

Original Article

Investigating the Protective Effect of Resveratrol on Hepatotoxicity and Oxidative Stress Caused by 3-monochloropropan-1,2-diol in Rats



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ABSTRACT

Background and Objectives: 3-monochloropropan-1,2-diol (3MCPD) is a chlorohydrin glycerol known as a toxic substance in food processing. This substance can cause toxicity in various organs, such as the kidney, liver, reproductive system, etc. This study investigates the protective effect of resveratrol on hepatotoxicity and oxidative stress caused by 3MCPD in rats.

Methods: A total of 30 male adult rats were obtained and kept under standard conditions. Animals were divided into five groups of 6 rats, including the control group (normal saline), 3MCPD group (10 mg/kg), 3MCPD+resveratrol group (25 mg/kg), 3MCPD+resveratrol group (50 mg/kg), and 3MCPD+resveratrol (100 mg/kg) group. Injections were done intraperitoneally for 14 days. Then, 24 h after the last injection, the liver tissue was removed to evaluate oxidative parameters.

Results: 3MCPD could increase reactive oxygen species and decrease glutathione levels and mitochondrial activity; however, no significant lipid peroxidation was observed in the group receiving 3MCPD. Also, the simultaneous administration of resveratrol could reduce the level of reactive oxygen species and lipid peroxidation and increase the level of glutathione and mitochondrial activity.

Conclusion: 3MCPD can cause toxicity in the liver of rats by inducing oxidative stress. Also, resveratrol, having antioxidant properties, can inhibit this toxicity.

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Introduction

3-monochloropropane-1,2-diol (3-MCPD) is a source of food contamination found in various foods, including products derived from natural grains to grilled cheese and coffee. Since high concentrations of 3-MCPD in food have been reported to have adverse health effects, scientists have recently paid more attention to predicting this substance in the food and the mechanism of toxicity caused by it, along with its derivatives [1]. Based on the toxicological data, in short-term exposure, the rat kidney is the target organ with the highest sensitivity to 3-MCPD toxicity [2]. In chronic and sub-chronic exposure, other organs (kidneys, liver, reproductive system, and heart) are susceptible [3]. The International Agency for Research on Cancer (IARC) classifies 3-MCPD as a class 2B carcinogen for humans [4]. In addition, the European Scientific Committee on Food, held in 2001, classified 3-MCPD as a non-genotoxic threshold carcinogen with an acceptable daily intake of 2 mg/kg/day for humans [5]. According to recent studies, oxidative stress and cellular redox imbalance may be involved in 3-MCPD toxicity. Although the toxicity of 3-MCPD has been evaluated in some studies, the molecular mechanisms underlying its cytotoxicity and tumorigenesis are still not elucidated [6]. The possible mechanism of gonadal and renal toxicity with 3-MCPD is due to the inhibition of glycolysis and oxidative stress [3].

Resveratrol (RES) with the chemical formula 3,5,4'-trihydroxytrans-stilbene is a polyphenolic phytoalexin derived from plants that are produced in response to environmental stresses, such as climate change exposure to ozone, sunlight, and heavy metals. Meanwhile, the enzyme stilbene synthase produces infectious agents, such as pathogenic microorganisms [7]. RES has strong antioxidant properties. This substance has other biological effects, including anti-inflammatory, anti-platelet, hypoglycemic, anti-apoptotic, and anti-mutagenic impacts, which make it a possible aid for the prevention and treatment of various disorders, including cardiovascular diseases, cancer, obesity, and neurodegenerative diseases [8, 9].

Accordingly, this study investigates the protective effect of RES on hepatotoxicity and oxidative stress caused by 3-MCPD in rats.

Materials and Methods

Study design

Chemicals and reagents

3-monochloropropane-1,2-diol

3-MCPD was obtained from CAS NO:96242 (Sigma chemical company -Germany) Sigma chemical company (Germany) CAS NO:501-36-0 (wise-powder). All other chemicals and reagents used in this study were obtained from the Sigma chemical company (Germany), with the highest available commercial grade.

Preparation of resveratrol

We dissolve RES powder in normal saline with a few drops of toluene 80 and put it in a shaker until it becomes homogeneous.

Grouping of animals

This was an experimental study, and 30 adult male rats weighing 200-250 g were obtained from the Laboratory Animal Breeding Center of Mazandaran University of Medical Sciences and kept under standard conditions. In this experiment, the animals were randomly selected and divided into five groups of 6 rats:

1. Control group (normal saline);
2. The group receiving 3MCPD (10 mg/kg) [10];
3. The group receiving 3MCPD+RES (25 mg/kg);
4. The group receiving 3MCPD+RES (50 mg/kg);
5. The group receiving 3MCPD+RES (100 mg/kg)

Injections were done intraperitoneally for 14 days. Animals were euthanized 24 h after the last infusion under complete ether anesthesia and were subsequently euthanized. Liver tissue was removed for oxidative studies.

Measurement of tissue protein

Protein concentration was measured by the Bradford method. This method's protein concentration range is 250-2000 µg/mL. First, by using the Coomassie Blue stock solution, different standard concentrations of protein were prepared in the range of 250-2000 µg/mL, then 100 µL of standards, sample, and blank (buffer) were poured into separate tubes, and 5 mL of diluted reagent

color was added to the mix and vortexed. The absorbance was read against a blank between 10 min and 1 h at a wavelength of 595 nm [11].

Measurement of reactive oxygen species by the fluorimetry method

The reactive oxygen species (ROS) amount was measured using the dichloro-dihydro-fluorescein diacetate (DCFH-DA) reagent. After tissue protein determination, 20 μ L of DCFH-DA was added to 2000 μ L of the sample and kept at 4°C for 15 min. The absorption was measured at the excitation wavelength of 312 nm and emission at 420 nm [12, 13].

Measurement of lipid peroxidation

To measure the amount of lipid peroxidation by the thiobarbituric acid method, 0.1 mL of thiobarbituric acid (TBA) reagent (including 0.5 M HCl, 15% TCA, and 0.3% TBA) was added to 0.2 mL of tissue suspension and mixed well. The solution was incubated in a hot water bath at 100 c for 30 min. After cooling, 0.4 mL of n-butanol was added and shaken well. Subsequently, the solution was centrifuged at 3500 rpm for 10 min. The n-butanol layer was separated, and its absorbance was read at a wavelength of 532 nm with a spectrophotometer. The amount of MDA was calculated from the standard curve [12, 13].

Measurement of glutathione

An amount of 0.25 mL of 20% trichloroacetic acid was added to 1 mL of tissue suspension, vortexed, and centrifuged at 1000 rpm for 20 min. Meanwhile, 2 mL of disodium hydrogen phosphate 0.3 M and 0.5 CC 5,5'-di-thiobis (DTNB) 0.04% were added to 1 mL of the supernatant solution obtained from the centrifuge. Then, it was vortexed and incubated for 15 min until the reaction was complete. A spectrophotometer measured the absorbance at a wavelength of 412 nm. Glutathione concentration was calculated from the glutathione standard curve in nm/mL [12, 13].

MTT assay

First, 1 mL of the sample was poured into a microtube with 25 μ L of MTT solution and placed in a bain-marie at 37 degrees for 30 min. Then, it was centrifuged at 1000 rpm for 10 min. The supernatant solution was discarded, and 1000 μ L of dimethyl sulfoxide (DMSO) was added to the sediment. Then, with the help of pipetting and vortexing, the residue was well dispersed in DMSO. The resulting mixture was centrifuged for 10 min at

1000 rpm. The supernatant solution was removed, and 150-200 μ L of the supernatant solution of each sample was poured into each well of a 96-well plate. Then, the reading was done with the help of the Elisa reader device. The percentage change in enzyme activity was calculated by measuring the groups' absorption against the absorption of the control sphere [12, 13].

Statistical calculations

All statistical calculations were done using the Prism software, version 8. Data comparison was made with the one-way analysis of variance (ANOVA) and the related post-test exams (Tukey-Kramer multiple comprehension tests). The same graphics program drew the graphs.

Results

Results of reactive oxygen species fluorimetry

The results of fluorimetry studies to measure the amount of ROS are summarized in diagram 3.1.

Results of lipid peroxidation measurement

The results of lipid peroxidation measurements are summarized in diagram 3.2.

Results of glutathione measurement

The results of glutathione measurement to measure the amount of glutathione in rats' liver cells are summarized in Figures 1, 2, 3 and 4.

Results of the MTT test

The results of the MTT test are summarized in diagram 3.4.

Discussion

In this study, we investigated the protective effect of RES on hepatotoxicity and oxidative stress caused by 3-MCPD in rats.

The data obtained from this study clearly showed that 3MCPD could cause toxicity in rat liver cells by inducing oxidative stress. 3MCPD is a significant source of contamination during the processing of some foods and ingredients [14]. Several in vivo toxicological studies have identified 3MCPD as a rodent carcinogenic compound. Histopathological data obtained from chronic exposure to 3MCPD in rodents showed that the kidney, liver, testis, brain, and heart are the target organs for the toxicity

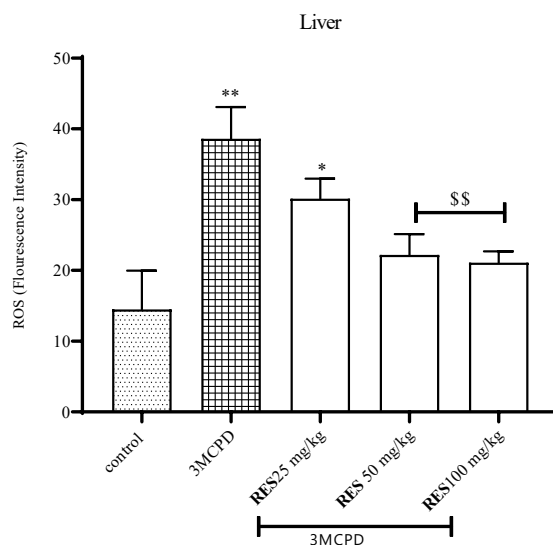


Figure 1. The results of examining the amount of reactive oxygen species in different studied groups

Notes: *P<0.05, **P<0.01, compared to the control group, §§P<0.01 compared to the 3-monochloropropan1-2diol group.

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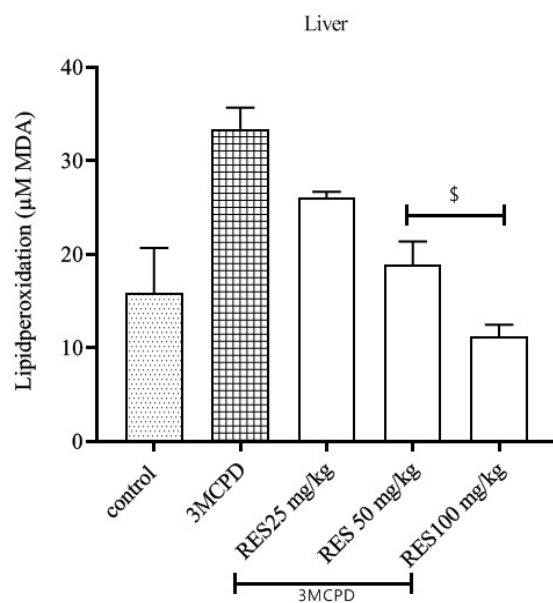


Figure 2. The results of examining the amount of lipid peroxidation in the studied groups

§P<0.05 compared to the 3-monochloropropan1-2diol group.

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of this substance [4, 14]. Previous studies reported that 3MCPD could induce apoptosis without increasing ROS levels and decreasing mitochondrial membrane potential (MMP) and adenosine triphosphate (ATP) synthase gene expression. It also caused a significant decrease in the Bcl2/Bax ratio in HEK-293FT cells, implying that mitochondrial dysfunction could be the main pathway of 3MCPD-induced toxicity [15, 16]. The results of these

two studies differ from ours in terms of increasing the amount of oxygen free radicals.

In comparison, these studies confirm the results obtained from examining mitochondrial function in the present study. Nazari et al. evaluated the toxicology of 3MCPD as a significant food contaminant in three laboratory models: Involvement of oxidative stress and cell

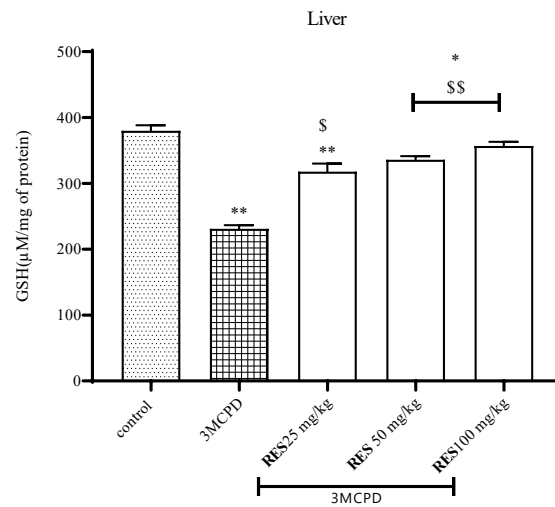


Figure 3. The results of measuring glutathione levels in the studied groups

*P<0.05, **P<0.01 compared to the control group, §P<0.05, §§P<0.01 compared to the 3-monochloropropan1-2diol group.

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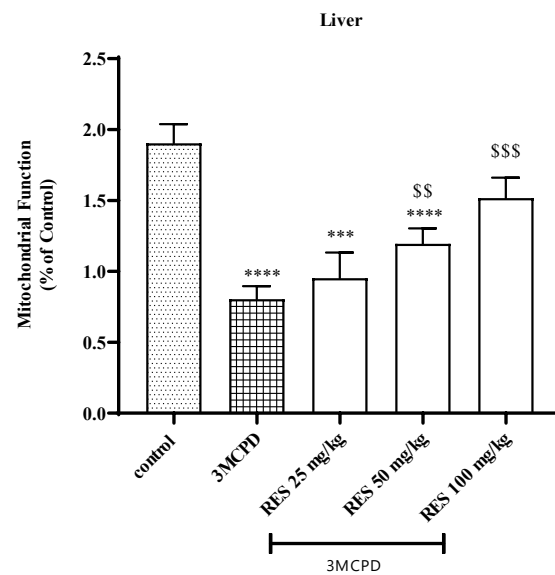


Figure 4. The results of the MTT test in the studied groups

P<0.001 and *P<0.0001 compared to the control group, SSSP<0.001, §§P<0.01 compared to the 3-monochloropropan1-2diol group.

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death signaling pathway. In this study, the studied factors were compared in isolated mitochondria and rat liver and kidney cells, which showed that the amount of ROS in rat liver and kidney cells was increased compared to the samples containing isolated mitochondria. According to the data obtained from these studies, it can be concluded toxicity of 3MCPD is related to the metabolites produced from this compound, which require a longer incubation time [17]. Previous studies confirmed that the

oxidative metabolism of 3MCPD leads to the production of three metabolites, such as β-chlorolactaldehyde, β-chloroacetic acid, and oxalic acid calcium oxalate in the proximal renal tubule, which was a critical possible nephrotoxic mechanism for 3MCPD. In addition, the production of 3MCPD metabolites inhibited the glycolysis pathway, which was introduced as another toxic pathway of this substance [4, 18]. Accordingly, using isolated mitochondria (as observed in the two studies by Zhang et

al. [15] and Peng et al. [16]) is not a suitable model to determine the mechanism of toxicity of 3MCPD and other compounds that must be metabolized to induce toxicity.

Oxalic acid, as the primary metabolite of 3MCPD, inhibits the glycolysis pathway and creates acidic conditions in isolated cells by creating redox potentials in the ETC, participating in the Haber-Weiss reaction, producing more superoxide and hydroxyl radicals. It disrupts the mitochondrial Krebs cycle and induces mitochondrial dysfunction [17].

Regarding lipid peroxidation in the present study, no significant difference was observed between the 3MCPD receiving and control groups. However, the results of our study showed that the amount of lipid peroxidation in the groups receiving RES with concentrations of 50 and 100 mg/kg was significantly reduced compared to the 3MCPD group ($P < 0.05$). Accordingly, RES can inhibit lipid peroxidation in rat liver cells. Considering that the treatment with 3MCPD did not show a significant difference in lipid peroxidation from the control group, it cannot be concluded that 3MCPD caused lipid peroxidation inhibited by resveratrol.

The results of a 28-day study by Schultrich et al. in 2019 showed that exposure to 3MCPD could not cause lipid peroxidation in all male rats. The authors stated that the lack of lipid peroxidation in kidney cells of male mice exposed to 3MCPD is insufficient ROS [19]. On the other hand, mitochondria, as one of the primary sources of ROS production, can induce lipid peroxidation in hepatocytes after exposure to 3MCPD. Also, a high level of polyunsaturated fatty acids (PUFA) in mitochondrial membranes enables lipid peroxidation only in high levels of ROS [20-22]. Based on this, the lack of lipid peroxidation in the present acute study can be due to insufficient ROS produced in the presence of 3MCPD to overcome the PUFA present in the mitochondrial membranes. The development of this complication is due to requiring longer exposure time and higher amounts of ROS.

RES is a biologically active phytoalexin with antioxidant properties and multiple phenolic groups. As a polyphenolic compound, RES can act as a free radical scavenger and trap them. In addition, many of the protective effects of resveratrol in the body are mediated by gene regulation. RES regulates the expression and activity of several redox enzymes, thereby inhibiting ROS production and facilitating ROS detoxification. This substance exerts its antioxidant effects by increasing the regulation of superoxide dismutase enzymes, increasing

the expression of GPx1 and catalase genes, influencing the Nrf2 pathway, and increasing the regulation of γ -glutamylcysteine synthetase, and increasing cellular glutathione. This substance can also prevent the production of oxygen-free radicals in mitochondria [23-25]. According to the above explanations, it can be concluded that RES can inhibit lipid peroxidation by directly and indirectly inhibiting free radicals.

The present study showed that simultaneous exposure to 3MCPD and RES with doses of 50 and 100 mg/kg could reduce the amount of oxygen free radicals produced in the liver cells of rats ($P < 0.01$). Furthermore, these data indicate that resveratrol at doses of 50 and 100 mg/kg can inhibit the increase in ROS caused by exposure to 3MCPD.

3MCPD can disrupt the mitochondrial electron transport in the cell, which is related to the disruption of the mitochondrial oxidative phosphorylation system, the increase of ROS level by its active metabolites, and the failure of MMP, leading to the release of cytochrome c from the mitochondria to the cytosol and Finally, cell death signal is activated [17]. Also, RES, having antioxidant properties, can directly inhibit ROS and trap existing free radicals. In addition, as explained above, this substance can help prevent the accumulation of free radicals in the cell by activating other antioxidant pathways.

The amount of glutathione in the groups receiving resveratrol with concentrations of 50 and 100 mg/kg was significantly higher than the group receiving 3MCPD ($P < 0.01$) and lower than the control group ($P < 0.05$).

The above results show that resveratrol has essentially prevented the reduction of cellular glutathione caused by exposure to 3MCPD in the liver of rats. Still, it cannot ultimately compensate for the decrease in cellular glutathione.

Glutathione is one of the essential bio-functional peptides that play an important role in xenobiotic metabolism. This protein can detoxify the body by directly conjugating many toxic substances and playing an antioxidant role by trapping free radicals. Free 3MCPD can also be directly conjugated with glutathione and thus excreted through urine. In a study conducted by Yang et al. in 2020, it is stated that glutathione family proteins are decreased in 3MCPD-induced intoxication. By conjugating free 3MCPD and its metabolites, these proteins can neutralize this substance's toxic effects and help eliminate it through urine [26]. In addition, 15 different metabolites of 3MCPD have been found so far, one of

which is glycidol. According to previous studies, the primary mechanism of inhibiting glycidol is the conjugation of this substance with glutathione [14, 18, 27]. On the other hand, glutathione could help control oxidative stress in cells by neutralizing free radicals. In this way, RES can help increase the amount of glutathione in liver cells exposed to 3MCPD by trapping free radicals and having antioxidant properties.

The results of the MTT test show that RES with concentrations of 50 and 100 mg/kg can improve mitochondrial function in rat liver cells exposed to 3MCPD.

The study by Peng et al. showed that 3MCPD induced a significant reduction of MMP. Similar findings have been reported concerning MMP decay in R2C Leydig cells after exposure to 3MCPD [28-30]. 3MCPD down-regulates Bcl-2 and regulates Bax, which is associated with 3MCPD-induced MMP reduction and breaks the balance between Bcl-2 and Bax. Therefore, the decrease in Bcl-2/Bax value will cause the reduction in occurred MMP and induce apoptosis. Furthermore, 3MCPD can induce mitochondria-mediated apoptosis by regulating the expression of Bcl-2 family proteins at the protein and mRNA expression level in HEK293FT cells [16, 31, 32]. Previous studies using the proteomic approach show that 3MCPD causes respiratory chain disorder in rat kidneys [33-36]. In the study of Peng et al., human cells treated with 3MCPD showed a significant impairment of oxidative phosphorylation, especially reduction of complex III and ATP synthases [16]. Decreased protein synthesis of electron transport chain complex II (SDHA), complex IV (COX17), and incredibly complex III (UQCRCQ) caused by 3MCPD can lead to a decrease in the pH gradient and electrical potential across the membrane, thus reducing the mitochondrial membrane potential. Another vital pathway contributing to the reduction of ATP production is the uncoupling of oxidative phosphorylation by loss of membrane potential required for ATP synthesis. Depletion of ATP can eventually lead to mitochondrial dysfunction and cell death [10, 37, 38].

Conclusion

The findings showed that 3MCPD could cause toxicity, disrupt mitochondrial function, and reduce glutathione by inducing oxidative stress and increasing the amount of ROS in rat liver cells. Also, resveratrol can reduce the toxic effects of 3MCPD due to its direct and indirect antioxidant properties.

Ethical Considerations

Compliance with ethical guidelines

All experimental procedures were conducted according to the ethical standards and protocols approved by the Committee of Animal Experimentation of [Mazandaran University of Medical Sciences](#) (Code: IR.MAZUMS.REC.1400.173).

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Authors' contributions

Study administration: Mohammad Shokrzadeh, Farzaneh Motafeghi; Study design, data collection, data analysis, review, and editing: Farzaneh Motafeghi; Manuscript preparation: Mahsa Deylamian.

Conflict of interest

The authors declared no conflict of interest.

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