Abstract. Background/Aim: The clinical use of arsenic trioxide (As$_2$O$_3$) is hampered due to its cardiotoxicity. Therefore, it is critical to prevent As$_2$O$_3$-induced loss of endothelial integrity. The purpose of this study was to examine As$_2$O$_3$-induced endothelial dysfunction and evaluate the efficacy of crocetin on reversing As$_2$O$_3$-induced cardiotoxicity.

Materials and Methods: Cultured human umbilical vein endothelial cells (HUVECs) were used to examine As$_2$O$_3$-induced oxidative stress, apoptosis, production of reactive oxygen species (ROS) and DNA adducts. In addition, the impact of crocetin on As$_2$O$_3$-induced cardiotoxicity was evaluated. Results: As$_2$O$_3$ decreased the viability of HUVEC cells and led to apoptosis. Additionally, As$_2$O$_3$ elevated NADPH oxidase activity, and the levels of intracellular ROS. Furthermore, the formamidopyrimidine DNA-glycosylase- and endonuclease III-digestible adducts were induced by As$_2$O$_3$. Crocetin treatment reversed the As$_2$O$_3$-induced reduction in cell viability, the induction of apoptosis, the activation of NADPH oxidase activity, ROS levels and DNA adducts. Conclusion: Crocetin protects from As$_2$O$_3$-induced cardio-toxicity.

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reducing oxidative stress (18), atherosclerosis (19), hypertension (20) and cardiac hypertrophy (21, 22). Crocetin has also been reported to significantly enhance glutathione peroxidase and superoxide dismutase activities (18). In addition, crocetin can regulate various myocardial enzymes, and collaborate with them to reduce cardio-toxicity and apoptosis (18, 23). However, the effect of crocetin on the DNA level has never been studied.

Based on the aforementioned findings, the current study aimed to evaluate the effects of As$_2$O$_3$ exposure on HUVEC cells in relation to apoptosis, oxidative stress, and DNA adducts, and to investigate whether and how crocetin reverses As$_2$O$_3$ toxicity.

**Materials and Methods**

**Cell line and chemicals.** Human umbilical vein endothelial cells (HUVECs) (American Type Culture Collection, CRL-1730, Manassas, VA, USA) were cultured in RPMI-1640 (Hyclone, UT, USA) containing 10% fetal calf serum. As$_2$O$_3$, crocetin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Company (St. Louis, MO, USA).
Cell viability assay. Cell viability of HUVEC cells was tested by MTT assay as previously published (24, 25). After drug treatments, the cells were treated with MTT, and the plates were incubated in the dark for 4 h at 37˚C. The intensity was measured with a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland). Each experiment was repeated at least thrice.

Measurement of cell apoptosis. Cell apoptosis of HUVEC cells was examined as previously published (26, 27). After drug treatments, HUVEC cells were ethanol-fixed and incubated with propidium iodide buffer for 30 min in the dark at 37˚C. After the fixing and staining processes, HUVEC cells were filtered through a 40-μm nylon filter and the percentage of HUVEC cells in the sub-G1 phase was analysed by flow cytometry using a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA). Each experiment was repeated at least thrice.

Intracellular ROS production. Intracellular ROS production was measured as previously described (26, 27). After drug treatments, HUVEC cells were harvested, re-suspended in 10 μM DCFH-DA, incubated at 37˚C for 30 min, and analyzed by flow cytometry (BD Biosciences). Results are expressed as fold of the untreated control and each experiment was repeated at least thrice.

NADPH oxidase activity. The NADPH oxidase activity of HUVEC cells was checked as previously described (28). After drug treatments, HUVEC cells were harvested, re-suspended in 10 mM DCFH-DA, incubated at 37˚C for 30 min, and analyzed by flow cytometry (BD Biosciences). The reaction buffer was composed of 100 mM of Tris-HCl, 1 mM of EDTA and 0.2 mM of NADPH. The NADPH oxidase activity correlated with the decrease in absorbance at 340 nm. Each experiment was repeated at least thrice.

Comet assay for oxidative DNA adducts. The FPG- and endonuclease III-digestible adducts of HUVEC cells were examined as previously described (28). Briefly, after preparation of typical 3-layer agarose gel slides, the slides were lysed, washed, and incubated at 37˚C for 30 min. Then, formamidopyrimidine DNA-glycosylase (FPG) or endonuclease III (Trevigen, Gaithersburg, MD, USA) together with the enzyme reaction buffer were added and further incubated at 37˚C for 2 h. Then, the slides were put at 4˚C for 18 h, followed by incubation in an alkaline solution for 20 min, and then electrophoresed and stained by SYBR green I. The comet moment was quantified with the formula \(\sum 0^{-n} \times \text{[amount of DNA at distance X]} \times \text{(distance X)}] / \text{total DNA}. \) For each sample, at least 50 cells were detected.

**Figure 2.** The effects of As₂O₃ and crocetin on HUVEC cell apoptosis. (A) The effects of 24-h treatment of 0~32 μM As₂O₃ on HUVEC cells. Apoptotic cells were detected by flow cytometry with sub-G1. Data were present as mean±SD for at least 3 experiments. *Statistically significant (p<0.05) compared with untreated group. (B) Treatments of various doses of crocetin with 32 μM (◆), 16 μM (▲) or 0 μM (●) As₂O₃ for 24 h. Data were present as mean±SD for at least 3 experiments. *Statistically significant (p<0.05) compared with the control (As₂O₃ alone) group.

**Figure 3.** The effects of As₂O₃ and crocetin on ROS production in HUVEC cells. Treatments of 4, 8, or 16 μM As₂O₃ with or without 10 or 20 μM crocetin for 12 h. Data are presented as mean±SD of at least three experiments. *Statistically significant (p<0.05) compared with the control (As₂O₃ alone) group.
Statistical methodology. Results are shown as the mean±SEM for each repeated data. Statistical significance was assessed by the Student’s t-test or one-way ANOVA with post hoc test using the SPSS (version 15.0) software (SPSS Inc., Chicago, IL, USA). p-Values<0.05 were considered statistically significant.

Results

As₂O₃-induced cytotoxicity was reversed by crocetin. The ability of As₂O₃ to induce cytotoxicity in HUVEC cells was analyzed. Treatment with 0, 4, 8, 16, and 32 μM of As₂O₃ induced a dose-dependent decrease in cell viability as visualized by microscopy (Figure 1A). In detail, cell viability of HUVEC cells treated with 4 μM As₂O₃ was not significantly affected (Figure 1B). However, treatment with 8, 16, and 32 μM of As₂O₃ for 24 h, led to 83.6, 58.4, and 21.5% cell viability, respectively (Figure 1B). Treatment with crocetin reversed the 16 and 32 μM As₂O₃-induced suppression in cell viability (Figure 1C).

As₂O₃-induced apoptosis was reversed by crocetin. The ability of As₂O₃ to induce cell apoptosis in HUVEC cells was then analyzed. Treatment of HUVEC cells with As₂O₃ concentrations higher than 8 μM for 24 h led to apoptosis (Figure 2A). At 8, 16, and 32 μM, As₂O₃ induced 21.7, 38.3, and 66.1% of HUVEC cells to undergo apoptosis, respectively (Figure 2A). Treatment with crocetin reversed the 16 and 32 μM As₂O₃-induced cell apoptosis (Figure 2B).

As₂O₃-induced oxidative stress was suppressed by crocetin. As₂O₃ treatment induced the production of ROS dose-dependently at the range of 4–16 μM at 24 h (Figure 3). The 4, 8, and 16 μM As₂O₃-induced ROS could be suppressed by treatment with 10 and 20 μM of crocetin, respectively (Figure 3).

As₂O₃-activated NADPH oxidase was suppressed by crocetin. Four, 8, 16, and 32 μM As₂O₃ treatment activated NADPH oxidase activity by 2.4-, 3.0-, 3.3- and 4.6-fold, respectively (Figure 4A). Treatment with crocetin suppressed the As₂O₃-activated NADPH oxidase activity in a dose-dependent manner (Figure 4B).

As₂O₃-induced FPG- and Endonuclease III-digestible DNA adducts was suppressed by crocetin. As₂O₃ treatment induced FPG- and endonuclease III-digestible adducts dose-dependently at 4, 8, and 16 μM (Figure 5 and Figure 6). The oxidative adducts were effectively reduced by treatment with 10 and 20 μM of crocetin, and the reducing efficacy was higher at 20 μM (Figure 5 and Figure 6).

Discussion

Increased apoptosis of HUVEC cells may be closely related to loss of endothelial integrity, leading to vascular disorders (29, 30). Our results confirmed that As₂O₃ can induce HUVEC cells to undergo apoptosis, while crocetin can effectively rescue the As₂O₃-induced HUVEC programmed cell death (Figure 2B). In this study, As₂O₃-induced apoptosis was not as severe as that in Yu’s experiments, where 5 μM As₂O₃ induced about 40% apoptosis (31). Our results are consistent with those of Ma’s findings, which showed that 5 μM As₂O₃ was able to induce about 20%
apoptosis in HUVEC cells (32). The differences may be probably due to different culturing passages of HUVEC cells. Treatment with crocetin was found to effectively reverse the As2O3-induced apoptosis in HUVEC cells (Figure 2B). The As2O3-induced ROS production started to increase at 30 min, and could be sustained for 12 h (data not shown). Here, we only show the ROS status at 12 h (Figure 3). Treatment with crocetin did not alter the levels of ROS (Figure 3), but suppressed the As2O3-induced induction of ROS levels, and 20 μM was more effective than 10 μM of crocetin (Figure 3). In a rat model, crocetin has been reported to not only induce the activities of superoxide dismutase, glutathione-peroxidase, and catalase, but also decrease the levels of malondialdehyde and ROS (33).

A number of cardiovascular disorders, such as atherosclerosis and hypertension, have been closely related to increased vascular ROS levels, which has been designated as oxidative stress (34, 35). Our studies showed that As2O3 activated NADPH oxidase, and that treatment with crocetin can effectively reverse it (Figure 4B). This may be the major mechanism related to As2O3-mediated intracellular ROS generation. There is no doubt that As2O3-induced ROS production could be due to different mechanisms other than NADPH oxidase, and could cause different consequences. For instance, As2O3 has been reported to induce the loss of mitochondrial membrane potential and ROS formation, leading to DNA damage and cell apoptosis (36, 37). Moreover, ROS formation has been found to associate with autophagy and other programmed cell death mechanisms (37, 38).

One of the novel findings of this study is the measurement of crocetin-induced suppression of As2O3-induced FPG-digestible adducts and endonuclease III-digestible adducts. The subtle alterations in DNA adducts are one of the end points for understanding how ROS induced intracellular damage and cell death. Consistent with previous findings (28), treatment of As2O3 was capable of inducing FPG-digestible endonuclease III-digestible DNA adducts dose-dependently at the range of 4–16 μM (Figure 5 and Figure 6). As previously reported, FPG can specifically digest oxidized purines, such as 8-oxoguanine, 5-hydroxycytosine, 5-hydroxuryracil, 2,6-diamino-4-hydroxy-
5-N-methylformamidopyrimidine, and 4,6-diamino-5-formamidopyrimidine (35). However, endonuclease III can specifically digest oxidized pyrimidines, such as thymine glycol, 5,6-dihydrothymine, 5-hydroxydihydrothymine, 5-hydroxycytosine, 5-hydroxyuracil, and uracil glycol (39). This study showed for the first time that treatment with crocetin could reverse the formation of these As₂O₃-induced oxidative DNA adducts (Figure 5 and Figure 6) dose-dependently, although the detail mechanisms require further investigation. We did not however examine whether As₂O₃ can induce lipid peroxidation, and whether crocetin can reverse it. In 2017, Ma et al. found that As₂O₃ can increase lipid peroxidation in HUVEC cells (32), while the effects of crocetin on this requires further investigation. A scheme summarizing the effects of crocetin on the As₂O₃-induced NADPH oxidase activity, ROS levels and oxidative DNA adducts is shown in Figure 7.

In conclusion, crocetin is capable of reversing the As₂O₃-induced apoptosis, the activation of NADPH oxidase, and the production of ROS and oxidative DNA adducts in HUVEC cells. The antioxidant capacities of crocetin can aid cardiovascular disease prevention in clinical practice.

Conflicts of Interest

The Authors declare no conflicts of interest in regard to this study.

Authors’ Contributions

Tsai CL, Tsai CW and Chang WS conceived and designed the experiments. Tsai CW and Chang WS performed the experiments. Lin JC and He JL analyzed the data. Shih LC and He JL contributed reagents/materials/analysis tools. Tsai CW and Bau DT wrote and revised the article.

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