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Isfahan University of Medical Sciences

Original Article

Antioxidant and genoprotective effects of osthole against cadmium-induced DNA damage: an *in vitro* study using comet assay

Ehsan Vahidifar[1], Seyed Ebrahim Sajjadi[2], and Mahmoud Etebari[1,*]

[1]Department of Pharmacology and Toxicology and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.
[2]Department of Pharmacognosy and Isfahan Pharmaceutical Sciences Research Center, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

Abstract

Background and purpose: Osthole, a plant-derived coumarin, has shown numerous pharmacological effects. However, its genoprotective effects against cadmium-induced DNA damage have not been determined yet. Therefore, this project aimed to assess the effectiveness of osthole against genotoxicity caused by cadmium.
Experimental approach: For this purpose, human umbilical vein endothelial cells (HUVECs) were incubated with various concentrations of osthole (40, 60, 80, and 120 µM) 24 h before cadmium chloride ($CdCl_2$) treatment (40 µM), and then DNA damage was evaluated by comet assay. Furthermore, DPPH and free thiol group assays were applied to evaluate reactive oxygen species scavenger and antioxidant capacities of osthole.
Findings / Results: In the present study, all concentrations of osthole significantly decreased $CdCl_2$-induced DNA damage. Furthermore, the antioxidant properties of the osthole were confirmed by DPPH and free thiol assays.
Conclusion and implications: Overall, the findings of this project revealed that osthole could ameliorate cadmium-induced genotoxicity probably by its antioxidant activity.

Keywords: Cadmium; Comet assay; DNA damage; Genotoxicity, Osthole; Oxidative stress.

INTRODUCTION

As a heavy metal, cadmium can continuously, be added to the environment because of its abundant industry application (1). It can quickly enter plants and the food chain due to its high solubility. Food materials and cigarette smoke are some of the primary sources of the mentioned metal. Cadmium's main exposure routes are inhalation, oral, and dermal for animals and humans (2). The absorption amount of cadmium depends on the exposure way. Three to ten percent of cadmium absorption is related to the oral route, whereas 50% is inhalation. After absorption, cadmium has a rapid and complete distribution to various body organs. The half-life of cadmium in humans is 15-20 years (3). The amount of cadmium in different tissues is associated with metallothionein (MT) content, which indicated a tremendous binding affinity to cadmium. Despite reduced production and application of cadmium in countries, it is a primary concern for human health due to its high stability and durability in the organism body and milieu (4). Cadmium is a toxic metal whose toxicity is shown in plants, animals, and humans. Cadmium's toxic effects are induced on human organs, including the liver, kidney, heart, lung, and brain, through various mechanisms such as oxidative stress, inflammation, and apoptosis (5).

*Corresponding author: M. Etebari
Tel: +98-9125154823, Fax: +98-3136680011
Email: etebari@pharm.mui.ac.ir

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The risk of cancer and mortality has been shown to increase with low cadmium levels. Previous studies have shown that the mechanism of cadmium toxicity is the induction of oxidative stress and mitochondrial dysfunction (6-8), and cadmium bound to sulfhydryl groups of proteins causes glutathione depletion, decreasing reactive oxygen species (ROS)-detoxification capacity. ROS accumulation is associated with knocking down cellular molecules such as proteins, lipids, and DNA (9, 10). Other researchers have confirmed cadmium-induced DNA damage (10). Cadmium not only has a potent ability to induce genotoxic effects such as sister chromatid exchanges, DNA strand breaks, DNA-protein crosslinks, and chromosomal aberrations but also can suppress the DNA repair process (9). Cadmium genotoxicity associated with some cancers such as lung, kidney, testes, and prostate has been shown in the literature (3, 11). Therefore, it is essential to maintain organism cells from cadmium's destructive effects on cellular macromolecules, especially DNA.

Osthole or 7-methoxy-8-(3-methyl-2-butenyl) coumarin has been extracted from medicinal plants, including *Cnidium monnieri* (L.) Cusson, (12). Osthole has numerous pharmacological functions such as anti-inflammatory, antioxidative, antiapoptotic, anti-diabetic, anti-allergic, and anticancer activities (13, 14). In addition, it is reported that osthole, as a potent antioxidant agent, can scavenge ROS and suppress lipid peroxidation (15). However, its possible protective effects against cadmium-induced DNA damage have not been evaluated yet.

Single-cell gel electrophoresis (SCGE) or comet assay method as a reliable technique is used to evaluate DNA damage. Simplicity in use, sensitivity, ability to achieve quantitative results, short time, and low costs are advantages of the comet method, which can apply in any eukaryotic cell to determine genotoxicity (16, 17).

According to our surveys, there is no information about the protective effects of osthole against cadmium-induced genotoxicity. Therefore, the present study aimed to examine osthole genoprotective effects against cadmium-induced DNA damage in human umbilical vein endothelial cells (HUVECs).

MATERIAL AND METHODS

Chemicals

Cadmium chloride ($CdCl_2$), low-melting-point agarose (LMA), ethidium bromide, Tris-HCL, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich, USA. Osthole is a gift from Dr. Sajjadi. Dulbecco's modified eagle's medium (DMEM) was achieved by Bioidea company, Iran, and Biosera, France supplied fetal bovine serum (FBS) and penicillin/streptomycin. H_2O_2, NaCl, KH_2PO_4, and KCL were procured from Merck Company, Germany. All reagents were of analytical grade.

Osthole

Professor Sajjadi donated the crystallized form of the osthole. A plant sample is deposited at the herbarium of the Department of Pharmacognosy, School of Pharmacy, Isfahan University of Medical Sciences, Iran (Voucher No. 1126).

Cell culture

The Iranian National Center for Genetic and Biologic Resources presented the HUVEC cell line. DMEM medium containing 10% FBS and 100 U penicillin/streptomycin was used for cell culture in a CO_2 incubator at 37 °C. First, HUVEC cells (40×10^4) were incubated for 24 h in a 12-well plate. After that, different concentrations of $CdCl_2$ (5, 10, 20, 40 µM) and osthole (40, 60, 80, and 120 µM) were added to cells, and after incubation for 24 h, the comet assay method was carried out to achieve the minimum genotoxic and safe concentrations of $CdCl_2$ and osthole, respectively.

Comet assay

The single-cell gel electrophoresis (comet assay) was performed according to the previous studies (18). HUVEC cells (40×10^4 cells) were exposed to a selected concentration range of osthole (40, 60, 80, and 120 µM) for 24 h, thereafter the culture medium was changed, and incubation with $CdCl_2$ at 40 µM was continued for a further 24 h. Then,

the HUVEC cells were washed with PBS two times and suspended in it (1 mL). After that, cells were dissolved in 1% LMA and spread onto slides preloaded with normal agarose (1.5%). The slides were plunged in cold lysing buffer containing 2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100 (pH 10) for 1 h. Then, samples were maintained in a gel electrophoresis tank full of cold electrophoretic buffer containing 1 mM Na_2EDTA and 300 mM NaOH (pH 13) for 20 min, and after that, the samples were electrophoresed at 300 mA and 20 V for 30 min. The slides were neutralized by 1 mL of 0.4 M Tris base (pH 7.5) three times for 5 min each. Fifty µL of ethidium bromide (20 µg/mL) were spread onto the slides. The image of slides was taken at ×400 magnification using fluorescent microscopy. One hundred randomly selected cells of each slide were analyzed by the Comet Score software project (CASP). Three factors, including tail length, percent of DNA in the tail, and tail moment, were evaluated to measure the degree of DNA damage.

Thiol assay

Thiols play a prominent role in maintaining cells from free radical's damage. In the present study, free thiol groups were evaluated by a commercial kit using the spectrometry method (KTHI96, Kiazist, Iran) according to the manufacturer's protocol.

DPPH assay

2,2-Diphenyl-1-picrylhydrazyl (DPPH) method is used to evaluate the antioxidant activity of natural components. DPPH, as a free radical agent, has been shown hydrogen acceptor ability towards antioxidants. DPPH assay was performed by a method previously explained (19). In brief, 2 mg of DPPH was dissolved in 50 mL methanol. Different concentrations of osthole (40, 60, 80, and 120 µM) were added to a 96-well plate. Then, 100 µL of DPPH solution was added to the various concentrations of osthole. Plate incubation was performed at ambient temperature and dark conditions for 30 min. After that, the absorbance of samples was measured by an ELISA reader at 517 nm.

Experimental design

In this research, the following steps have been performed: (1) determination of the minimum genotoxic concentration of $CdCl_2$, (2) determination of the safe concentration range of osthole, (3) determination of genoprotective effect of different concentrations of osthole against the minimum genotoxic concentration of cadmium, and (4) investigation of the effect of cadmium and osthole on the amount of thiol and ROS.

Statistical analysis

GraphPad Prism software (9.0.0) was used for analyzing all data. One-way analysis of variance (ANOVA) followed by the Tukey-Kramer post hoc test was used for data analysis. The results are expressed as mean ± SEM. *P* values < 0.05 were considered significant.

RESULTS

Antioxidant activity of osthole

Figure 1 indicates the findings obtained by the DPPH radical scavenging activity, which is a method for radical-scavenging activity. Antioxidant activity of osthole increased with its concentration.

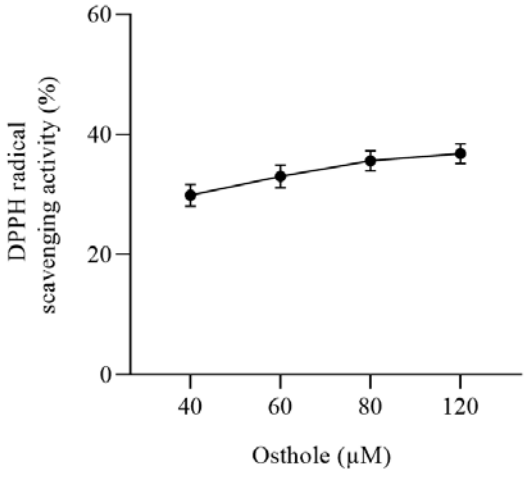

Fig. 1. Percent of DPPH radical scavenging activity of different concentrations of osthole. Data are expressed as the mean ± SD. DPPH, 2,2-Diphenyl-1-picrylhydrazyl.

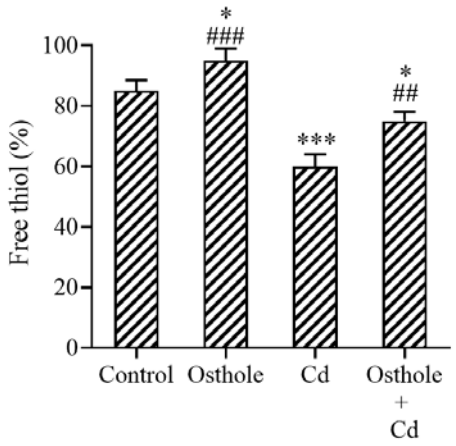

Fig. 2. Osthole (100 µM) and CdCl2 (40 µM) affect free thiol groups. Results are expressed as the mean ± SEM. **P* < 0.05 and ****P* < 0.001 indicates significant differences compared to the control group; ##*P* < 0.01 and ###*P* < 0.001 versus Cd group. Cd, cadmium.

Thiol assay

As shown in Fig. 2, free thiol groups were decreased in the cadmium group compared to the control. In addition, pretreatment cells with osthole at 100 µM before $CdCl_2$ administration increased free thiol groups in comparison with $CdCl_2$ alone (40 µM).

Genotoxicity of cadmium and safety of osthole

To determine the genotoxic potential of cadmium and the non-genotoxic concentration of the osthole, we used the single-cell gel electrophoresis (comet assay). Our findings showed no significant changes in the evaluated concentration range of the osthole compared to the control group (Table 1). However, as shown in Table 2, cadmium-induced genotoxic effects in HUVEC cells in all used concentrations.

The effects of osthole on DNA damage of $CdCl_2$

The possible genoprotective effect of osthole has been evaluated by comet assay. The software scoring and percentage of total DNA damage induced by $CdCl_2$ and prevention by osthole were shown in Fig. 3. A significant increase in DNA damage was observed in the $CdCl_2$ group compared to the control group. We used 40 µM of $CdCl_2$ as optimum genotoxic concentration, and osthole in the pretreatment conditions decreased significantly the level of DNA fragmentation compared to the $CdCl_2$ group.

Table 1. Evaluation of the safety of the osthole. All three parameters were not significantly different from the control group. Data express mean ± SEM.

Treatments	Tail length (pixels)	% DNA in tail	Tail moment
Negative control	2.42 ± 0.08	4.77 ± 0.2	0.16 ± 0.01
Osthole (40 µM)	2.73 ± 0.07	6.01 ± 0.21	0.21 ± 0.01
Osthole (60 µM)	2.54 ± 0.12	5.84 ± 0.24	0.18 ± 0.01
Osthole (80 µM)	2.48 ± 0.10	5.44 ± 0.32	0.18 ± 0.01
Osthole (120 µM)	2.41 ± 0.09	4.82 ± 0.14	0.16 ± 0.01

Table 2. Evaluation of the genotoxicity of cadmium. ****P* < 0.001 indicates significant differences compared to the negative control group. Data express mean ± SEM.

Treatments	Tail length (pixels)	% DNA in tail	Tail moment
Negative control	2.48 ± 0.26	5.02 ± 0.01	0.26 ± 0.01
$CdCl_2$ (5 µM)	13.48 ± 1.14***	18.17 ± 0.52***	2.17 ± 0.12***
$CdCl_2$ (10 µM)	16.47 ± 1.29***	21.34 ± 0.30***	3.87 ± 0.2 ***
$CdCl_2$ (20 µM)	18.03 ± 1.19***	22.91 ± 0.62 ***	4.62 ± 0.45***
$CdCl_2$ (40 µM)	24.04 ± 1.39***	26.03 ± 0.74***	6.26 ± 0.95***

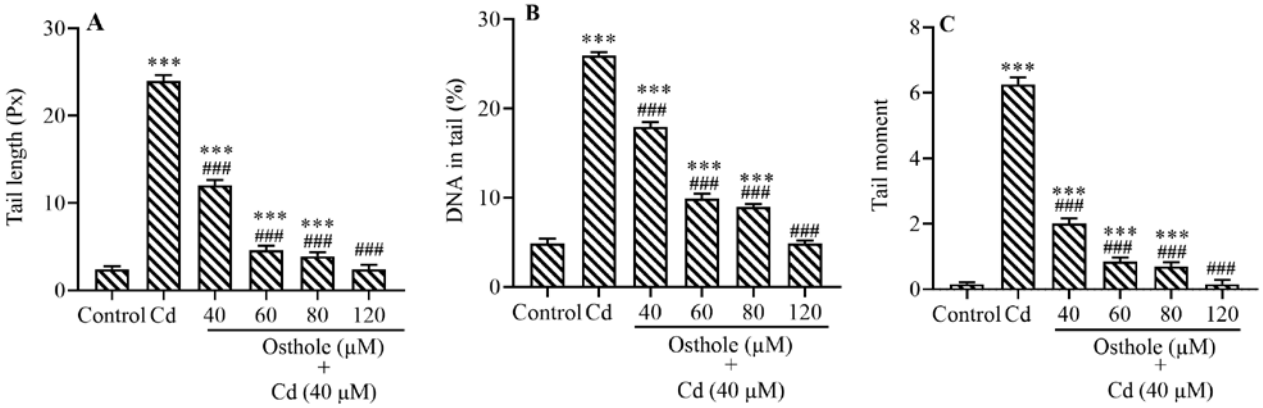

Fig. 3. The genoprotective effect of osthole against $CdCl_2$-induced DNA damage. Results are expressed as the mean ± SEM. ***$P < 0.001$ indicates significant differences compared to the control group; and ###$P < 0.001$ versus Cd group. Cd, cadmium.

DISCUSSION

Many of natural compounds such as osthole have been acknowledged worldwide with a rising study on folk medicine. Chinese medicine and its practical components are considered harmless, dependable, and reasonable (14). The various reported properties of osthole, such as antioxidant, anti-inflammatory, and anti-apoptotic effects, confirm the long history of its use in traditional Chinese medicine (20). The present study demonstrates the genoprotective effect of osthole against $CdCl_2$ on HUVEC cells.

Several reports have confirmed the genotoxic potential of cadmium in animals and mammalian cells (21,22). The results of the present study revealed that osthole has no genotoxic effects on HUVEC cells, while treatment with $CdCl_2$ for 2 h induced genotoxicity and oxidative stress on HUVEC cells. $CdCl_2$ induced a significant genotoxic effect indicated by an increased rate of comet assay parameters. Many studies have demonstrated similar results. Rozgaj *et al*. showed that cadmium compounds caused chromosome damage in Chinese hamster cells in an animal experiment (23). In another study, clastogenicity and cytotoxic effects of cadmium were reported in the rat hemopoietic system (24). Previous studies showed that cadmium causes cell death and DNA damage in rat bone marrow at long-term treatment (25).

Kasuba's study showed a dose-dependent increase in micronucleus and chromosomal aberrations after exposure to $CdCl_2$ in human lymphocytes (26) and the effect of fetal calf serum on cadmium clastogenicity in Chinese hamster ovary cells investigated by Wang and Lee. In addition, they found that $CdCl_2$ harms genetic material (27). In the current study, DNA damage was observed in HUVEC cells after treatment with $CdCl_2$ by comet assay, and also, we measured free thiol groups to evaluate the role of oxidative response in the genotoxicity of $CdCl_2$. The finding of this research confirmed a significant decrease in free thiol groups level after treatment with $CdCl_2$.

Cadmium generated free radicals such as superoxide anion, hydrogen peroxide, hydroxyl radical, and lipid radicals through indirect mechanisms such as glutathione depletion, Kupffer cells, inflammation, and iron involvement in the Fenton reaction. Adaptive mechanisms, including the induction of metallothionein, glutathione, and cellular antioxidants, reduce cadmium-induced oxidative stress. Cadmium carcinogenesis causes DNA damage and changes in DNA methylation status (28). Other researchers reported preventive effects of chelating agents or antioxidant compounds such as N-acetylcysteine, vitamin E, catalase, and superoxide dismutase against the cytotoxicity of cadmium in cellular and animal models (27). The intense antioxidative properties are one of the approved mechanisms of action of osthole. Reports have shown that unnecessary and extreme intracellular ROS generation has a crucial role in genotoxicity. ROS causes disturbance in various biological molecules, such as membrane lipids, DNA, and proteins, leading to biological membrane damage.

Furthermore, ROS has a determining role in the apoptotic process (29, 30). Free radicals can damage DNA. Hydroxyl radicals cause single-stranded or double-stranded breaks and fragmentation of DNA (31). As a potent antioxidant agent, osthole has been reported to scavenge ROS and inhibit lipid peroxidation.

Transcription of some portions of the glutathione and thioredoxin antioxidant systems and enzymes involved in biotransformation and detoxification of exogenous and endogenous products, NADPH regeneration, and heme metabolism were regulated by Nrf2 (32-34). It, therefore, characterizes a vital regulator of the cellular defense mechanisms against xenobiotic and oxidative stress. Remarkably, osthole has an antioxidant effect, and *in vivo* - *in vitro* studies have revealed its action in triggering Nrf2 signaling, which protects against ROS-mediated damage (33). Osthole has a considerable capacity for scavenging free radicals (35). The potential phenolic hydroxyl group in the osthole's structure may be related to its antioxidant activity. Yan and colleagues showed that the activities of SOD, GPx, and CAT were enhanced dramatically by osthole in a dose-dependent manner and suppressed the formation of the lipid peroxidation products, MDA, and 4-HNE, in the injured heart tissues. The effects of osthole are related to the decrease and increase in the expression of proinflammatory cytokines and anti-inflammatory cytokines, respectively, and the inhibition of the production of HMGB1 and phosphorylated IκB-α and NF-κB proteins (36). Liu *et al.* reported inhibition of cell death, lactate dehydrogenase, and cytochrome c release, overproduction of ROS, morphological changes in nuclei, DNA fragmentation, damage to antioxidant enzymes, Bax/Bcl-2 ratio, and induction of caspase-3 expression in MPP+-treated PC12 cells (37).

Comet assay is a proper method for measuring single- and double-stranded DNA fragments at the cellular level. This method has been widely used in studies on genetic toxicity. Although comet assay has limitations such as not exhibiting DNA-DNA cross-linking and other cellular changes, (38) it is a sensitive test for detecting various types of DNA lesions for cadmium toxicity.

CONCLUSION

we used osthole to reveal its capability against $CdCl_2$-induced genotoxicity. Our results showed attenuation of the oxidative stress injury and genotoxicity induced by CdCl2 in HUVEC cells by osthole. These effects were confirmed with thiol and DPPH assay. Osthole significantly decreased DNA damage in a concentration-dependent manner. On the other side, the influence of osthole against $CdCl_2$-induced oxidative damage was determined by measuring free thiol groups. However, further studies are necessary to determine the exact genoprotective mechanisms before drawing definite conclusions.

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Conflict of interest statement

All authors declared no conflicts of interest in this study.

Authors' contribution

All authors contributed equally to this work.

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