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Influence of Isoflavones on Cadmium-induced Adverse Effects in Vascular Endothelial Cells (ECV 304)

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Objective To study the possible intervention of isoflavones in cytotoxicity induced by cadmium in vascular endothelial cells. **Methods** An ECV 304 cell line derived from human umbilical vein endothelial cells was adopted. Genistein / daidzein was added prior to or simultaneously with $CdCl_2$, cell viability was determined by MTT assay, and metallothionein mRNA expression was monitored by RT-PCR method. **Results** Cell viability was higher in isoflavone and $CdCl_2$ co-treated groups than that in $CdCl_2$ treated group, with $CdCl_2$ concentration at 10, 20, 40, and 80 μmol/L, respectively. However this increase was not observed in the group treated with $CdCl_2$ at a concentration of 60 μmol/L. Isoflavones (10^{-10} mol/L to 10^{-5} mol/L) were added 24 h before cells were challenged with 80 μmol/L $CdCl_2$ for 24 h or simultaneously with 80 μmol/L $CdCl_2$. Genistein increased cell viability only at 10^{-5} mol/L, while daidzein caused a dose-dependent increase from 10^{-10} mol/L to 10^{-5} mol/L in co-treatment with $CdCl_2$. In pre-treatment, genistein (10^{-7} to 10^{-5} mol/L) increased cell viability whereas only 10^{-5} mol/L of daidzein exerted protection. Apparent protection could be found when the cells were pre-treated with 10^{-5} mol/L isoflavones for over 12 h, whereas 24 h incubation was required in such a co-treatment, with the exception of daidzein that had a significant protection in only 3 h. Isoflavones (10^{-6} mol/L) incubated for 3 h to 24 h, increased MT IIA and MT IF mRNA expression, but the induction could not last for more than 24 h. Co-treatment with isoflavones could induce an additional induction of MT IIA mRNA expression in cells exposed to cadmium. However, the additional induction of MT IIA and MT IF mRNA was not seen when pre-treatment was carried out with isoflavones, with the exception of an increase in MT IIA mRNA expression in the daidzein pre-treated group. **Conclusion** Genistein/daidzein could reverse the cytotoxicity of cadmium either in pre-treatment or in co-treatment. The protection is the strongest in 10^{-5} mol/L of isoflavones with a dose-dependent pattern. There are differences between genistein and daidzein in their protective effects. Whether the protection of isoflavones is related to their capacity of inducing MT mRNA expression remains to be elucidated.

Key words: Isoflavone; Cadmium; Cytotoxicity; Metallothionein; Vascular endothelial cells

INTRODUCTION

The relationship between environmental pollutants, cadmium, and cardiovascular diseases has been studied epidemiologically[1] and experimentally. Cadmium causes atherosclerosis[2] and hypertension[3] *in vivo* and vasoconstriction *in vitro*[4]. Nolan and Shaikh[5] suggested that the initial effect of acute cadmium administration is on the integrity and permeability of the vascular endothelium. Vascular endothelial cells might be the first target of cadmium in vascular tissue. Actually cadmium has been found to induce occurrence of de-endothelialized areas in cultured endothelial cell layer[6]. On the other hand, agents like zinc might induce a tolerance to hepatotoxicity of cadmium, which is mediated by metallothionein induction. Since isoflavones have been shown to be an inducer of metallothionein in cultured Caco-2 cells[7] and to be protective against endothelial damage *in vivo*[8] through uncertain channels, it appears to be possible that isoflavones may induce a tolerance to cadmium cytotoxicity in vascular endothelial cells. To examine this possibility, confluent cultures of ECV 304 cells were exposed to cadmium and isoflavone, and the cell viability and the expression of MT mRNA were investigated.

MATERIALS AND METHODS

Materials

Genistein, daidzein, phenol red -free Dulbecco's modified essential medium (DMEM) were purchased from Sigma-Aldrich (USA), ICI 182 780 was from Tocris (UK). All reagents used for the cell culture were from Invitrogen (USA) except for fetal bovine serum, which was purchased from Shanghai Pufei Biochemistry Co. (China). ECV 304 cells were from

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the Chinese Academy of Sciences (China). TRIzol reagent was from Life Technologies (USA), two-step RT-PCR kit was from Fermentas (USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was from Fluka (USA). Trypsin was from Shanghai Huamei Biochemistry Co. (China). Cadmium chloride was from Shanghai No.2 Chemical Reagent Factory (China). and all other reagents were purchased from local manufactories in China.

Cell Culture and Treatment

Cells were routinely propagated in DMEM supplemented with 10% fetal bovine serum in humidified 5% CO_2 / 95% air at 37℃. The cells were normally split when reaching confluence, and trypsinized with 0.25% trypsin solution. For maintenance of the cell line, cells were seeded at the density of 2×10^4 cells/cm^2 in flasks. For experiments, cells were seeded at the density of 4×10^4 cells/cm^2 in plates. Cadmium concentration in DMEM without any supplement was 0.3890±0.013 μg/L, assessed by atomic absorption spectrum method, with three duplicate measurements. Stock solutions were prepared as follows. Genistein and daidzein were dissolved in 100% DMSO at a concentration of 10 mmol/L, cadmium chloride was dissolved in 0.9% sodium chloride at a concentration of 1 mmol/L. All stock solution was stored at -20℃. Cells were treated with serum-free DMEM for 24 h before challenged. Designated chemicals were diluted with serum-free DMEM before each experiment, with the final concentration of DMSO less than 0.1%.

Assay of Cell Viability

The cell viability rate was examined by MTT assay. The cells were seeded in a 96-well plate. At the end of treatment, 15 μL 0.5% MTT was added into each well. Following further incubation in 5 % CO_2 at 37℃ for 4 hours, cultural medium was discarded and replaced by 150 μL DMSO. The values of absorbance at 490 nm were detected with a microplate autoreader (EL309; Bio-Tek, USA).

Cell relative viability=Mean value of absorbance in exposure group/ Mean value of absorbance in media control group.

Semi-quantification of MT II A and MT IF mRNA Expression

Levels of MT II A and MT IF mRNA expression were monitored with RT-PCR method, and at the same time the level of GAPDH mRNA expression was measured for normalization.

Total cellular RNA was extracted from cells pooled from 1 well (6-well plate) using TRIzol reagent. Concentrations of RNA were quantitated by absorbance at 260 nm (Bio-Rad, SmartspecTM 3000 spectrophotometer) and stored at -70℃ until further use. Complementary DNA was synthesized from 1 mg of total cellular RNA. Amplification was carried out on a DNA thermocycler (Perkin-Elmer-Cetus 9600), reverse transcription was performed following the guideline of the kit. A total of 25 cycles of PCR for MT IIA and GAPDH, and a total of 31 cycles of PCR for MT IF were performed, each at 94℃ for 15 s, at 55℃ for 60 s, at 72℃ for 30 s, and a final extension at 72℃ for 10 min. PCR products were electrophoresed (Phamacia Biotech, Eps 1000) and visualized (Bio-Rad, Gel Doc., 2000). For semi-quantization, Quantity-one 4.01 software (BioRad) was used to analyze the abundance of each gene. Relative abundance of targeted gene = abundance of targeted gene/ abundance of GAPDH. Oligonucleotide primers were synthesized by Sangong Biological Engineering and Technology Service Co. Ltd (China), primers for MTIIA and MT IF genes were referred to a previous report[9], primers for GAPDH gene were determined using Primer3 software (http://www.genome.wit.edu./cit-bin/primer/) from the complete sequence of human GAPDH cDNA (GI: 40226183) in Genebank (http//www.ncbi.nlm.nih.gov). The length of PCR production and sequences of the primers were as follows:

MT IIA (259 bp): 5′ primer, 5′- CCG ACT CTA GCC GCC GCC TCT T -3′

3′primer, 5′- GTG GAA GTC GCG TTC TTT ACA-3′ :

MT I F (232 bp): 5′ primer, 5′- AGT CTC TCC TCG GCT TGC-3′

3′primer, 5′- ACA TCT GGG AGA AAG GTT GTC-3′

GAPDH (307 bp): 5′ primer, 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′

3′ primer, 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′ .

Statistical Analysis

Data were analyzed using one way analysis of variance (ANOVA) by SPSS software. Results were expressed as $\bar{x}\pm s$. *P* values less than 0.05 were considered statistically significant.

RESULTS

Cell Viability

Protective effect of isoflavone on cell viability

Our previous study showed that cadmium decrease the relative viability of ECV in a time-and dose-

dependent manner, with the ID_{50} for 24 h about 50 μmol/L to 100 μmol/L[10]. Isoflavone ranging from 10^{-10} mol/L to 10^{-5} mol/L, incubated for 24 h, had no obvious effect on cell viability (data not shown). However, co-treatment with isoflavone increased the viability of cells exposed to cadmium, except for the 60 μmol/L $CdCl_2$ group (Fig. 1). The highest dose of $CdCl_2$ (80 μmol/L) was selected in subsequent studies.

Dose-response curve of isoflavone's protective effect Co-treatment with daidzein had a dose-dependent protection with the maximal effect obtained at 10^{-5} mol/L, while co-treatment with genistein had a milder effect, with cell viability increased only at 10^{-5} mol/L. However, in pre-treatment, genistein seemed to be more potent than daidzein, since the protection was observed at a dose above 10^{-7} mol/L in genistein, but could only be seen at 10^{-5} mol/L in daidzein (Fig. 2).

Time-response curve of isoflavone's protective effect Genistein and daidzein had the highest effect at the highest dose both in pre-treatment and co-treatment groups, so the highest dose (10^{-5} mol/L) was chosen to investigate the time-course of their effects. Co-treatment with daidzein increased the relative viability at 3 h, while it had no obvious protection at 6 h and 12 h. Both genistein and daidzein increased the relative viability at 24 h. Pre-treatment with isoflavone for over 12 h could increase the relative viability (Fig. 3).

Influence of ER antagonist on isoflavone's protective effect One μmol/L ICI 182 780 alone had no effect on cell viability, neither did it affect the viability of cells exposed to 80 μmol/L cadmium.

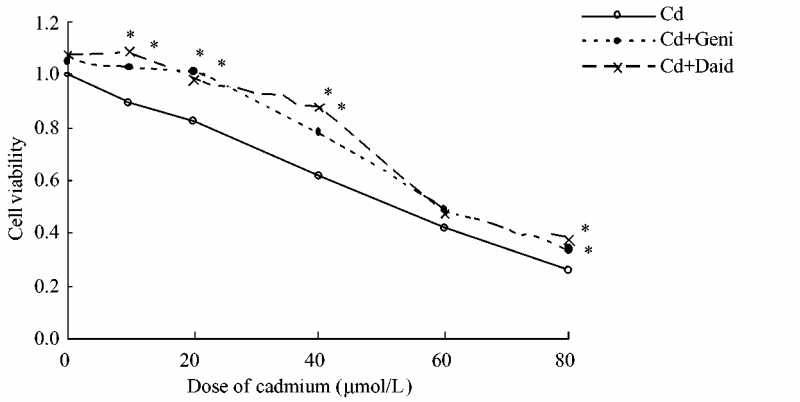

FIG. 1. Cell viability after co-treatment with isoflavone and $CdCl_2$ for 24 h (n=8).Cells were treated with $CdCl_2$ alone (Cd) or in the presence of genistein (Cd+geni), daidzein (Cd+daid) for 24 h, final doses of $CdCl_2$ were 0, 10, 20, 40, 60, 80 μmol/L respectively, final dose of genistein/daidzein was 10 μmol/L. *P<0.05 compared with sole cadmium treated group.

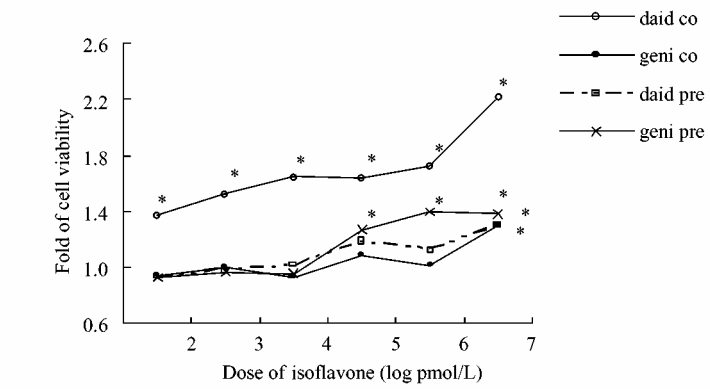

FIG. 2. The influence of isoflavones on viability of cell exposed to $CdCl_2$ (n=8). Cells were treated with $CdCl_2$ alone or in the presence of genistein (geni co), daidzein (daid co) for 24 h. Cells were pre-treated with serum-free media, genistein (geni pre) or daidzein (daid pre) for 24 h, then the culture media was discarded, cells were washed with PBS twice and challenged with $CdCl_2$ for another 24 h. Final doses of genistein/daidzein were 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} mol/L respectively, final dose of $CdCl_2$ was 80 μmol/L. Control group was treated with serum-free media. Fold of viability=Cell viability in isoflavone treated group/ Cell viability in sole cadmium treated group. *P<0.05 compared with sole cadmium treated group.

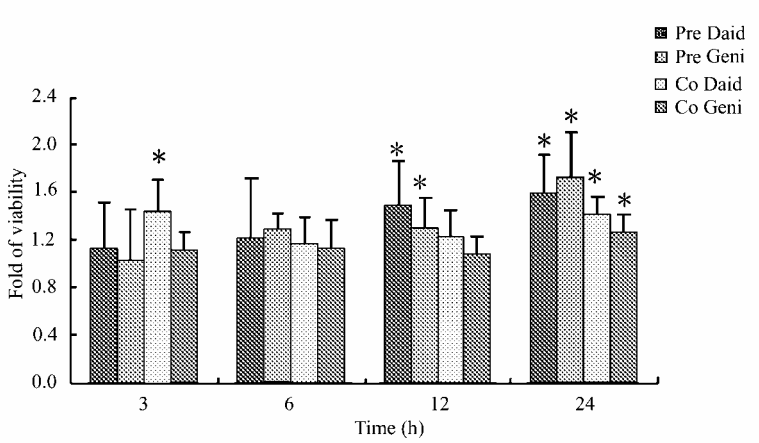

FIG. 3. The time course of isoflavones' effect on cell viability (n=8). Cells were treated with $CdCl_2$ alone (Cd) or in the presence of genistein (Co Geni) , daidzein (Co Daid),the exposure time was 3, 6, 12, 24 h, respectively. Cells were pre-treated with serum-free media, genistein (Pre Geni) or daidzein (Pre Daid) for 3, 6, 12, 24 h, respectively, then the culture media was discarded, cells were washed with PBS twice and challenged with $CdCl_2$ for another 24 h.Control group was treated with serum-free media. Final dose of $CdCl_2$ was 80 μmol/L, final dose of genistein/daidzein was 10 μmol/L. $^{*}P$<0.05 compared with sole cadmium treated group.

Pre-treatment with 1 μmol/L ICI 182 780 for 24 h blocked the effect of genistein but not daidzein. Co-treatment with ICI 182 780 blocked both the effect of daidzein and genistein (Fig. 4).

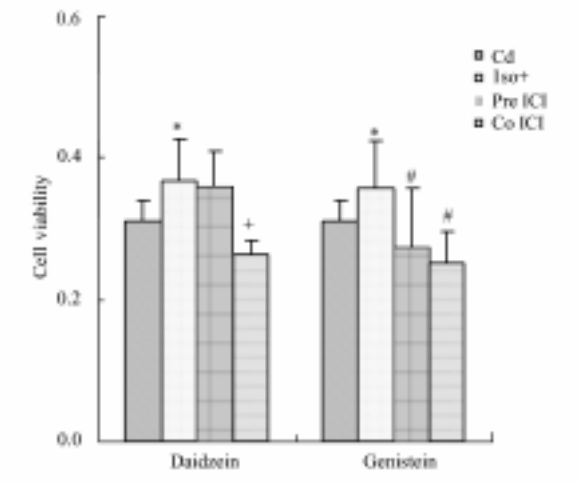

FIG. 4. Cell relative viability after co-treated or pre-treated with ICI 182,780 for 24 h (n=8). ICI 182, 780 was added 24 h before cells being challenged with genistein/daidzein plus $CdCl_2$ for another 24 h (Pre ICI). Or ICI 182, 780 was added together with genistein/daidzein and $CdCl_2$ (Co ICI), Iso+ stands for isoflavone and $CdCl_2$ co-treatment group. The final concentration in the corresponding group was ICI 182, 780, 1 μmol/L; genistein/daidzein, 10μmol/L; $CdCl_2$, 80 μmol/L. Control group were exposed to serum-free media for 24 h or 48 h. $^{*}P$<0.05 compared with sole cadmium treated group, ^{+}P<0.05 compared with daidzein co-treated group, $^{\#}P$<0.05 compared with genistein co-treated group.

MT IIA and MT IF mRNA Expression

Genistein up-regulated MT IIA and MT IF mRNA expression in a time-dependent manner from 3 h to 24 h, with the maximum induction reached at 24 h. But daidzein did not induce an apparent expression of MT IF mRNA until 24 h. MT IIA expression was increased by 3 h treatment with daidzein, reduced at 6 h and 12 h, but was still higher than that of control, with the highest induction at 24 h (Figs. 5 and 6).

Co-treatment with isoflavone and $CdCl_2$ caused an additional increase in MT IIA expression compared with sole $CdCl_2$ exposure. MT IIA expression in the genistein and daidzein co-treated groups was 1.19, 1.11, respectively of that in the sole $CdCl_2$ treated group (Fig. 7).

Pre-treatment with isoflavone also up-regulated MT IIA and MT IF mRNA expression, however the MT mRNA induction in the isoflavone pretreated groups was not higher than that in the sole $CdCl_2$ treated group, except that MT IIA expression in the daidzein pretreated group was 1.50 of that in the sole $CdCl_2$ treated group. When treated with isoflavone for 24 h followed by incubation with media for another 24 h, the MT expression returned to the basic level. Compared with media control, no increase was observed except for MT IF expression in the genistein treated group (Fig. 8).

ICI 182 780 alone had no appreciable impact on MT gene induction, but it blocked the MT IF mRNA up-regulation by isoflavone. However, MT IIA induction by isoflavone was not influenced by ICI 182 780 (Fig. 9).

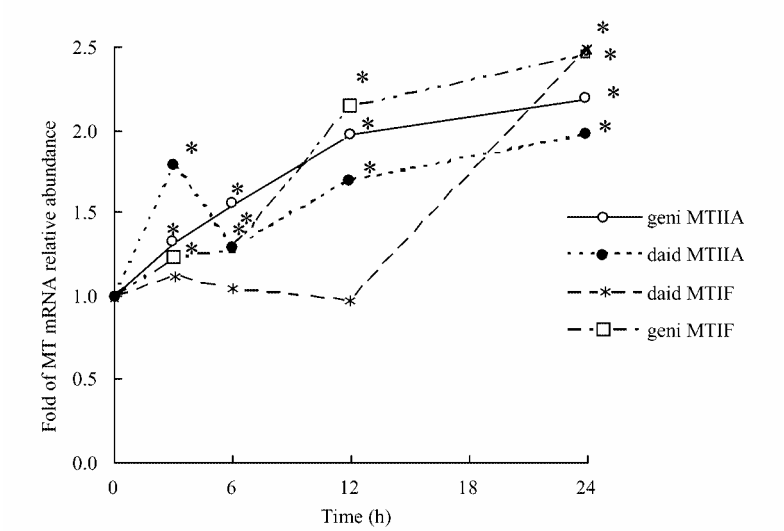

FIG. 5. MT mRNA induction when exposed to isoflavone (n=3).Cells were exposed to genistein or daidzein for 3, 6, 12, 24 h respectively, control group was incubated with serum-free media for 24 h. Fold of abundance=Relative abundance of MT gene in Treated group/ Relative abundance of MT gene in control group. geni MT IIA / daid MT IIA : MT IIA mRNA expression after incubated with genistein / daidzein, geni MT IF / daid MT IF : MT IF mRNA expression after incubated with genistein / daidzein, $^{*}P<0.05$ compared with control group.

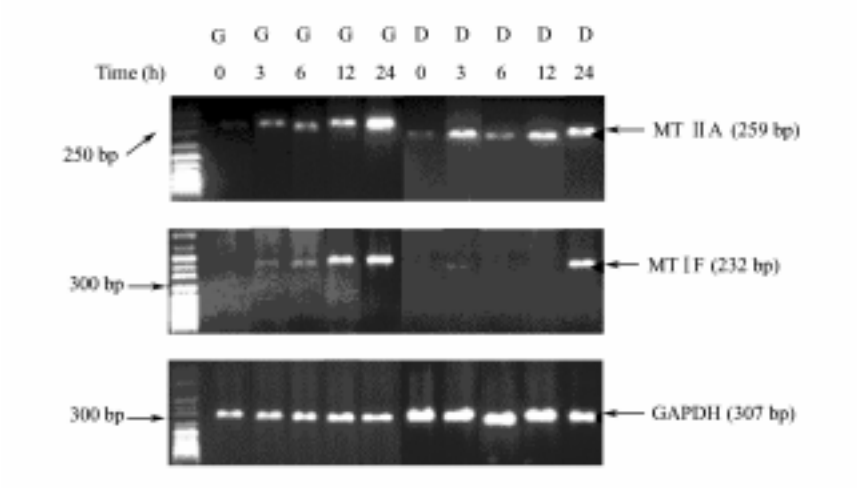

FIG. 6. MT mRNA expression after exposure to isoflavone for 3 to 24 h.

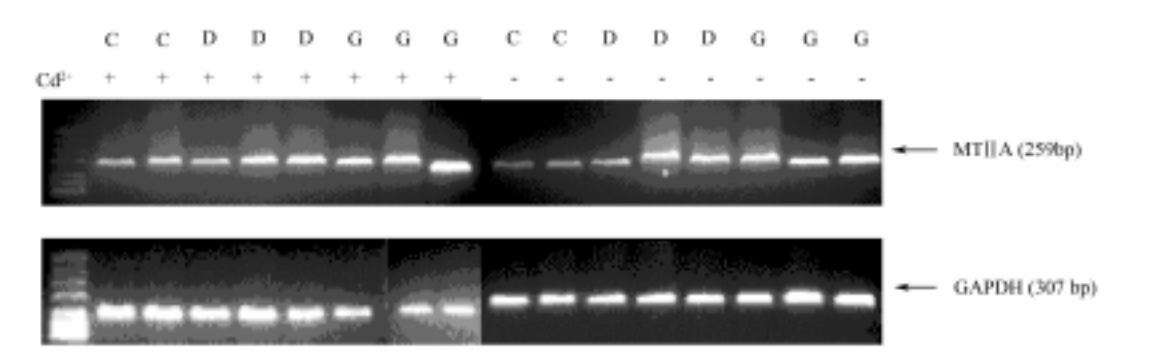

FIG. 7. MT IIA expression after co-treated with isoflavone and $CdCl_2$ (n=3). Cells were treated with Media (C), genistein(G), daidzein(D) in the presence (Cd^{2+}+) or absence (Cd^{2+}-) of $CdCl_2$ for 24 h, final dose of genistein/daidzein was 10 μmol/L, final dose of $CdCl_2$ was 10 μmol/L.

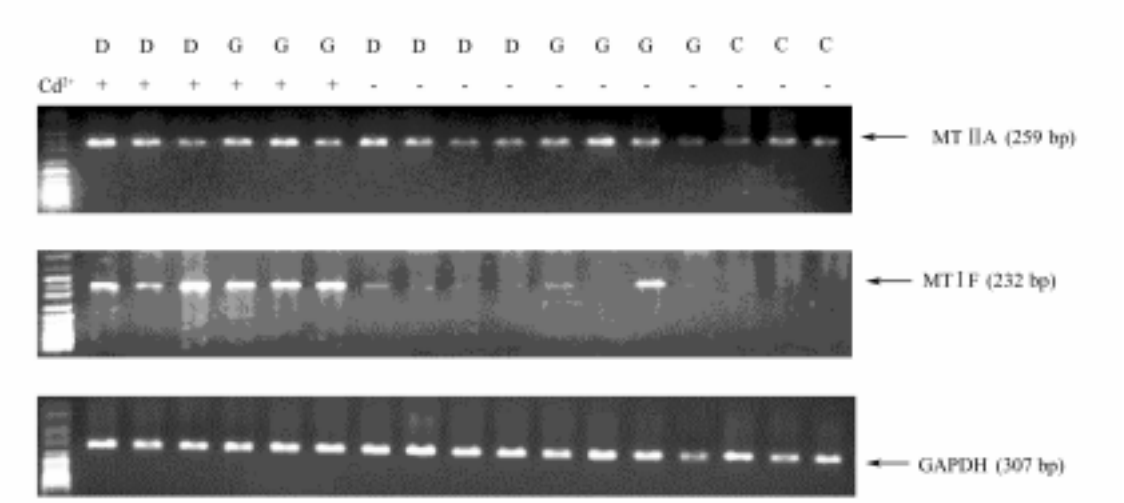

FIG. 8. MT expression after pre-treated with isoflavone (n=3). Cells were pre-treated with media (C), genistein (G) or daidzein (D) for 24 h, then the culture media was discarded, cells were challenged with $CdCl_2$ (Cd^{2+}+) or incubated with media (Cd^{2+}-) for an additional 24 h. Final dose of $CdCl_2$ was 10 μmol/L, final dose of genistein/daidzein was 1 μmol/L.

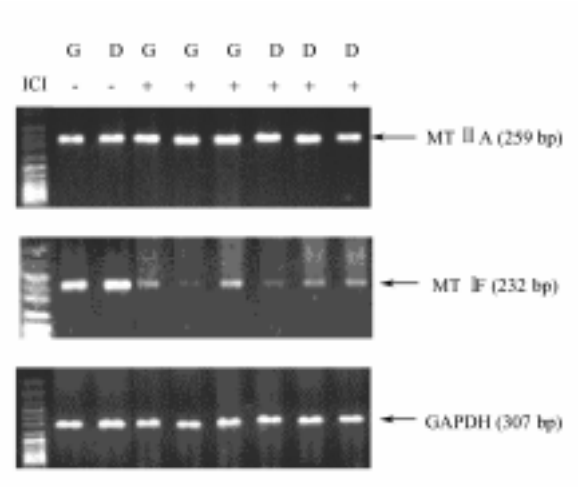

FIG. 9. Effect of ICI 182 780 on the induction of MT expression by isoflavones (n=3). Cells were co-treated media (C), 1 μmol/L genistein (G) , 1 μmol/L daidzein (D) in presence (ICI+) or absence (ICI-) of 1 μmol/L ICI 182 780 for 24 h.

DISCUSSION

Genistein and daidzein are two of primary components in isoflavone, rich in soy products. High soy diet in human can generate a plasma genistein or daidzein concentration of about 10^{-6} mol/L,while the concentration in Western populations is about 10^{-7} mol/L[11]. Since genistein and daidzein have a relative short biological half-life, around 6-8 h, the accumulation in organs is minor[12]. So in the present study, 10^{-5} mol/L was supposed to be the maximum concentration *in vivo*, physiologically. Genistein or daidzein at the dose ranging from 10^{-10} mol/L to 10^{-5} mol/L has no detectable effect on cell viability, indicating that the growth of vascular endothelial cells is independent of isoflavone. However, both genistein and daidzein could reverse the cytotoxicity of cadmium. The protective effect is dependent on the dose of cadmium and isoflavone. The maximum effect could be achieved at the highest dose of isoflavone. Isoflavone could exert the protective effect at all other tested doses of cadmium, except for 60 μmol/L. The underlying factor remains to be elucidated. The difference in potency between genistein and daidzein could also be observed, with daidzein possessing a stronger protection in co-treatment. The mechanism for this is still unclear. Our data suggest that daidzein may induce a quick response of cells as seen in the time-course study. Co-treatment with daidzein was more potent than pre-treatment, but such a difference was not seen in genistein, indicating that the slight difference in structure of these two isoflavones may lead to different biological effecs, as could also be seen in other studies[13]. In the co-treatment groups both rapid and direct effect and chronic and indirect effect may play a role, but in the pretreatment group, chronic and genomic way might be dominant. So we assume that both rapid non-genomic way and chronic genomic way are responsible for isoflavone's effect. However, during the pre-treatment, at least 12 h were required for a significant protection, for the co-treatment, 24 h were needed, except for daidzein which exerted protection in 3 h. Thus, the effect of genistein may not involve a direct interaction with cadmium, but is associated with modulation of protective factors in the cells. In contrast, daidzein might have alternative

ways that allow a quick and strong protection in the co-treatment group. Furthermore, the protective effect of isoflavone was blocked by complete ER antagonist ICI 182 780, suggesting that it is ER related.

Genistein and daidzein also demonstrated different patterns in inducing MT expression. Daidzein up-regulated MT IIA expression at 3 h, but had no effect at 6 h and 12 h, which was consistent with the increase of cell relative viability at 3 h, suggesting that the induction of MT gene is attributable in part to daidzein's protective effect. The two subtypes of MT gene responded differently to isoflavone, and the different response of MT subtypes to the same inducer has been reported elsewhere[14]. MT IF induction by isoflavone might be associated with the typical ER pathway as the up-regulation is blocked by ER antagonist. But MT II A induction seemed to be unrelated with the typical ER pathway. The finding that isoflavone could interact with the promoter of MT gene through ER may explain our observation. Although the induction of MT by cadmium can be maintained for 60 h[15], the induction of MT by isoflavones could last for no more than 24 h.

Isoflavones have been reported to induce MT IIA synthesis in a time and dose dependent manner in Caco-2 cells at the tested dose of 10 μmol/L to100 μmol/L[16]. The present study has shown that 1 μmol/L isoflavone is sufficient to induce MT mRNA expression, but 10 μmol/L isoflavone is needed to counteract the toxicity of cadmium. The MT induction at 1 μmol/L could not meet the need to reverse the toxicity, or other ways require a higher dose to be activated. Dietary achievable isoflavone could induce endogenous protective factors like MT, which may be protective against the damage. However, the explanation of isoflavone's protective effect on cadmium toxicity is entirely speculative at this time.

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