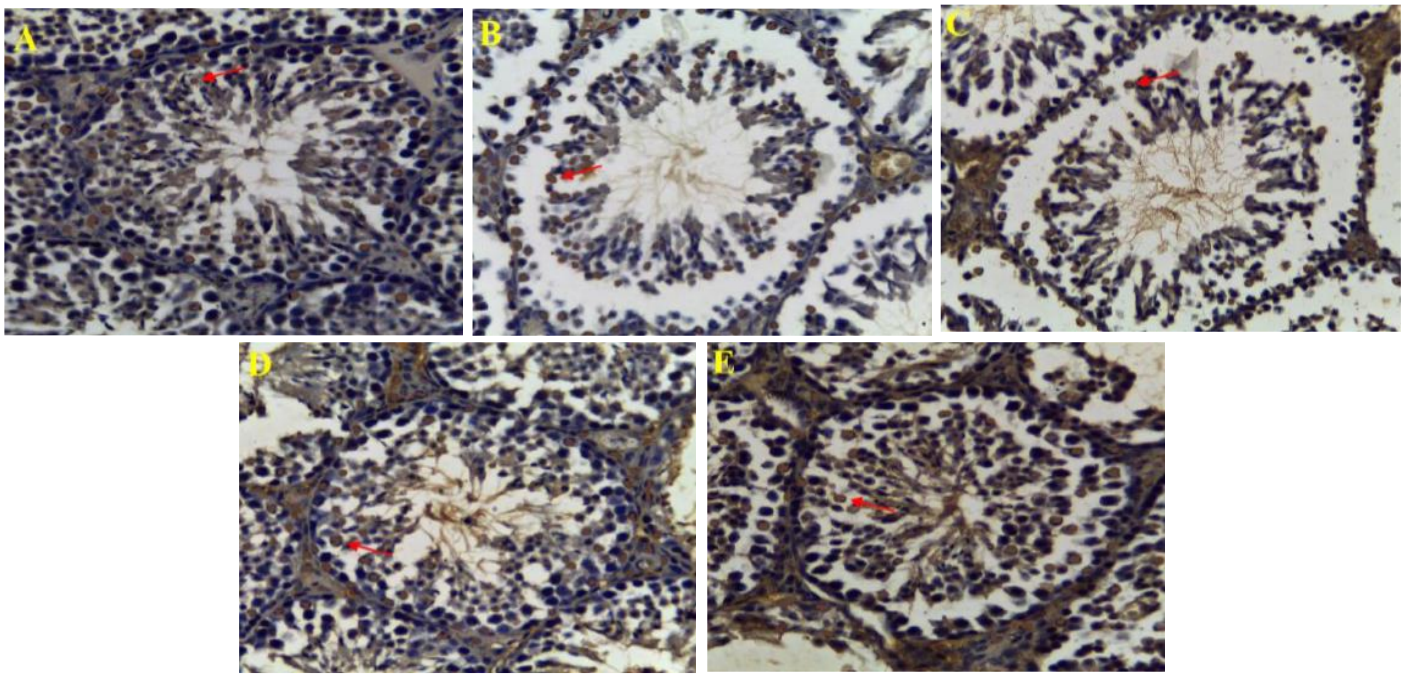

Figure 2: Immunohistochemical study of xanthone on MDA expression (indicated by red arrows) on 2-methoxyethanol-decreased Sertoli cell number. Control group (a); 2-methoxyethanol group (b); mice treated with xanthone 60 mg/kg BW; 120 mg/kg BW, and 240 mg/kg (c-e)

Table 3 and Figure 3 showed the results of xanthone on the expression of SOD in protective 2-ME-decreased Sertoli cell number. The administration of 2-ME on mice caused a significant decrease in the expression of SOD and Sertoli cell number compared to the control group. The treatment xanthone increase the expression of SOD and Sertoli cell number in a dose-dependent manner**.**

Table 3: Protective effect of xanthone on SOD expression in against 2-ME-decreased Sertoli cell number

Group	SOD in Sertoli cell (Mean ± SD)
Control group	$6.51^{a} \pm 0.53$
2-ME group	$3.89^{b} \pm 0.30$
Xanthone 60 mg/kg BW	$3.90^{b} \pm 0.34$
Xanthone 120 mg/kg BW	$3.96^{c} \pm 0.26$
Xanthone 240 mg/kg BW	$4.96^{c} \pm 0.39$

^{a,b,c}Different superscript within each column differ significantly ($P < 0.05$)

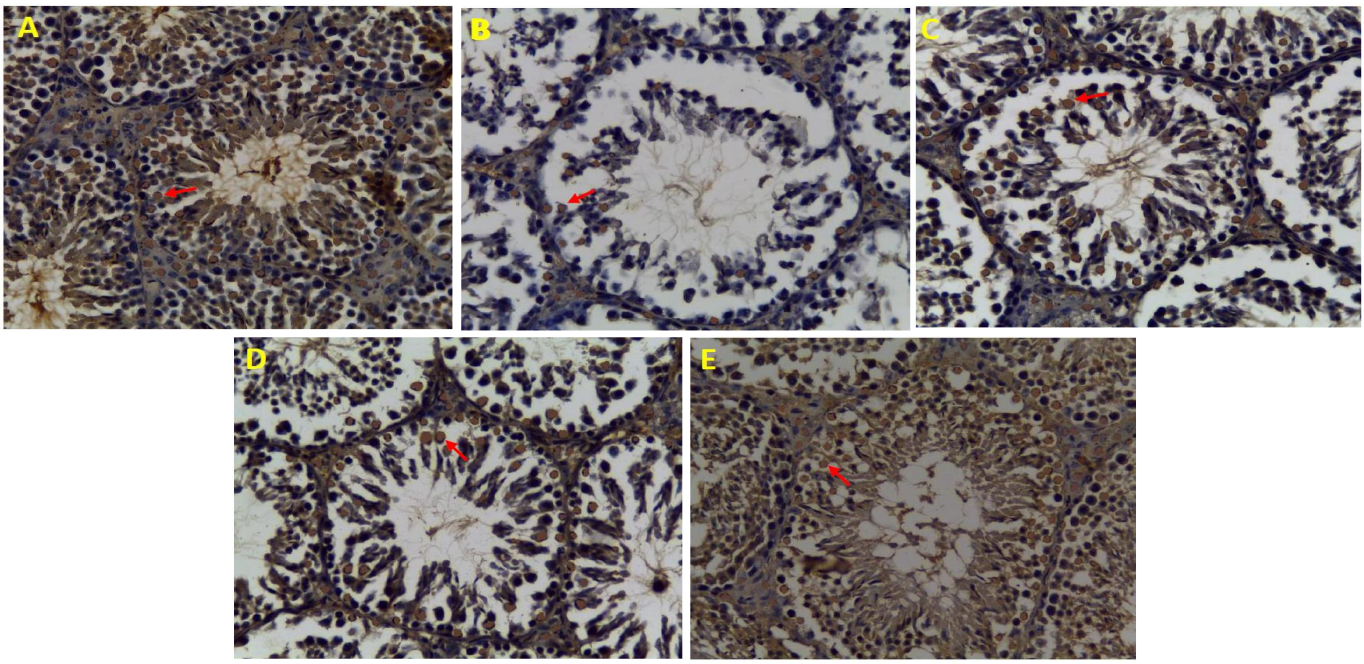

Figure 3: Immunohistochemical study of xanthone on SOD expression (indicated by red arrows) on 2-methoxyethanol-decreased Sertoli cell number. Control group (a); 2-methoxyethanol group (b); mice treated with xanthone 60 mg/kg BW; 120 mg/kg BW, and 240 mg/kg (c-e)

Table 4 and Figure 4 showed the results of xanthone on the expression of GPx in protective 2-ME-decreased Sertoli cell number. The administration of 2-ME on mice caused a significant decrease in the expression of GPx and Sertoli cell number compared to the control group. The treatment xanthone increase the expression of GPx and Sertoli cell number in a dose-dependent manner.

Table 4: Protective effect of xanthone on GPx expression in against 2-ME-decreased Sertoli cell number

Group	GPx in Sertoli cell (Mean ± SD)
Control group	$5.54^{a} \pm 0.59$
2-ME group	$3.30^{b} \pm 0.37$
Xanthone 60 mg/kg BW	$3.41^{b} \pm 0.49$
Xanthone 120 mg/kg BW	$3.87^{c} \pm 0.45$
Xanthone 240 mg/kg BW	$4.11^{c} \pm 0.34$

^{a,b,c}Different superscript within each column differ significantly ($P < 0.05$)

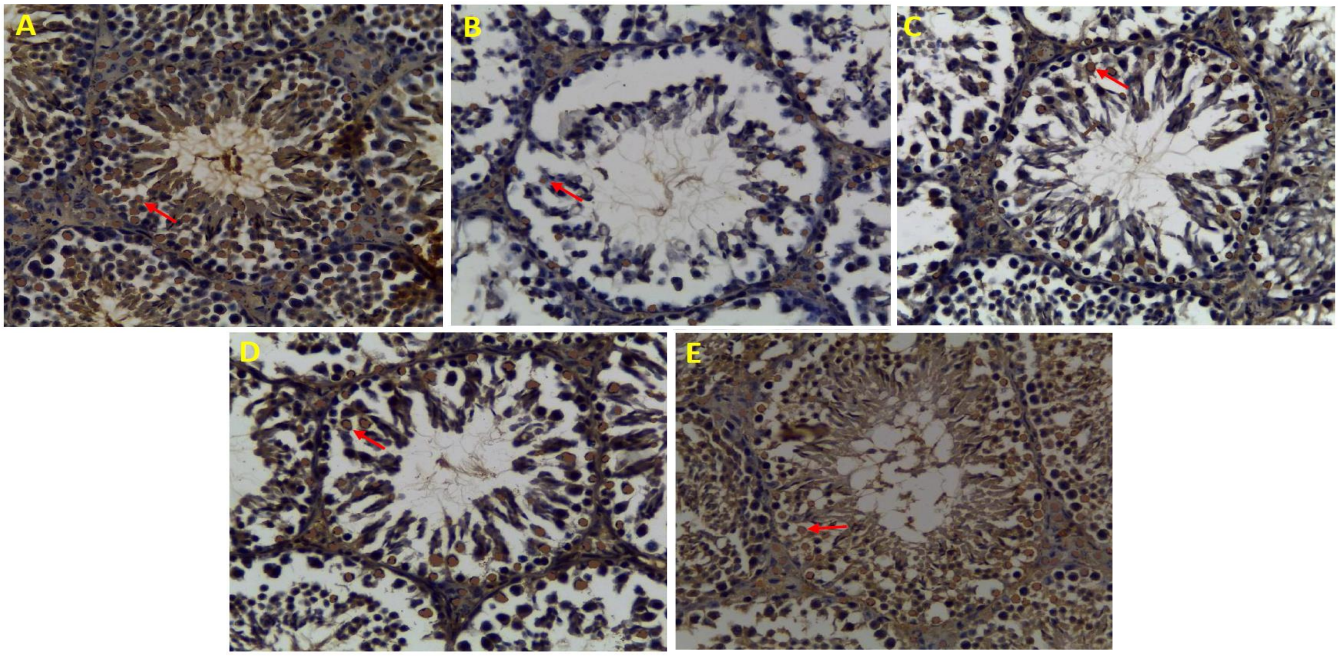

Figure 4: Immunohistochemical study of xanthone on GPx expression (indicated by red arrows) on 2-methoxyethanol-decreased Sertoli cell number. Control group (a); 2-methoxyethanol group (b); mice treated with xanthone 60 mg/kg BW; 120 mg/kg BW, and 240 mg/kg (c-e)

DISCUSSION

2-ME may induce oxidative stress leading to the generation of free radicals and alteration in oxygen free radical scavenging enzyme system or antioxidant such as Catalase, SOD and GPx (Devi Sampath and Vijayaraghavan, 2007; Pomierny et al., 2014). In the current study, we evaluated the protective mechanism of xanthone against the oxidative stress changes in the Sertoli cell resulting from the administration of 2-ME in mice. The biochemical mechanisms decreased in the Sertoli cell number of 2-ME were studied by measuring the MDA expression and by screening the activities of primary antioxidant enzymes such as SOD and GPx expression in immunochemical studies. It also Sertoli cell number was investigated for histopathological studies.

The present study showed that 2-ME administration significantly decreased the SOD, GPx and increased MDA levels. 2-ME also decreased the Sertoli cell number. 2-ME-decreased Sertoli cell number have been attributed, at least in part, to toxicant-induced oxidative stress. It results suggest that 2-ME stimulates the formation of ROS, thus causing oxidative damage to Sertoli cell resulting in decrease of cell number. Long-term exposure to 2-ME increases MDA or lipid peroxidation and causes inhibition of SOD and GPx activity inducing oxidative damage in testicular cell (Adedara and Farombi, 2010; Kumar and Shing, 2018). The various toxic effects induced by 2-ME in biological systems have been linked to increased MDA or lipid peroxidation, as an early and sensitive consequences of 2-ME exposure. 2-ME toxicity leads to the generation of free radical damage by two separate pathways, including hydroperoxides, singlet oxygen, and hydrogen peroxides, evaluated by MDA expression as the final products of lipid peroxidation, and the direct depletion of antioxidant reserves (Sudjarwo et al., 2017). The present investigation resulted in significantly increased of MDA expression in the Sertoli cell of 2-ME-treated mice in comparison to the control. This means that it increased the oxidative stress in the 2-ME-treated mice. Therefore, the significantly lower expression of MDA in the Sertoli cell of xanthone treated groups as compared with the 2-ME group indicate attenuation of lipid peroxidation. It is known that 2-ME-induced oxidative stress and Sertoli cell damage could be caused by two mechanisms including increased generation of ROS and by causing direct depletion of antioxidant reserves (Matsuyama et al., 2018). Intense lipid peroxidation caused by 2-ME exposure may affect the mitochondrial and cytoplasmic membranes, causing more severe oxidative damage in the cell and consequently releasing lipid hydroperoxides into circulation which reflects the induction of oxidative stress (Adedara and Farombi, 2010). The xanthone, which behaves as a powerful antioxidant and free radical scavenger, can decrease the MDA expression perturbed by 2-ME in mice Sertoli cell, as observed in this study. Treatment of mice with xanthone at a dose of 240 mg/kg BW prevented the expression of MDA to rise when the mice were challenged with 2-ME. This means that xanthone minimized the toxic effect of 2-ME via its antioxidant activity. The antioxidant protective mechanism decreases the oxidative stress and scavenges the free radical responsible for the Sertoli cell damage and thus inhibit the lipid peroxidation as measured by MDA expresion. The findings of this study suggest that xanthone could attenuate oxidative stress by decreasing the lipid peroxidation (MDA expression) in the 2-ME-treated Sertoli cell. A similar result has shown that antioxidants derived from plants such as Tribulus terrestris, Withania somnifera, Mucuna pruriens; Garcinia kola, and Garcinia mangostana enhanced the

antioxidant status and inhibited lipid peroxidation in rats with 2-ME induced testis injury (Zheleva-dimitrova *et al.*, 2012;Tania and winarni, 2015; Hayati *et al*., 2017; Kumar and Sing, 2018)..

SOD and GPx are important antioxidant enzymes. The enzyme SOD plays a vital role in protection from oxidant damage produced by ROS in terms of dismutation of highly toxic superoxide anion radicals into less toxic hydrogen peroxide, which is then neutralized into oxygen and water by catalase. Further, GPx catalyzes the reduction of lipid peroxides and hydrogen peroxide using glutathione to protect against accumulation of lipid peroxides and other oxidants, thereby preventing oxidant damage (Adewoyin et al., 207). The observed reduction in activities of antioxidant defenses demonstrates the failure of the primary antioxidant system to act against 2-ME-induced oxidant stress. Therefore, the activities of SOD and GPx have been used to assess oxidative stress in cells (Devi Sampath and Vijayaraghavan, 2007; Pomierny et al., 2014). In the present study, the activity of SOD and GPx in sertoli cell number was decreased by 2-ME treatment. This decreased SOD and GPx activities with 2-ME treatment is in agreement with previous studies. This suggested that 2-ME exposure induced oxidative stress by inhibiting the activity of this antioxidant enzyme. Interestingly, the administration of xanthone increased the activities of SOD and GPx in the Sertoli cell of 2-ME-treated mice, which might be due to the ability of xanthone to reduce the accumulation of free radicals. xanthone acts as a scavenger for the oxygen-derived free radicals, thus protecting from Sertoli cell damage (Martinez et al., 2012; Gutierrez-Orozco and Failla, 2013).

The decrease in lipid peroxidation due to xanthone has been attributed to alterations in the antioxidant defense system which includes enzymes such as catalase (CAT), SOD and GPx which normally protect against free radical toxicity. The primary mechanism of action of xanthone may involve the scavenging of free radicals which can inhibit free radical formation (Matinez et al., 2012; Thonga et al., 2015). It has been found a decrease MDA expression and an increase in the antioxidant enzyme parameters including SOD, CAT, and GPx in the plasma and tissue such as hearth, testis, and brain of animals that were administered xanthone (Pomierny et al., 2014; kumar and shing, 2018; Ernawati et al., 2019).

Histopathological results demonstrating structural changes in testis tissue of 2-ME were reported by some researchers. In the present study, histopathological view of testis sections in the 2-ME treated group showed decreasing of the Sertoli cell number, as compared to the control group. The decrearesing were considerably mild in the groups treated with xanthone 240 mg/kg.

In conclusion, our results indicate that *xanthone* as antioxidant agent is able to increase Sertoli cell number in mice treated with 2-ME through decreased MDA expression, and increased both SOD and GPx expression

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Conflicts of interest
There are no conflicts of interest

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