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The protective effects of glycyrrhizic acid on heavy metal ions-induced reproductive toxicity in Chinese hamster ovary cells

Liang Gao[a], Pai Liu[b], Tianqi Du[a], Jian Yin[b,c,*], Hong Li[a,*]

[a] Center of Reproduction and Genetics, Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Suzhou 215002 China
[b] CAS Key Laboratory of Biomedical Diagnostics, Suzhou Institute of Biomedical Engineering and Technology, Chinese Academy of Science, Suzhou 215163 China
[c] Jinan Guo Ke Medical Technology Development Co., Ltd, Jinan 250100 China

*Corresponding authors, e-mail: yinj@sibet.ac.cn, hongliszivf@163.com

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ABSTRACT: In this work, Chinese hamster ovary (CHO) cells were used to examine the protective effects of glycyrrhizic acid (GA) on the reproductive toxicity of heavy metal ions, including Cd^{2+} and Cu^{2+}. As a result, both metal ions induced significant toxicity in CHO cells after 48-h treatment, as revealed by a severe decrease in cell viability. GA and glutathione (GSH) largely reduced the toxicity caused by Cd^{2+} and Cu^{2+}, which could be due to their recovery of GSH levels and superoxide dismutase (SOD) activities in CHO cells. In addition, GA and GSH significantly up-regulated the gene expressions of *glutathione S-transferase (gst)*, *sod*, and *heme oxygenase (ho)-1*, which could be another explanation for their protective effects. More importantly, GA exhibited comparable protective effects as GSH but at much lower concentrations (50–100 μM v.s. 500–1000 μM). Therefore, GA could be effective for the alleviation of reproductive toxicity of Cd^{2+}/Cu^{2+}, which needs further investigation in animal models.

KEYWORDS: Chinese hamster ovary cells, Cd^{2+}, Cu^{2+}, glycyrrhizic acid, oxidative stress

INTRODUCTION

So far, the toxicity of heavy metal ions, like Cd^{2+} and Cu^{2+}, are being paid more and more attentions, as they are nondegradable and inevitably released from human activities like mining, smelting, and manufacturing [1–4]. Free Cd^{2+} and Cu^{2+} have been found to be toxic to rat, mice, and various fishes; causing weight loss, developmental abnormality, and even death [5–7]. More importantly, both metal ions can significantly alter the reproductive capability of treated animals without causing significant damages [8]. In this respect, it is important to illustrate the mechanism on the reproductive toxicity of metal ions and to screen potential detoxification agents.

Various experiments have been conducted on the reproductive toxicity of metal ions. Their results indicated that the toxicity might occur in testicle, fetus, spermatozoa, and ovary [9–13]. The involving mechanisms, like impairment of steroid hormones and cell apoptosis, have been proposed in different literatures, but oxidative stress was most widely accepted as the common reason [14, 15]. Production of glutathione (GSH) and the induction of anti-oxidative enzymes like glutathione S-transferase (GST), catalase (CAT), superoxide dismutase (SOD), and heme oxygenase (HO)-1 were important in the protection of organisms from the toxicity of metal ions [16, 17]. Unfortunately, significant toxicity still occurred when the oxidative stress exceeded the limits of these anti-oxidative systems. In this respect, it should be useful to develop reducing agents for the treatment of metal ions-induced reproductive toxicity.

Nowadays, many herbal medicines have been tried for the treatment of reproductive diseases, and most of them have detoxification functions [18, 19]. Liquorice is one of such herbs to reduce chemical-induced reproductive toxicity in animals [20, 21]. The main bioactive compound of liquorice, glycyrrhizic acid (GA), is well known for its ability to eliminate reactive free radicals and to stabilize cell membrane [10, 22]. Several studies revealed that GA exhibited a greater protective effect than liquorice [23, 24]. Nevertheless, the use of GA mostly focuses on the reduction of drug-induced toxicity, and its effects on the toxicity of metal ions are still rare, especially the reproductive toxicity.

The present study aimed to investigate the effects of GA on the reproductive toxicity of heavy metal ions, including Cd^{2+} and Cu^{2+}, in Chinese hamster ovary (CHO) cells. To assess the relating mechanism in such interactions, the alteration of glutathione (GSH) level, superoxide dismutase (SOD) activity, apoptosis status, as well as the expressions of anti-oxidative enzymes caused by metal ions and metal ions-GA/GSH mixtures were evaluated. GSH was used here as a positive control, since it was the major antioxidant produced by the cells, protecting them from free radicals [25].

MATERIALS AND METHODS

Chemicals

CHO cells were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum were purchased from Gibco (Gaithersburg, USA). Methyl thiazolyl tetrazolium (MTT), glutathione (GSH), and glycyrrhizic acid (GA) were got from Sigma-Aldrich (St Louis, MO, USA). $CuCl_2$ and $CdCl_2$ were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Annexin V-FITC apoptosis detection kit and enhanced cell counting kit-8 (CCK-8) were purchased from Shanghai Biyuntian Biotechnology Company (Shanghai, China). First strand cDNA synthesis and qPCR kits (SYBR Green Method) were got from Thermo Fisher Scientific (San Jose, CA, USA). Primers were synthesized by Genewiz (Suzhou, China). Ultrapure water used in this study was produced by the Milli-Q Water System (Millipore Corp., Bedford, MA, USA). All other chemicals purchased from local reagent companies and of reagent grade.

Cell treatment

CHO cells were cultured in 24-well plates and maintained in DMEM medium containing 10% fetal bovine serum. The cells were kept at 37 °C, 5% CO_2 in a humidified atmosphere until reaching 80% confluence. After then, cells were washed with phosphate buffer solution (PBS, PH=7.4) and treated with medium containing a series of concentrations of $CuCl_2$, $CdCl_2$, and $CuCl_2$/$CdCl_2$+GA/GSH.

To investigate the cytotoxicity of heavy metal ions, CHO cells were treated with $CdCl_2$ and $CuCl_2$ at the concentrations of 0, 0.01, 0.1, 1, 10, 100, and 1000 μM. Cell viability was determined after 48 h treatment. To determine the apoptosis status, oxidative stress level, and transcriptional alterations caused by each treatment, cells were washed with PBS and cultured in 1 ml medium containing $CuCl_2$ (50 μM)/$CdCl_2$ (5 μM) with or without GA (50 and 100 μM)/GSH (500 and 1000 μM). After 48 h treatment, cells in each group were collected for further analysis.

Cell viability assay

Cell viability was evaluated with CCK-8 assay according to the manufactures' instructions. After each treatment, cells in each well were washed with PBS and treated with 0.5 ml DMEM medium containing 10 μl of CCK-8 reagents. After 2-h incubation at 37 °C, the optical density of each well at 450 nm was detected with a Synergy 2 microplate reader (BioTek Instruments, Winooski, VT, USA). Viability of treated cells was expressed as the percentage of untreated control.

GSH and SOD detection

At 48 h, cells in each group were washed with PBS, scraped by a rubber policeman, and collected in 500 μl PBS. After that, the collected cells were lysed by sonication (40 kHz, 900 W, 5s) twice. Subsequently, the lysates were centrifuged (10 000 g, 4 °C, 10 min) to get rid of cell fragments and stored at 4 °C for further analysis. The reduced GSH levels and SOD activities in each group were then detected using commercial kits (Beyotime Institute of Biotechnology, Jiangsu, China) as described before [26]. Among them, GSH levels were determined by the formation of 5-thio-2-nitrobenzoic acid (412 nm) and expressed as pmol of GSH presented in 10^6 cells (pmol/10^6 cells). SOD activities were detected by nitroblue tetrazolium/riboflavin photometric quantitative methods (420 nm). The obtained results were expressed as U/10^6 cells, as well.

Apoptosis detection

After each treatment, cells in 24-well plates were detached with Trypsin-EDTA solution (0.25% Trypsin and 0.02% EDTA, m/v, dissolved in PBS). The collected cells from each well were suspended in 0.5 ml PBS and stained with Annexin V-FITC apoptosis detection kit for 10 min. The fluorescence in each group was detected by a Synergy 2 microplate reader (BioTek Instruments, Winooski, VT, USA) at 485/530 nm. The obtained results were normalized to cell viability and expressed as multiples of untreated control.

RT-PCR analysis

Total RNA was extracted from CHO cells with commercial kits (Axygen Scientific, Inc., USA) after each treatment. The quality of RNA was determined by 260/280 nm absorption using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., USA). The obtained RNA was reversely transcribed into cDNA and amplified with commercial kits according to the manufactures' instructions. PCR reactions were conducted in a real-time PCR system (Stepone Plus, Applied Biosystems, CA, USA) with cycling parameters of 95 °C for 30 s, 40 cycles of 95 °C, for 5 s and 60 °C for 34 s. Relative expressions of each gene were obtained with $2^{-\Delta\Delta C(t)}$ method and normalized to untreated control groups. All the primers are shown in Table S1 and *β-actin* was used as a house-keeping gene [5].

Statistical analysis

All the results in this experiment were the average values (mean ± SD) of three independent experiments. Statistical analysis was carried out with SPSS v 15.0 (SPSS Inc., Chicago, Illinois, USA). One-way ANOVA post-hoc tests were used to analyze the differences among multiple groups. Dunnett's test was applied for the comparison between control and treated groups. Significance was obtained when $p < 0.05$.

RESULTS

Toxicity of Cd^{2+} and Cu^{2+}

After exposure to Cd^{2+} and Cu^{2+} for 48 h, concentration-dependent damages in CHO cells were noticed (Fig. 1). A slight reduction of cell viability was found after the treatment of 10 µM Cd^{2+} for 48 h, which was 80.10 ± 6.73%. Nearly all the cells died after the exposure of 500 µM Cd^{2+} (Fig. 1A). On the other hand, the survival rate of CHO cells after the treatment of 10–1000 µM Cu^{2+} decreased from 99.43 ± 5.14% to 1.04 ± 0.43% (Fig. 1B). According to the concentration-response curve, median-lethal concentrations were determined to be 42.5 µM and 287.5 µM for Cd^{2+} and Cu^{2+}, respectively. These concentrations were also used in the subsequent experiments.

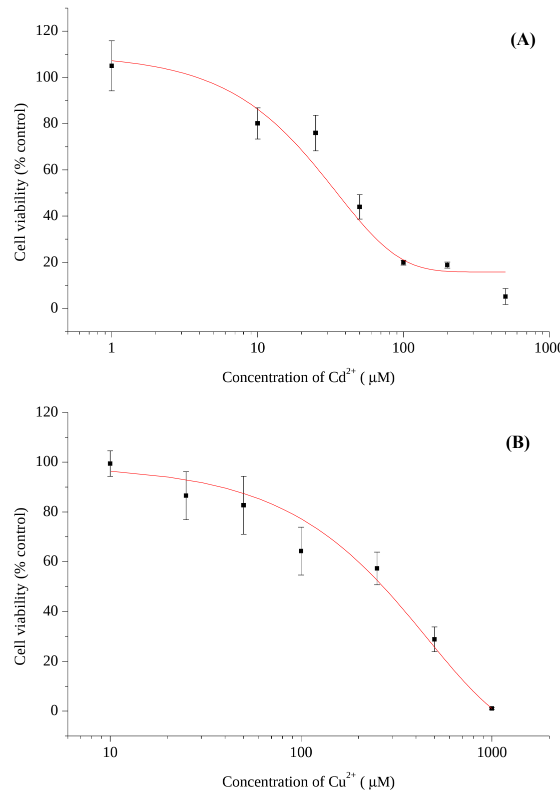

Fig. 1 Concentration-dependent mortality caused by Cd^{2+} and Cu^{2+} in CHO cells. After reaching 80% confluence, cells in each group were treated with different concentrations of $CdCl_2$ and $CdCl_2$. Cell viability was determined after 48 h treatment using MTT assay and expressed as the percentage of untreated control groups. Data were represented as mean values ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with untreated control.

Effects of GA and GSH on the toxicity of metal ions

As shown in Fig. 2, GA (50 and 100 µM) and GSH (500 and 1000 µM) significantly reduced metal ions-induced cell death, in a concentration-dependent manner. For instance, 42.5 µM Cd^{2+} caused a cell viability of 50.28 ± 8.68%, and the values increased to 75.92 ± 8.68% and 92.36 ± 8.02% after the co-exposure of 50 and 100 µM GA, respectively. The recovering effects of GSH were more obvious, as the cell viability were 103.57 ± 13.06% and 106.07 ± 10.04% after the addition of 500 and 1000 µM GSH, respectively. Similar phenomena were also found with Cu^{2+}, and the highest cell viability was 93.52 ± 2.83%, after the co-treatment

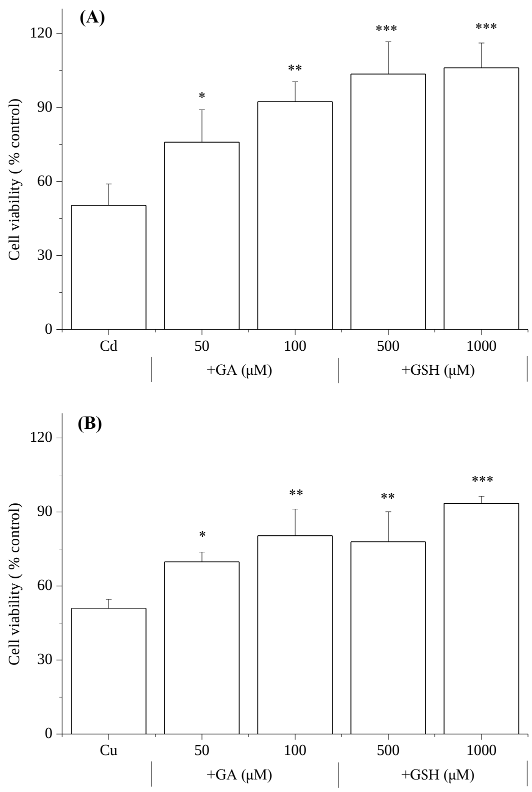

Fig. 2 Effects of GA and GSH on metal ions-induced death in CHO cells. GA and GSH were added together with metal ions, and cell viability were recorded at 48 h using MTT assay and expressed as the percentage of untreated control groups. Data were represented as mean values ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with groups treated with metal ions alone.

of 287.5 μM Cu^{2+} and 1000 μM GSH.

Changes in the levels of GSH and SOD

To illustrate the possible detoxification mechanisms of GA and GSH, alteration of reduced GSH level and SOD activities were evaluated after the treatment of metal ions with or without GA/GSH (Fig. 3). As a result, both Cd^{2+} and Cu^{2+} caused significant reduction of SOD and GSH after 48-h treatment. The reduction could be recovered by the co-treatment of GA and GSH. The reduced GSH levels after the treatments of Cd^{2+} and Cu^{2+} were 1.33 ± 0.20 pmol/10^6 cells and 1.54 ± 0.09 pmol/10^6 cells, respectively; which were significantly lower than the values of the untreated control (4.93 ± 0.32 pmol/10^6 cells, $p < 0.001$). The highest GSH level, 3.00 ± 0.50 pmol/10^6 cells, was obtained after the co-treatment of 287.5 μM Cu^{2+} and 1000 μM GSH. The value was also higher than those of the groups co-treated with 42.5 μM Cd^{2+} and 1000 μM GSH (2.63 ± 0.55 nmol/10^6 cells). For SOD activities, they were reduced from 1.14 ± 0.12 U/10^6 cells to 0.60 ± 0.06 U/10^6 cells and 0.62 ± 0.06 U/10^6 cells, after the addition of 42.5 μM Cd^{2+} and 287.5 μM Cu^{2+} ($p < 0.001$), respectively. The values could be recovered to the highest at 0.97 ± 0.12 U/10^6 cells after the co-treatment of 287.5 μM Cu^{2+} and 1000 μM GSH.

Changes in the apoptosis status

Accompanying the alterations in cell viability, treatment of Cd^{2+} and Cu^{2+} caused significant induction of apoptosis in CHO cells, as reflected by the dramatic elevation of Annexin V-FITC fluorescence levels (Fig. 4). The fluorescence levels for 10^6 cells were 6347.67 ± 1149.39 and 5643.50 ± 938.28 after the treatments of 42.5 μM Cd^{2+} and 287.5 μM Cu^{2+}, respectively. The values were much higher than that of the control group, which was 2503.05 ± 407.40 ($p < 0.001$). The fluorescence could be largely reduced by the addition of GA and GSH. The lowest value was 2116.67 ± 364.52, which occurred after the co-treatment of 287.5 μM Cu^{2+} and 1000 μM GSH.

Altered gene expressions of anti-oxidative stress enzymes

As the inner mechanism for the detoxification effects of GA and GSH, gene expressions of *gst*, *sod*, *cat*, and *ho-1* were detected (Fig. 5). As a result, down-regulations of *gst* and *sod* were noticed after the treatment of Cd^{2+} or Cu^{2+}, but they were recovered or even induced to a higher extent after the addition of GA/GSH. Although *ho-1* was unaffected by Cd^{2+} or Cu^{2+}, its expressions were still induced by the addition of GA and GSH. These inductions were in a concentration-dependent manner, and the most obvious induction was found in *ho-1* after the co-treatment of 42.5 μM Cd^{2+} and 1000 μM GSH, which was 8.50 ± 0.71 folds of the control group level. For *gst* and *sod*, their expressions were respectively reduced to 24.02% and 39.51% of the control group after the treatment of 42.5 μM Cd^{2+}, but the values increased to 3.74 ± 0.69 and 2.24 ± 0.60 folds of the control group after the co-treatment of 42.5 μM Cd^{2+} and 1000 μM GSH. After the treatment of 287.5 μM Cu^{2+}, the expressions of *gst* and *sod* were reduced to 36% and 41% of the control group values, respectively. These values increased to 2.07 ± 0.33 and 3.90 ± 0.81 folds of the control

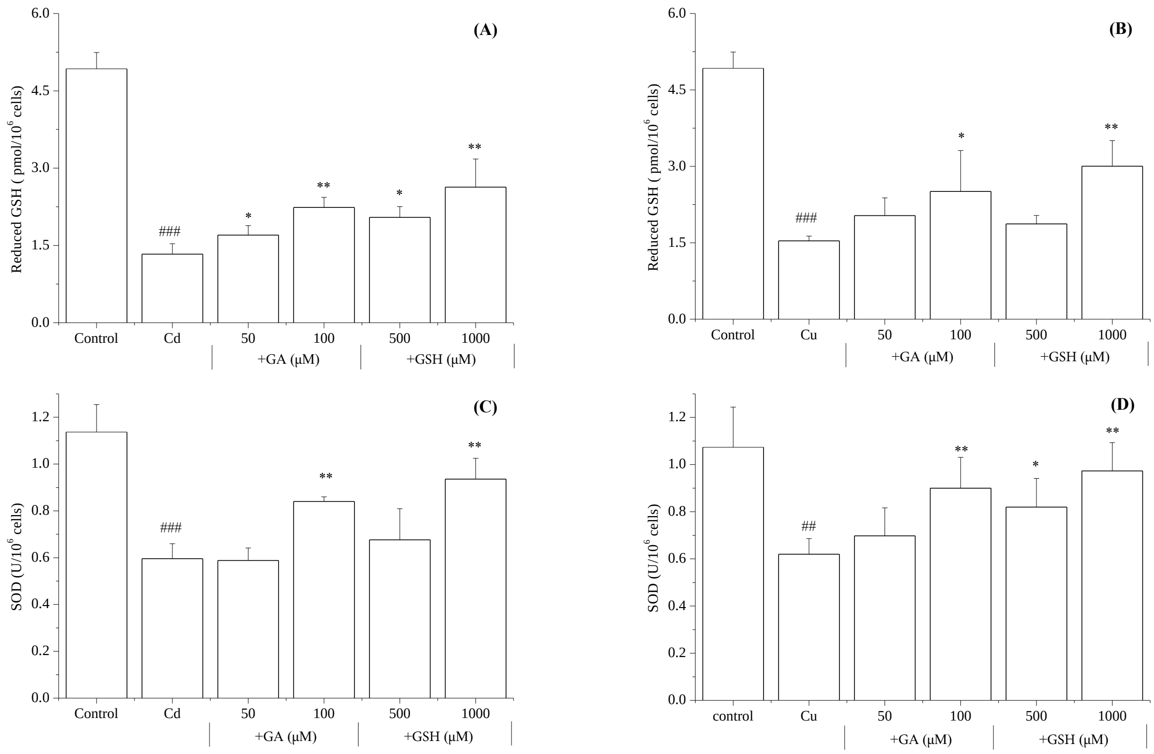

Fig. 3 Effects of GA and GSH on metal ions-induced reduction of GSH and SOD in CHO cells. GA and GSH were added together with metal ions, and the reduced GSH levels and the SOD activities were recorded at 48 h with commercial kits. Data were represented as mean values ± SD of three independent experiments. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with untreated control. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with groups treated by metal ions alone.

groups after the addition of 1000 μM GSH. Finally, only the expressions of *cat* were unaltered after the treatment of either metal ions or metal ions-GA/GSH mixtures.

DISCUSSION

Supplementation with GA has been proved to be useful in reducing the toxicity of clinical drugs like methotrexate and cocklebur [10, 27], but its applicability in the treatment of metal ions-induced toxicity has not been verified. In this respect, this study used CHO cells to investigate the effects of GA on the reproductive toxicity of Cu^{2+} and Cd^{2+}, with GSH as a positive control.

Both Cd^{2+} and Cu^{2+} caused significant reduction of cell viability in CHO cells, and the toxicity could be eliminated by the co-treatment of GSH and GA (Figs. 1 and 2). Although the toxicity of metal ions has been attributed to various elements like disruption of cell membrane, damages of DNA, dysfunction of mitochondrial, as well as endoplasmic reticulum stress, induction of oxidative stress is the most widely-accepted reason [28, 29]. In response to such toxicity, production of GSH is elevated to eliminate metal ions-induced reactive oxygen species (ROS) [30]. Therefore, the protective effects of oxidative stress scavenger like GA and GSH are reasonable (Fig. S1) [10, 31]. To further explore the involving mechanism, GSH levels, SOD activities, apoptosis status, and the gene expressions of anti-oxidative stress enzymes (including *gst*, *sod*, *cat*, and *ho-1*) were evaluated, after the treatment of Cd^{2+}/Cu^{2+} with or without GA/GSH.

At first, the protective effects of GA and GSH were reflected by the recovering of anti-oxidative function in CHO cells (Fig. 3). So far, glutathione S-transferase conjugation of GSH was believed to be important in the detoxification of Cd^{2+} and Cu^{2+} *in vitro* and *in vivo* [32, 33]. Meanwhile, SOD was involved in the elimination of ROS produced by metal ions [17, 34]. However, when the concentrations of Cd^{2+} and Cu^{2+} were high beyond the capacity of these enzymes, severe cell death and reduction of these enzymes were found [35]. In this respect, the elevation of GSH and SOD could be considered as the recovering of self-protective

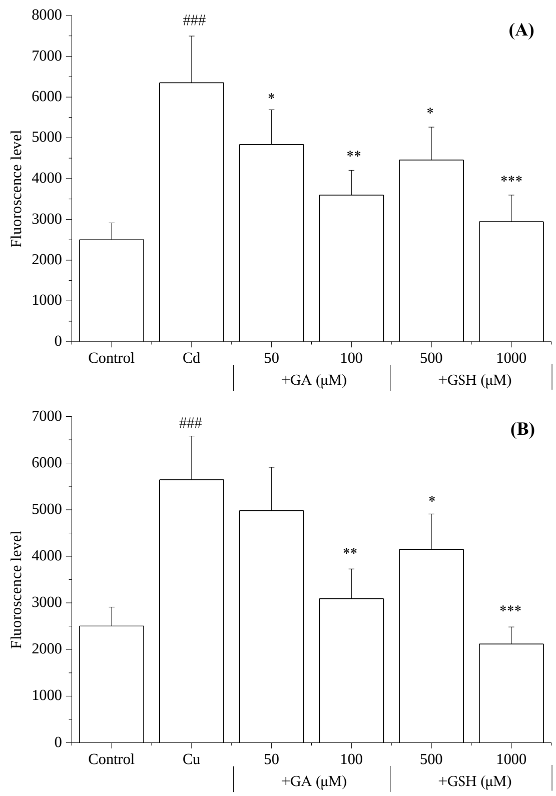

Fig. 4 Effects of GA and GSH on metal ions-induced apoptosis in CHO cells. GA and GSH were added together with metal ions, and the apoptosis status was recorded at 48 h with commercial kits. Data were represented as mean values ± SD of three independent experiments. $^{\#\#\#}p < 0.001$ compared with untreated control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with groups treated by metal ions alone.

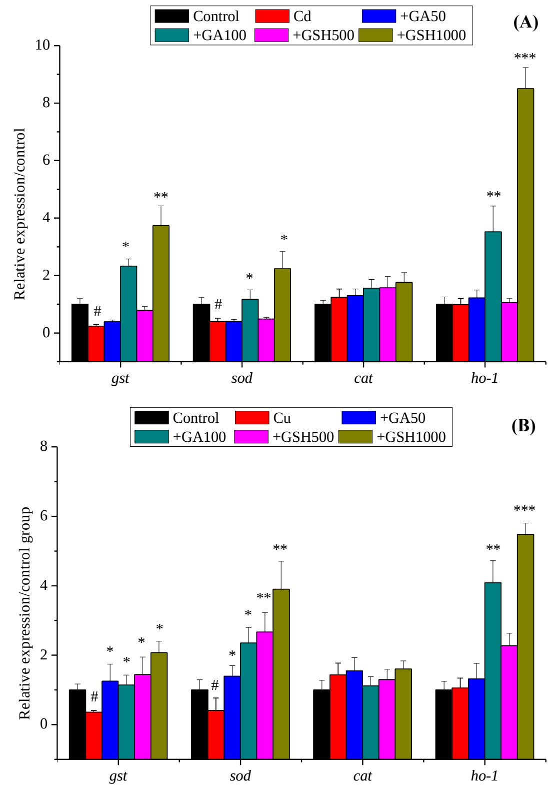

Fig. 5 Altered gene expressions of anti-oxidative stress enzymes including *gst*, *cat*, *sod*, and *ho-1* after the treatments of Cd^{2+}/Cu^{2+} with or without GA/GSH. Gene expressions were detected with RT-PCR and expressed as the average values of three independent experiments. $^{\#}p < 0.05$ compared with untreated control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with groups treated by metal ions alone.

functions, which could be a novel finding for the protective functions of GA. More importantly, GA exhibited a comparable detoxification effects even at much lower concentrations than GSH (50–100 μM v.s 500–1000 μM), indicating a fact that more inner mechanisms remained to be elucidated.

Secondly, apoptosis was found to play an important role in the toxicity of metal ions, which was also largely reduced by the co-treatment of GA and GSH (Fig. 4). Such phenomena could be explained by the elevation of GSH and SOD activities. Although the reproductive toxicity of metal ions has been widely reported [10, 36], their relationship with apoptosis-inducing effects are still rare. Our study not only confirmed the role of apoptosis in the reproductive toxicity of Cd^{2+} and Cu^{2+}, but also showed that GA and GSH could reduce such toxicity by preventing the occurrence of apoptosis in CHO cells.

Finally, treatments of GA and GSH induced the expressions of *gst*, *sod*, and *ho-1*, which could be another reason for the protective effects of the two reducing reagents (Fig. 5). Enzymes, including Gst, SOD, and Ho-1, have been widely accepted as anti-oxidative enzymes, which help in the defense of ROS in living organisms [16, 17, 23]. Therefore, the induction of these anti-antioxidative stress enzymes suggested a promotion of self-protection function in CHO cells. In addition, elevation of *gst* could be caused by the supplement of GSH from the treatment of GA and GSH, which could also be a partial reason for the induction of *sod* and *ho-1* [20]. Again, GA exhibited a comparable effect with GSH, although at much lower concentrations.

CONCLUSION

The results of this study indicated significant protective effects of GA on the toxicity of Cu^{2+} and Cd^{2+} in CHO cells. These effects were like GSH, occurring due to the promotion of anti-oxidative function. Such phenomenon needs confirmation from *in vivo* experiments. Moreover, efforts would be taken to screen more useful herbal medicines for reducing the reproductive toxicity of metal ions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2021.052.

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Appendix A. Supplementary data

Table S1 Primers used in this experiment.

		Primer (5′–3′)	Product size (bp)	Reference
β-actin	Forward	TCTTTCTTCGCCGCTCCAC	196	XM_007648665.3
	Reverse	GTAGGAGTCCTTCTGGCCCAT		
gst	Forward	GGACTTCTTGGCACGCTTTG	186	XM_003514942.4
	Reverse	GTCACAGAGCCACACCTGAG		
cat	Forward	CGATTTCTTACCCCGGGTGG	167	XM_003497440.4
	Reverse	GTGGTCAGGACATCGGGTTT		
sod	Forward	GACTGACTGAAGGCCAGCAT	156	XM_007642000.1
	Reverse	CAGTCACATTGCCCAGGTCT		
ho-1	Forward	GCATAGCCGGGAGCCTAAAG	141	XM_003511957.4
	Reverse	GCCTGGATATGCACCTCCTT		

Specific primers for each gene were designed with Primer Premier 5.0 software (Premier Biosoft Inc., Palo Alto, CA, USA).

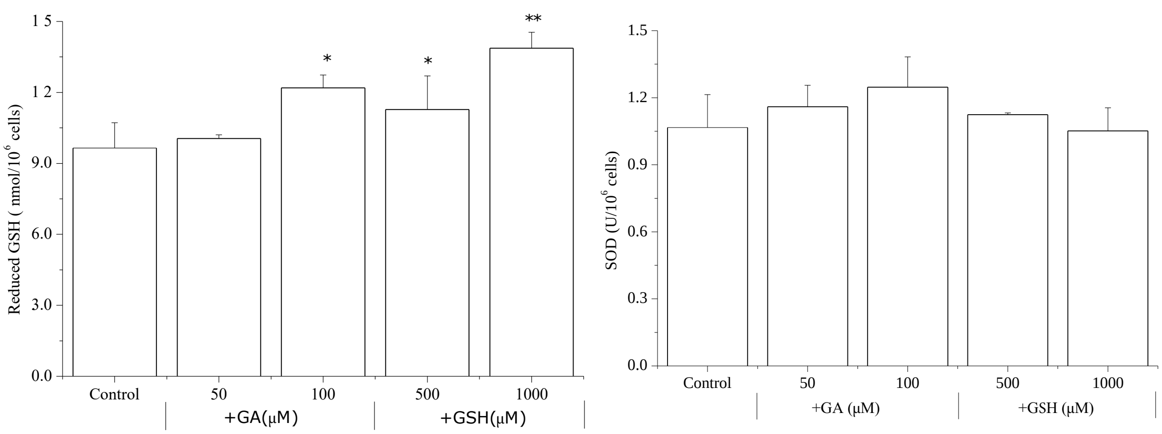

Fig. S1 Effects of GA/GSH treatments on the GSH and SOD levels of CHO cells. Data were represented as mean values ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$ compared with untreated control.