

Protective Activity of Chitosan Nanoparticle against Cadmium Chloride Induced Gastric Toxicity in Rat

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ABSTRACT

Objective: This study was carried to investigate the role of chitosan nanoparticle in protecting against cadmium chloride-induced gastric toxicity in the rat. **Methods:** The 50 male rats were divided into 5 group: negative control (Rats were given daily with aquadest) ; positive control (Rats were given daily with cadmium chloride 5 mg/kg BW orally once in a day for 28 days) and the treatment group (Rats were given the chitosan nanoparticle 150 mg; 300 mg; 600 mg/kg BW orally once in a day for 32 days and on 4th day, were given cadmium chloride 5 mg/kg BW one hour after the chitosan nanoparticle administration for 28 days). On day 32, the rats were sacrificed, and gastric tissues were collected to measure Malondialdehyde (MDA), Superoxide Dismutase (SOD), Glutathione Peroxidase (GPx) and histological evaluations. **Results:** Oral administration of cadmium chloride 5 mg/kg BW for 28 days significant induced gastric mucosal hemorrhagic lesions, increase MDA, decrease SOD and GPx, and also caused necrosis of gastric mucosal epithelial cell in the rat. Treatment with the chitosan nanoparticle 600 mg/kg BW but not 150 mg/kg BW and 300 mg/kg BW

significantly improved gastric injury, decreased MDA, increase in SOD and GPx levels, and also improved necrosis of gastric mucosal epithelial cell as compared to positive control group. **Conclusion:** From the results of this study concluded that the chitosan nanoparticle could be a potent natural product provide a promising gastroprotective effect against cadmium chloride induced gastric toxicity in rats.

Key words: Chitosan nanoparticle, Cadmium chloride, Gastric ulcer, MDA, SOD, GPx.

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INTRODUCTION

Cadmium, a toxic heavy metal and a widespread environmental pollutant which induces severe alterations in the body tissues of both humans and animals. It has been reported the physiological, biochemical, and behavioral effects of this toxic cadmium in the animal, including disorders of the cardiovascular system, kidney, liver, reproductive system and gastrointestinal system.^{1,2}

The mechanism of cadmium gastric toxicity is the oxidative stress and there is an imbalance between the scavenging capacity of antioxidants and the generation of reactive oxygen species (ROS) in the gastric. Recently study shown the reactive oxygen species (ROS) or free radicals such as superoxide ion (O₂⁻), hydroxyl radical (OH⁻) and nitrogen oxide (NO) have a pivotal role in cadmium-induced gastric toxicity.^{1,3} MDA may be used as an indicator of cell membrane damage after exposure to reactive oxygen species and free radicals. The increase in MDA levels in gastric suggests that antioxidant failure to inhibit reactive oxygen species and formation of free radicals so can enhance lipid peroxidation causing gastric toxicity.^{2,4} The MDA level is the direct evidence of tissue injury processes caused by free radicals.⁵⁻⁶

It has been reported that the natural products having antioxidant properties by reducing free radical-induced tissue damage. Natural products have advantages over the conventionally used drugs that are much expensive and known to have harmful side effects for the treatment of various disease.⁷⁻⁸

Many authors tried antioxidant of a natural product like chitosan, quercetine; piperine; curcumin against heavy metal toxicity.⁹⁻¹⁰ Several studies have shown that chitosan has remarkable biological activities

such as antioxidant, anti-inflammatory and analgesic, antihypertensive, antitumoral, and antibacterial activities.¹¹⁻¹² Not only restricted to those activities, but also chitosan modification will enhance and open various ways to utilize chitosan. The rationale for this is that chitosan modification will keep the original biochemical and physiochemical properties of chitosan and make the new properties of the group introduced to them at the same time.¹³⁻¹⁴

Over the last decade, the modifications of chitosan have been reported. The modification of chitosan nanoparticle has received much attention in relation to their potential application, especially from a pharmaceutical viewpoint.¹⁵⁻¹⁶ Nanoparticle chitosan has attracted attention due to its biodegradability, biocompatibility, cost-effectiveness, high permeability and non-toxic property. Moreover, its ability to enhance the penetration of large molecules across a mucosal surface and its recognition as muco adhesivity chitosan.^{13,16} However, the gastroprotective effect of chitosan nanoparticles has seldom been reported elsewhere. The unique character of chitosan nanoparticles for their small size and quantum size effect could make chitosan nanoparticles exhibit superior activities. Chitosan nanoparticle very interesting to be developed as a new therapeutic drug because it has a good biodistribution, high sensitivity and low pharmacological toxicity.¹⁵⁻¹⁶

It has been reported that antioxidant activity or inhibition of generation of free radicals plays a crucial role in protection against cadmium toxicity. So, can be claimed that protective agents, such as antioxidants, may be useful therapeutic for against free radicals on heavy metal toxicity in gastric.² It has been reported that chitosan has an antioxidant effect.^{7,13}

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In the present study, the protective effects of chitosan nanoparticle was investigated on cadmium chloride-induced gastric damage in rats.

MATERIALS AND METHODS

Ethical approval

The study was conducted in the Department of Pharmacy-Biology, Faculty of Pharmacy, Hang Tuah University. All procedure employed was approved by the Ethical Clearance Committee for preclinical research, Faculty of Pharmacy, Hang Tuah University, Surabaya, Indonesia and obtained ethical clearance under No.88/UHT/6/2017.

Experimental animals

Male Wistar albino rat weighing approximately 200-250 g (2.5-3 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 $26 \pm 2^\circ\text{C}$ and 12 h alternates' light and dark cycles. The rats were given *ad libitum* with tap water and fed with a standard commercial rat.

Preparation of chitosan

The production of chitosan from shrimp shell consists of demineralization, deproteinization, and deacetylation.¹¹ The shrimp shells were demineralized by agitating continuously with 5% HCl at the ratio of 1:15 (w/v, shell to a solution) 36 h at room temperature. The demineralized shells were treated with 5% NaOH solution at the ratio of shell to a solution of 1:10 (w/v) at 90-95°C for 6 h. The deproteinized shells were filtered and washed with tap water until NaOH was removed completely, then dried overnight in an oven at 55-60°C. The shells were filtered and washed with tap water until became neutral. Then deacetylation of chitosan was carried out by hydrolyzing with 80% NaOH at the ratio of 1:20 (w/v, chitin to solvent) at 90-95°C for 5 h. This product was washed with tap water until it became neutral and dried overnight at 55-60°C. In the preparation of chitosan solutions, 1.0% (w/v) chitosans were dispersed in a 1.0% (v/v) acetic acid solution.

Preparation of chitosan nanoparticles

The ionotropic gelation method most frequently used to create chitosan nanoparticles which using sodium tripolyphosphate as a crosslinking agent.^{14,15} Initially in order to make the homogeneous chitosan solution, 1.5 g of chitosan dissolved in 200 ml of 2% acetic acid solution was kept under magnetic stirring process for about 20 min. The chitosan solution was added drop wise of 0.8 g of sodium tripolyphosphate dissolved in 107 ml of conductivity water and stirred well for about 30 min to reach equilibrium. A milky colored emulsion like a appearance of chitosan nanoparticles was formed upon the ionic cross-linking between the sodium tripolyphosphate and chitosan solution. After reaching equilibrium, The suspension was formed in above-mentioned conditions. The nanoparticles were separated by centrifugation at 20,000 g and 14°C for 30 min, freeze-dried and stored at $5 \pm 3^\circ\text{C}$.

Experimental design

Fifty male Wistar rats were divided randomly into five groups as the following: negative control group (rats were given daily with aquadest); positive control group (rats were given daily with cadmium chloride 5 mg/kg BW orally once in a day for 28 days) and the treatment group (rats were given the chitosan nanoparticle in dose of 150 mg, 300 mg, and 600 mg/kg BW orally by gastric gavages once in a day for 32 days and cadmium chloride 5 mg/kg BW were given on 4th day, one hour after the chitosan nanoparticle administration for 28 days. On day 32, all of the rats were sacrificed after anesthetization by diethyl ether inhalation, then their gastric were excised. Tissues of gastric were homogenized in ice-cold 50 mM sodium phosphate buffer (pH 7.4) containing 0.1 mM ethylene diamine tetraacetic acid (EDTA). The supernatant was separated

by centrifugation at 1000 g for 20 min at 4°C. The supernatant was used for the analyzes of MDA and antioxidant enzymes (SOD and GPx). The gastric was removed and opened along the greater curvature to evaluate hemorrhagic erosions (gastric injury). The gastric was also fixed in a 10% neutral buffered formalin solution for histopathological examination

Macroscopic evaluation of gastric ulcer

The gastric ulcer was assessed on a scale of 0 to 3: 0= normal, 1= one to four petechiae, 2= five or more petechiae or hemorrhagic streaks up to 4 mm and 3= erosions longer than 5 mm or confluent hemorrhages.^{4,17}

The protection percentage (%) of gastric ulcer was calculated as follows:

$$\text{Inhibition (\%)} = [(Uc - Ut)/Uc] 100$$

Where Uc = ulcer of the control positive group and Ut = ulcer of the treatment group

Measurement of MDA level

MDA was determined in the supernatant of homogenate gastric tissue by the thiobarbituric acid (TBA) method which estimates the MDA formation. The concentration of MDA was measured at 532 nm and calculated by the absorbance coefficient of MDA-TBA complex.⁸ MDA is expressed as nanomoles MDA/g tissue.

Measurement of antioxidant enzymes

Tissue preparation for enzyme assay of rat gastric was rapidly thawed from -70°C at room temperature for 5 min and manually homogenized in cold phosphate buffer (pH 7.4) and debris removed by centrifugation at 3500 g for 10 min (Centrifuge 5415 R, Eppendorf AG, Hamburg, Germany). Supernatants were recovered and used for enzyme activity and protein assays.

The activity of SOD was measured with SOD detection kit according to the manufacturer's instructions from Cayman Chemicals (USA, Cat. No. 706002). The assay was based on the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the quantity of enzyme required to induce 50% dismutation of the superoxide radical. Read the absorbance at 440-460 nm using a plate reader. The results are expressed in $\mu\text{moles /mg protein tissue}$ for the gastric tissue.⁷

The activity of GPx was measured with GPx detection kit according to the manufacturer's instructions from Cayman Chemicals (USA, Cat. No. 703102). The GPx activity was measured indirectly by a coupled reaction with glutathione reductase, where the oxidized glutathione was produced upon the reduction of hydroperoxide by GPx. Read the absorbance at 340 nm using a plate reader. The GPx activity was expressed as $\mu\text{moles of GPx/mg protein tissue}$ for the gastric tissue.^{2,6}

Histopathological examination

The tissue of gastric was fixed in a 10% neutral buffered formalin solution, embedded in paraffin and used for histopathological examination with hematoxylin and eosin (H and E) stain.³

Statistical analysis

Data were presented as means \pm standard deviation. One-way 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 V. 17.0).

RESULTS

Gastroprotective activity of chitosan nanoparticle against cadmium chloride-induced gastric ulcer

Oral administration of cadmium chloride significant induced gastric mucosal hemorrhagic lesions when pre-treated only with aquadest (positive control). Pretreatment with 600 mg/kg BW but not 150 mg/kg

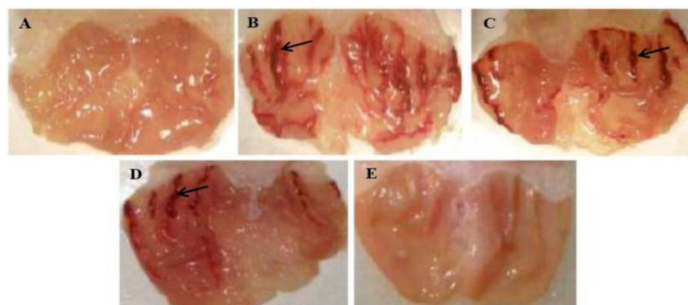


Figure 1: The macroscopic features of the rats cadmium chloride-induced gastric ulcer following treatment with chitosan nanoparticle. Negative control (A); Positive control (B); and chitosan nanoparticle at the concentrations of 150 mg / kg BW (C), 300 mg / kg BW (D) and 600 mg / kg BW (E). The hemorrhagic gastric ulcer was indicated by black arrows in positive control, chitosan nanoparticle at the concentrations of 150 mg / kg BW and 300 mg / kg BW.

Table 1: Gastroprotective effect of chitosan nanoparticle on cadmium chloride-induced gastric ulceration.

Group	Gastric Ulcer	Inhibition of Gastric Ulcer (%)
Negative Control	0 ^a ± 0	-
Positive Control	3.7 ^b ± 0.55	-
Chitosan Nano 150 mg/kg BW	3.4 ^b ± 0.69	8.2
Chitosan Nano 300 mg/kg BW	2.9 ^b ± 0.76	21.6
Chitosan Nano 600 mg/kg BW	0.8 ^c ± 0.35	78.4

^{abc}Different superscript within each column indicate significant difference between the means ($P < 0.05$)

BW and 300 mg/kg BW chitosan nanoparticle demonstrated significant ($p < 0.05$) reduction in gastric injury in a dose-dependent manner compared with positive control (Figure 1). Gastroprotective effect of chitosan nanoparticle at dose 150 mg/kg BW; 300 mg/kg BW and 600 mg/kg BW on cadmium chloride-inhibited gastric ulceration was 8.2; 21.6 and 78.4 %, respectively (Table 1).

Effects of chitosan nanoparticle on cadmium chloride-induced changes in MDA, SOD, and GPx in gastric tissue

Analysis of MDA has been done to evaluate the chitosan nanoparticle in cadmium chloride treated rats. The positive control group (cadmium chloride treated rats) showed significantly ($P < 0.05$) increase in MDA level comparing with the negative control group. In contrast, the groups pretreated with chitosan nanoparticle at dose 600 mg/Kg BW but not at dose 150 mg/Kg and 300 mg/kg BW showed significantly ($P < 0.05$) decreased MDA level with respect to the positive control towards normalization and close to the negative control group (Table 2).

Cadmium chloride enhances the intracellular formation of reactive oxygen species causing gastric damage. In the present study, we analyze the gastric levels of several antioxidants (SOD and GPx). Positive control (cadmium chloride treated rats) showed significant ($P < 0.05$) decrease in the level of SOD and GPx compared with negative control. Groups pretreated with chitosan nanoparticle at dose 600 mg/Kg BW but not at dose 150 mg/Kg and 300 mg/kg BW showed a significant ($P < 0.05$) increase in the level of SOD and GPx level compared with cadmium chloride treated rats, and close to the negative control (Table 2).

Effects of chitosan nanoparticle on cadmium chloride induce gastric injury in the histopathological study

Histopathological study was investigated using light microscopy. Microscopic examination of negative control showing intact mucosal epithelial

Table 2: Effects of Chitosan nanoparticle on cadmium chloride induced changes in the Malondialdehyde, Superoxide Dismutase and Glutathione Peroxidase.

Groups	Means ± Standard Deviation		
	SOD ($\mu\text{mol}/\text{mg}$ tissue)	GPx ($\mu\text{mol}/\text{mg}$ tissue)	MDA (nmol/mg tissue)
Negative Control	64.21 ^a ± 6.23	43.63 ^a ± 4.85	21.83 ^a ± 1.75
Positive Control	35.17 ^b ± 3.42	27.43 ^b ± 3.81	34.14 ^b ± 2.83
Chitosan Nano 150 mg/kg BW	33.32 ^b ± 4.53	29.32 ^b ± 4.11	35.23 ^b ± 3.12
Chitosan Nano 300 mg/kg BW	41.62 ^b ± 5.82	32.29 ^b ± 3.95	31.82 ^b ± 2.89
Chitosan Nano 600 mg/kg BW	54.67 ^c ± 4.67	39.82 ^c ± 4.36	25.12 ^c ± 2.42

^{abc}Different superscript within each column indicate significant difference between the means ($P < 0.05$).

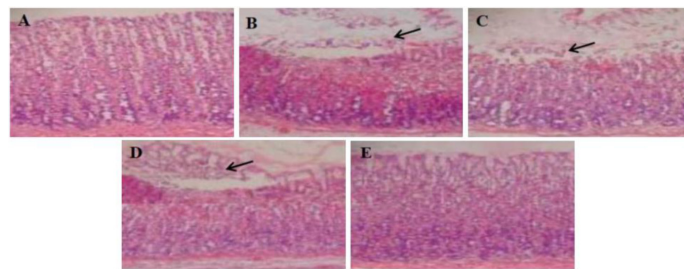


Figure 2: Histological study of pretreatment with chitosan nanoparticle on cadmium chloride induce gastric toxicity. Normal morphological of gastric sections in negative control group (A). The cadmium chloride treated group showed the necrosis in gastric mucosal epithelial cells (indicated by black arrows) (B). Rats treated with chitosan nanoparticle 150 mg/kg BW and 300 mg/kg BW showed necrotic changes (C and D). Rats treated with chitosan nanoparticle 600 mg/kg showed improvement in gastric mucosal epithelial cells (E). H and E $\times 400$.

cell. In cadmium chloride treated, gastric tissues showed necrosis mucosal epithelial cell indicates gastric injury. Pretreatment with chitosan nanoparticle at dose 600 mg/Kg BW but not at dose 150 mg/Kg and 300 mg/kg BW were significantly prevented necrosis mucosal epithelial cell compared with cadmium chloride treated rats, and close to the negative control (Figure 2). Observations indicate that the gastric toxic effects of cadmium chloride were reduced by chitosan nanoparticle.

DISCUSSION

In the current study, we evaluated the protective role of chitosan nanoparticle against the oxidative stress changes in the gastric tissue resulting from the administration of cadmium chloride in rats. These results showed that cadmium chloride administration significantly decreased the SOD, GPx and increased MDA levels. Cadmium chloride also induced gastric ulcer and altered histopathological gastric compared to the negative control group. Cadmium chloride-induced gastric damages have been attributed, at least in part, to toxicant-induced oxidative stress. It results suggest that cadmium chloride induces the formation of ROS, thus inducing damage of various tissues resulting in loss of membrane functions. Long-term exposure to cadmium increases MDA or lipid peroxidation and causes inhibition of SOD and GPx activity inducing oxidative damage in gastric.¹⁸⁻¹⁹ Cadmium chloride toxicity leads to the production of free radical, that consists of hydroperoxides, singlet oxygen, and hydrogen peroxides, which can increase MDA levels as the products of lipid peroxidation.² The present study is shown in signifi-

cantly increased MDA levels in the gastric of cadmium chloride-treated rats in comparison to the negative control. This means that it increased the oxidative stress in the cadmium chloride-treated rats. Therefore, the significantly lower levels of MDA in the gastric tissues of chitosan nanoparticle treated groups as compared with the cadmium chloride group indicate attenuation of lipid peroxidation. It is known that cadmium chloride-induced oxidative stress and tissue damage could be caused by increased production of free radicals and by causing a direct decrease of antioxidant reserves.¹⁻² Intense lipid peroxidation caused by cadmium exposure may affect the cytoplasmic membranes and mitochondrial, causing damage to the tissues and releasing lipid hydroperoxides into circulation which reflects the induction of oxidative stress.² The chitosan nanoparticle, which behaves as a powerful antioxidant and free radical scavenger, can decrease the MDA level perturbed by cadmium chloride in rats gastric, as observed in this study. Treatment of rats with chitosan nanoparticle at a dose of 600 mg/kg BW prevented the levels of MDA to rise when the rats were challenged with cadmium chloride. This means that chitosan nanoparticle minimized the toxic effect of cadmium chloride via its antioxidant activity. The antioxidant protective mechanism decreases the ROS and scavenges the free radical responsible for the gastric damage and thus inhibit the lipid peroxidation as measured by MDA levels.³ The findings of this study suggest that chitosan nanoparticle could attenuate oxidative stress by decreasing the lipid peroxidation (MDA level) in the cadmium-treated gastric.

A similar result has shown that vitamin C and vitamin E enhanced the antioxidant status and inhibited lipid peroxidation in rats with cadmium chloride toxicity. These findings indicate that the antioxidant activity of vitamin C and vitamin E are targeted primarily towards the lipid component of cells. Antioxidants such as vitamin C and vitamin E have been reported to inhibit the free radical formation and are effective in minimizing lipid peroxidation in several different biological systems.²⁰

The SOD and GPx are important antioxidant enzymes. They constitute a mutually supportive defense mechanism against free radical. SOD decomposes superoxide radicals (O_2^-) to produce H_2O_2 . GPx is a selenoenzyme which has played a major role in the decrease of H_2O_2 and hydroperoxide to produce nontoxic products. Therefore, the activities of SOD and GPx have been used to assess oxidative stress in cells.⁶⁻⁷ The cadmium chloride has a high affinity for Sulfhydryl (SH) groups in several enzymes such as SOD and GPx, thus it can alter antioxidant activities by inhibiting functional SH groups in these enzymes.^{1,19} In the present study, the activity of SOD and GPx in gastric rats was decreased by cadmium chloride treatment. This decreased SOD and GPx activities with cadmium chloride treatment are in agreement with previous studies. This suggested that cadmium chloride exposure induced oxidative stress by inhibiting the activity of this antioxidant enzyme. Interestingly, the administration of chitosan nanoparticle doses-dependent manner increased the activities of SOD and GPx in the gastric of cadmium-treated rats, which might be due to the ability of chitosan nanoparticle to reduce the accumulation of free radicals. Chitosan nanoparticle acts as a scavenger for the oxygen-derived free radicals which can inhibit free radical formation, thus protecting from gastric damage.^{13,21}

It has been found a decrease MDA levels and an increase in the antioxidant enzyme parameters including SOD, CAT, and GPx in the plasma and tissue such as liver, kidney, and brain of animals that were administered chitosan in association with heavy metal, in comparison to the group that was administered heavy metal alone.¹⁻²

Histopathological results demonstrating structural changes in the gastric tissue of heavy metal toxicity such as cadmium chloride were reported by some researchers. In the present study, histopathological view of gastric sections in the cadmium chloride treated group showed the gastric damage and necrosis of gastric mucosa epithelial cell as compared to the

negative control group. The rats pretreated with chitosan nanoparticle 600 mg/kg BW demonstrated significantly improved necrosis of gastric mucosal epithelial cell.

Further investigation of these promising protective effects of chitosan nanoparticle against cadmium chloride-induced gastric damage may have a considerable impact on developing clinically feasible strategies to treat patients with cadmium chloride-induced gastric ulcer.

CONCLUSION

It could be concluded that chitosan nanoparticle may exert its protective actions against cadmium-induced gastric injury in rats, possibly through its antioxidant mechanisms. Chitosan nanoparticle can be a future natural product for counteracting the cadmium chloride intoxication. These results showed that chitosan nanoparticle has a potential gastroprotective effect in a dose-dependent manner that minimizes or diminish the gastric toxicity effect of cadmium chloride.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

MDA: Malondialdehyde; **SOD:** Superoxide Dismutase; **GPx:** Glutathione Peroxidase; **ROS:** Reactive Oxygen Species; **CAT:** Catalase; **BW:** Body Weight.

SUMMARY

Administration of Cadmium chloride-induced gastric toxicity might be related to oxidative damage. Co-administration of chitosan nanoparticle lessened the effects of cadmium chloride-induced gastric toxicity possibly by inhibiting free radical-mediated process and increasing antioxidant (SOD and GPx) activity. Further investigation of these promising protective effects of chitosan nanoparticle against cadmium chloride-induced gastric damage may have a considerable impact on developing clinically feasible strategies to treat patients with cadmium chloride-induced gastric ulcer.

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