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Original Article

Evaluation of treatment for acute hexavalent chromium poisoning in human renal proximal tubular epithelial cells with 2,3-dimercapto-1-propanesulfonic acid (DMPS)

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Abstract: Objectives: Chelating agents have been utilized in clinical settings to counteract various heavy metal poisonings, although there are currently no established medical guidelines supporting the use of the chelating agent 2,3-dimercapto-1-propanesulfonic acid (DMPS) for treating acute hexavalent chromium (Cr(VI)) poisoning. Methods: Since systemic acute Cr(VI) poisoning has been associated with nephrotoxicity in clinical cases, this study utilized the immortalized human renal epithelial cell line HK-2 and primary proximal tubular epithelial cells (RPTEC) as *in vitro* models to explore potential treatments. Results: In the HK-2 cell model, cell viability dropped below 50% after exposure to 10 μmol/L Cr(VI) for 32 hours. Both simultaneous administration of 10 μmol/L Cr(VI) with 100 or 300 μg/mL DMPS and delayed treatment with DMPS after two hours effectively reduced Cr(VI)-induced cell death. Furthermore, in the RPTEC cell model, DMPS treatments ranging from 300 to 1000 μg/mL demonstrated significant protective effects on cell viability against Cr(VI) toxicity, mirroring the results observed in the HK-2 model. In contrast, meso-2,3-dimercaptosuccinic acid, another chelating agent, did not exhibit protective effects on HK-2 cells. Conclusions: These findings support DMPS as a promising antidote for acute Cr(VI) poisoning in kidney cells and provide valuable insights for potential clinical applications.

Keywords: Hexavalent chromium (Cr(VI)), 2,3-dimercapto-1-propanesulfonic acid (DMPS), meso-2,3-dimercaptosuccinic acid (DMSA), human renal proximal tubule epithelial cell, cell death, treatment

Introduction

Metallic elements characterized by densities exceeding 5 g/cm^3 and atomic weights greater than 40.04 are commonly classified as heavy metals [1]. Certain heavy metals, including cobalt, chromium, copper, magnesium, iron, molybdenum, manganese, selenium, nickel and zinc serve as vital nutrients necessary for human physiological functions. Nevertheless, high concentration of these elements can result in acute or chronic toxicity [2, 3]. Heavy metals such as iron, copper, chromium, arsenic, and mercury have been observed to exacerbate oxidative stress through the generation of free radicals [4-8].

The administration of antioxidant agents has demonstrated efficacy in mitigating oxidative damage induced by heavy metals. For instance, vitamin E has been shown to safeguard renal cells against oxidative stress caused by heavy metal exposure [9], while ascorbic acid (vitamin C) has exhibited protective effects against heavy metal-related renal toxicity [10]. Furthermore, N-acetyl-L-cysteine (NAC) has pro-

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ven its utility both as an antioxidant and as a metal-chelating agent in the management of heavy metal poisoning [11]. Additionally, British anti-lewisite (BAL or dimercaprol), the inaugural chelating compound formulated for arsenic exposure, is utilized for treating poisoning by various metals including arsenic, gold, mercury and lead [12, 13]. More recently developed analogues of BAL, notably 2,3-dimercapto-1-propanesulfonic acid (DMPS or unithiol) and meso-2,3-dimercaptosuccinic acid (DMSA or succimer), have been established as effective antidotes for mercury and arsenic toxicity [14-17]. These chelating agents represent significant advancements in the therapeutic approach to managing heavy metal poisoning.

Hexavalent chromium (Cr(VI)) represents a significant public health concern due to its toxicological impacts [18]. Exposure to Cr(VI) has been shown to induce the generation of reactive oxygen species (ROS), cause DNA damage, and trigger apoptosis in mammalian cells [19-21]. Moreover, systemic exposure to Cr(VI) has been linked to organ damage, particularly affecting the gastrointestinal system, liver and kidneys, as evidenced by clinical findings [22]. Despite the potential for metal chelators to serve as therapeutic or preventative agents for Cr(VI) toxicity, existing medical guidelines do not formally recommend the use of DMPS or DMSA for the treatment of Cr(VI) poisoning. Indeed, prior research has reported that DMPS administration does not significantly enhance chromium excretion in humans [23]. Nevertheless, an isolated clinical case described successful treatment of severe Cr(VI) poisoning with a combination of NAC, ascorbic acid and DMPS, suggesting the possible therapeutic value of DMPS in such cases [24]. To further elucidate this potential, the present study was designed to assess whether treatment with DMPS or DMSA could mitigate cellular damage caused by Cr(VI) exposure.

Due to the well-documented nephrotoxicity associated with systemic Cr(VI) poisoning [25] that can result in conditions such as acute tubular necrosis and diminished urine flow rate in clinical settings [26], this study aimed to evaluate the protective effects of DMPS and DMSA. Both chelating agents were tested individually and in combination using *in vitro* kidney cell models. Two human renal tubule cell lines were selected for this purpose: the immortalized proximal tubule epithelial cell line (HK-2) and primary renal proximal tubule epithelial cells (RPTEC). The study sought to determine the most effective therapeutic regimen involving DMPS and DMSA for managing Cr(VI)-induced nephrotoxicity.

Materials and methods

Chemicals

Sodium 2,3-dimercaptopropanesulfonate monohydrate (DMPS) (catalog number: D8016, Sigma-Aldrich) and meso-2,3-Dimercaptosuccinic acid (DMSA) (catalog number: D7881, Sigma-Aldrich) were used in this study. Stock solution of DMPS was prepared with sterile ddH_2O at 100 mg/mL and stock solution of DMSA was prepared with dimethyl sulfoxide (DMSO) at 10 mg/mL. The working solution of DMPS and DMSA used in this study was prepared by dilution with ddH_2O and DMSO, respectively.

Cell culture

Human primary renal proximal tubule epithelial cells (RPTEC) (PCS-400-01) and an immortalized proximal tubular epithelial cell line HK-2 (CRL-2190) were obtained from the American Type Culture Collection (ATCC). RPTEC were maintained in renal epithelial cell basal medium (PCS-400-03) supplied with renal epithelial cell growth kit (PCS-400-04) from ATCC and HK-2 were maintained in keratinocyte-serum-free medium (K-SFM) supplemented with bovine pituitary extract (BPE), human recombinant epidermal growth factor (EGF), 1% fetal bovine serum and 1% antibiotic/antimycotic (Gibco, 15240062). Both cells were incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO_2.

Cell viability assay

Cell viability of HK-2 and RPTEC cells were determined by AlamarBlue™ Cell Viability Reagent (catalog number: BUF012B, Bio-Rad) according to the manufacturer's instructions. Briefly, 100 µL of cells (3000 cells) were seeded in a 96 well plate overnight. For evaluating toxic effect of Cr(VI) on cell viability, the cell culture medium was replaced by the medium containing different concentrations of potassium dichromate

($K_2Cr_2O_7$, a hexavalent chromium) for an additional 32 hours and AlamarBlue reagent was subsequently added to wells for evaluation of cell viability. For evaluating the protective effect of DMPS or DMSA, the cell culture medium was replaced with the medium containing 10 or 30 μmol/L potassium dichromate along with different concentrations of DMPS or DMSA for an additional 32 hours. For evaluating the protective effect of delay treatment, the cells were treated with DMPS at 0 hours (h), 2 h, 4 h and 6 h after 10 or 30 μmol/L potassium dichromate intoxication (referred to as d0h, d2h, d4h and d6h). The d0h cells were cultured with medium containing 10 or 30 μmol/L potassium dichromate and different concentrations of DMPS or DMSA. The d2h, d4h and d6h cells were cultured with medium containing 10 or 30 μmol/L potassium dichromate for 2, 4 and 6 hours, and then the media were replaced with 10 or 30 μmol/L potassium dichromate and different concentrations of DMPS or DMSA. Cell viability was then evaluated 32 hours after exposure to potassium dichromate. The results were detected by using a microplate reader (BioTek Epoch Microplate Spectrophotometer).

Western blot

The HK-2 cells were lysed in a radioimmunoprecipitation (RIPA) lysis buffer with a protease inhibitor (Millipore). The concentrations of protein samples were determined by a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), with samples being run on a precast 4-15% gradient SDS/PAGE (Bio-Rad). Transferred polyvinylidene difluoride (PVDF) membranes (Millipore) were incubated with primary antibodies including anti-PARP1 (1:1,000, catalog number: 13371-1-AP, Proteintech) at 4°C overnight and followed by HRP-linked anti-rabbit IgG (1:4,000, catalog number: #7074, Cell signaling). β-actin served as an endogenous control by incubation with HRP-linked β-actin antibody (1:5,000, catalog number: tcba13655, Taiclone) at room temperature for 1 hour. The blots were visualized with Azure biosystems 300Q (Azure Biosystems, Inc., USA).

Statistics

The bar graphs were generated and statistics performed by GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). All data were expressed as means ± SD of at least three independent experiments. The statistical analysis of the protective effect of drug was carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons *post hoc* test. A p-value < 0.05 was considered to indicate a statistically significant difference when compared with the control group.

Results

The toxic effect of hexavalent chromium ($K_2Cr_2O_7$) in HK-2 cells

The toxic effects of Cr(VI) were assessed using the immortalized proximal tubule epithelial cell line (HK-2). These cells were treated with varying concentrations of $K_2Cr_2O_7$, a hexavalent chromium compound (**Figure 1A**). The findings indicated that exposure to $K_2Cr_2O_7$ at concentrations above 3 μmol/L significantly decreased cell viability and altered cell morphology, with the most pronounced effects observed at 10 and 30 μmol/L (**Figure 1B** and **1C**). Additionally, treatment with $K_2Cr_2O_7$ at 10 and 30 μmol/L elevated the protein levels of cleaved PARP-1, a well-known marker of cell death (**Figure 1D**). Consequently, concentrations of 10 and 30 μmol/L $K_2Cr_2O_7$ were selected to investigate the protective effects of DMPS and DMSA treatments in this study.

The protective effect of DMPS and DMSA in $K_2Cr_2O_7$-intoxicated HK-2 cells

Figure 2A illustrates that DMPS treatment at concentrations below 100 μg/mL and 600 μg/mL did not notably impact cell viability; however, an increase in cell viability was noted in HK-2 cells exposed to 300 μg/mL DMPS, while a decrease was observed at 1000 μg/mL. Following the co-administration of 10 μmol/L $K_2Cr_2O_7$ with varying concentrations of DMPS, cell viability of HK-2 in the 300 and 600 μg/mL DMPS-treated groups was not inhibited by $K_2Cr_2O_7$ when compared to the control group. Additionally, cell viability of HK-2 in the 100 to 1000 μg/mL DMPS-treated groups was higher than that of HK-2 cells exposed solely to 10 μmol/L (**Figure 2B**). Conversely, DMSA treatment did not significantly affect HK-2 cell viability, even when the DMSO concentration in the culture medium reached 3% (**Figure 2C**). Nonetheless, cell viability in all DMSA-treated groups was lower than that in the control group.

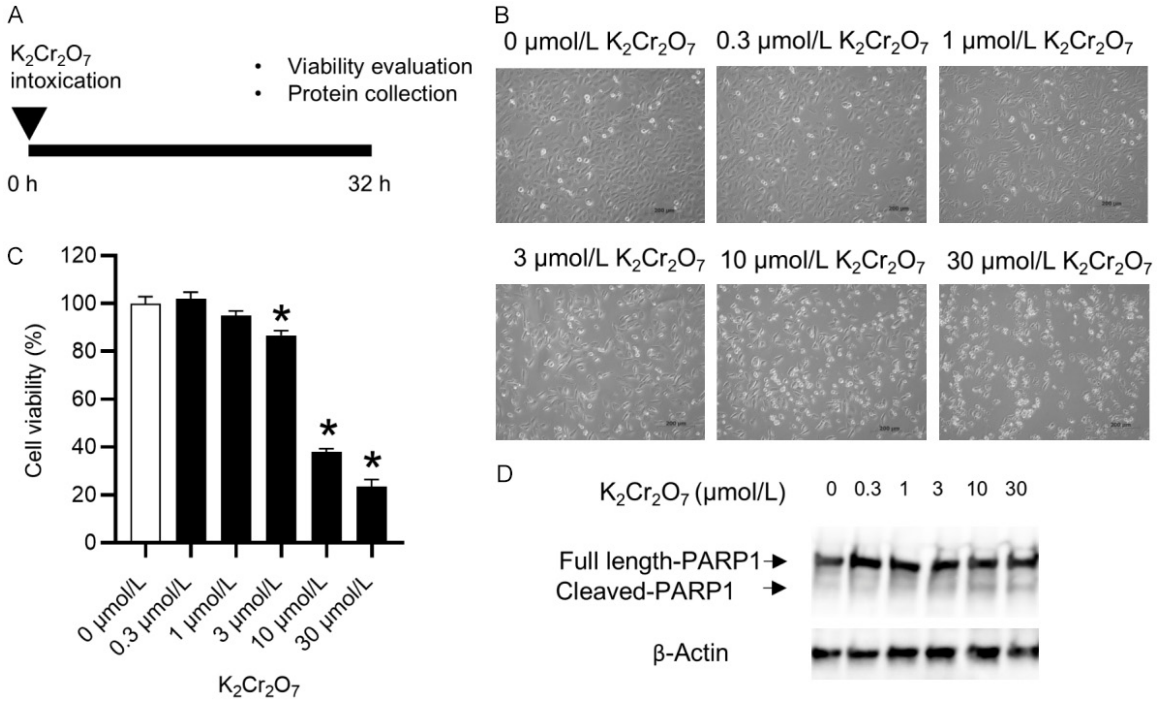

Figure 1. The toxic effect of Cr(VI) exposure at different concentrations. A. Scheme of Cr(VI) intoxication. Potassium dichromate ($K_2Cr_2O_7$) is a chemical compound of Cr(VI). B. The morphology of Cr(VI)-exposed HK-2 cells. C. The viability of Cr(VI)-exposed HK-2 cells. Data are presented as the mean ± SD. **p*-value < 0.05, when compared with 0 μmol/L group. D. The protein expression of poly(ADP-ribose) polymerase-1 (PARP-1) and an apoptotic marker, cleaved PARP-1.

Notably, cell viability in the groups treated with 100 and 300 μg/mL DMSA was higher than that of cells exposed only to 10 μmol/L $K_2Cr_2O_7$ (**Figure 2D**). These findings suggest that DMSA treatment alone provided minimal protective effects against Cr(VI)-induced toxicity in HK-2 cells. In summary, treatment with 300 or 600 μg/mL DMPS effectively shielded HK-2 cells from cell death caused by 10 μmol/L $K_2Cr_2O_7$.

The maximum time delay and optimal concentrations of DMPS treatment in $K_2Cr_2O_7$-intoxicated HK-2 cells

The investigation explored the maximum tolerable delay for effective DMPS treatment in HK-2 cells following exposure to 10 μmol/L $K_2Cr_2O_7$. Treatments with DMPS were administered at 0, 2, 4 and 6 hours post-intoxication (designated as d0h, d2h, d4h and d6h) as shown in **Figure 3A**. Observations of cell morphology demonstrated that HK-2 cells subjected to 10 μmol/L $K_2Cr_2O_7$ showed reduced damage when treated with 300 μg/mL DMPS within two hours (**Figure 3B**). **Figure 3C** highlights the protective effects of delayed DMPS treatment at 300 μg/mL after $K_2Cr_2O_7$ intoxication. Cell viability measurements indicated a significant improvement in the d0h and d2h groups compared to the untreated intoxicated group, while no significant benefit was observed in the d4h and d6h treatment groups. Nonetheless, cell viability in all delayed-treatment groups remained lower than that of completely untreated cells. Western blot analysis revealed an increase in cleaved-PARP1 protein levels starting at 4 hours after $K_2Cr_2O_7$ exposure (**Figure 3D**). These findings suggest that while 300 μg/mL delayed-DMPS treatment provided some protection against Cr(VI)-induced cell death in HK-2 cells, it was not sufficient to fully mitigate the toxic effects of Cr(VI).

Higher concentrations of DMPS were subsequently employed to assess its protective effects when administered as a delayed treatment. Notably, all groups treated with 600 and 1000 μg/mL DMPS exhibited significantly higher cell viability compared to the control group. However, no significant differences were observed in the cell viability of HK-2 cells between

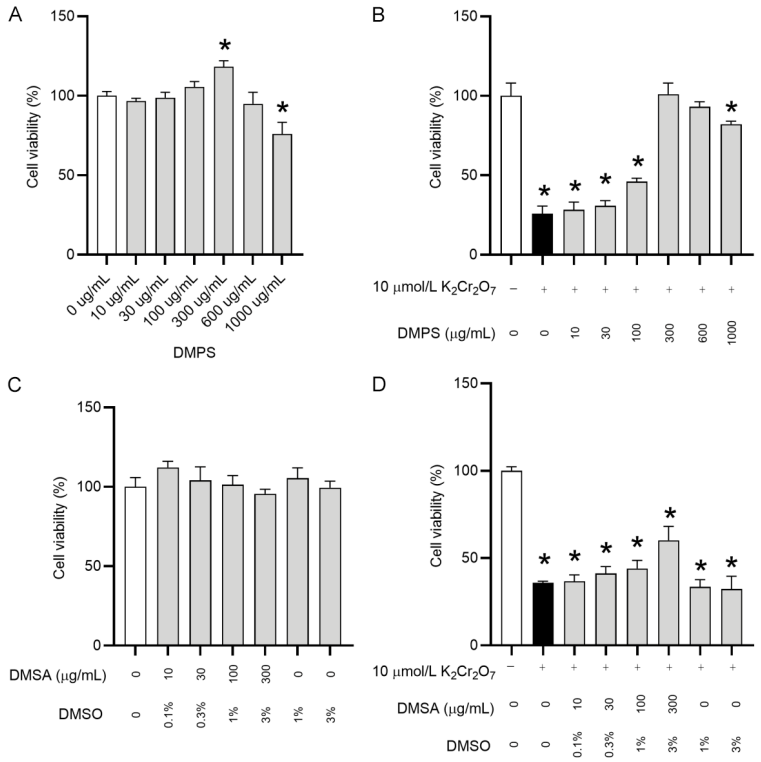

Figure 2. The cytotoxic and protective effects of 2,3-dimercapto-1-propanesulfonic acid (DMPS) and meso-2,3-dimercaptosuccinic acid (DMSA) in Cr(VI)-exposed HK-2 cells. A. The cytotoxicity of different concentrations of DMPS. B. The protective effect of different concentrations of DMPS on Cr(VI)-exposed cells. Cells underwent co-administration of Cr(VI) and DMPS. C. The cytotoxicity of different concentrations of DMSA. D. The protective effect of different concentrations of DMSA on Cr(VI)-exposed cells. Cells were co-administrated with Cr(VI) and DMSA. Data are presented as the mean ± SD. *p-value < 0.05, when compared with the control group (the group without Cr(VI) and DMPS treatments).

the 600 and 1000 μg/mL DMPS-treated groups, apart from the time point at d0h (**Figure 4A**). Although treatment with 1000 μg/mL DMPS alone significantly reduced HK-2 cell viability (**Figure 2A**), delayed treatment with 1000 μg/mL DMPS still demonstrated a positive impact, enhancing HK-2 cell viability following intoxication with 10 μmol/L $K_2Cr_2O_7$. Further investigation into the protective effects of DMPS was carried out on HK-2 cells intoxicated with 30 μmol/L $K_2Cr_2O_7$ (**Figure 4B**). Enhanced cell viability was evident in groups treated with 300, 600 and 1000 μg/mL DMPS at d0h. Additionally, cells treated with 600 and 1000 μg/mL DMPS at d2h also displayed higher viability compared to the 30 μmol/L $K_2Cr_2O_7$-intoxicated group. These findings suggest that high concentrations of delayed DMPS treatment offer significant protection to HK-2 cells against severe Cr(VI) intoxication.

To investigate whether combination of DMPS and DMSA served as a therapeutic strategy in acute $K_2Cr_2O_7$-exposed HK-2 cells

The administration of DMSA alone did not exhibit a statistically significant protective impact on HK-2 cells exposed to $K_2Cr_2O_7$. To assess whether the combination of DMSA and DMPS could yield an additive protective effect, extend the effective therapeutic window of DMPS while reducing its necessary protective dosage, the protective effects of DMSA and DMPS in combination therapy were investigated. **Figure 5A** illustrates the outcomes of combined treatment using 100 μg/mL of DMSA and DMPS, while **Figure 5B** illustrates the results for the combined treatment with 300 μg/mL of DMSA and DMPS. Notably, irrespective of dosage, the cell survival rates at both time points (d0h and d2h) were consistently higher across all groups receiving the combination treatment compared to those exposed to $K_2Cr_2O_7$ without intervention. The protective efficacy of the combination therapy utilizing 100 μg/mL each of DMPS and DMSA was superior to that observed with 100 μg/mL DMPS administered alone. Nevertheless, further analysis revealed that the combined treatment did not surpass the protective efficacy or extend the maximum therapeutic delay achievable with a higher concentration of 300 μg/mL DMPS alone. These findings suggest that while co-treatment with DMSA and 100 μg/mL DMPS could augment the protective effect relative to 100 μg/mL DMPS alone, it did not enhance the protective capacity of a higher dose (300 μg/mL) of DMPS in this HK-2 cell model.

The protective effect of DMPS in $K_2Cr_2O_7$-intoxicated human primary renal proximal tubule epithelial cells (RPTEC)

The study investigated the protective effects of DMPS on RPTEC, a primary proximal tubular

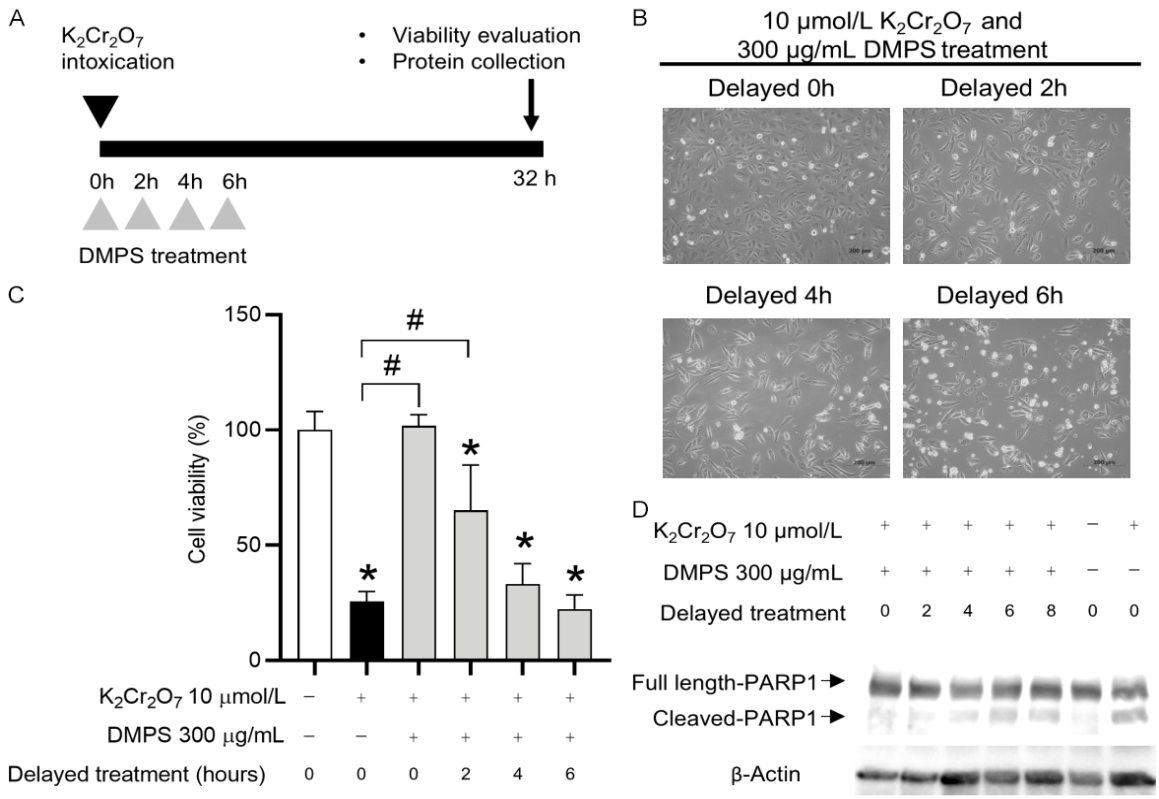

Figure 3. The viability in HK-2 cells with delayed 300 μg/mL DMPS treatment after exposure to 10 μmol/L Cr(VI). A. Scheme of delayed DMPS treatment after Cr(VI) intoxication. B. The morphology of 10 μmol/L Cr(VI)-exposed HK-2 cells after delayed 300 μg/mL DMPS treatment. C. The viability of Cr(VI)-intoxicated HK-2 cells with delayed DMPS treatment. HK-2 cells were treated with DMPS at 0 h, 2 h, 4 h, and 6 h after 10 μmol/L $K_2Cr_2O_7$ intoxication (referred to as d0h, d2h, d4h and d6h). Data are presented as the mean ± SD. * indicates *p*-value < 0.05, when compared with the control group. # indicates *p*-value < 0.05, when compared with the group exposed to 10 μmol/L Cr(VI) alone. D. The protein expression of PARP-1 and cleaved PARP-1.

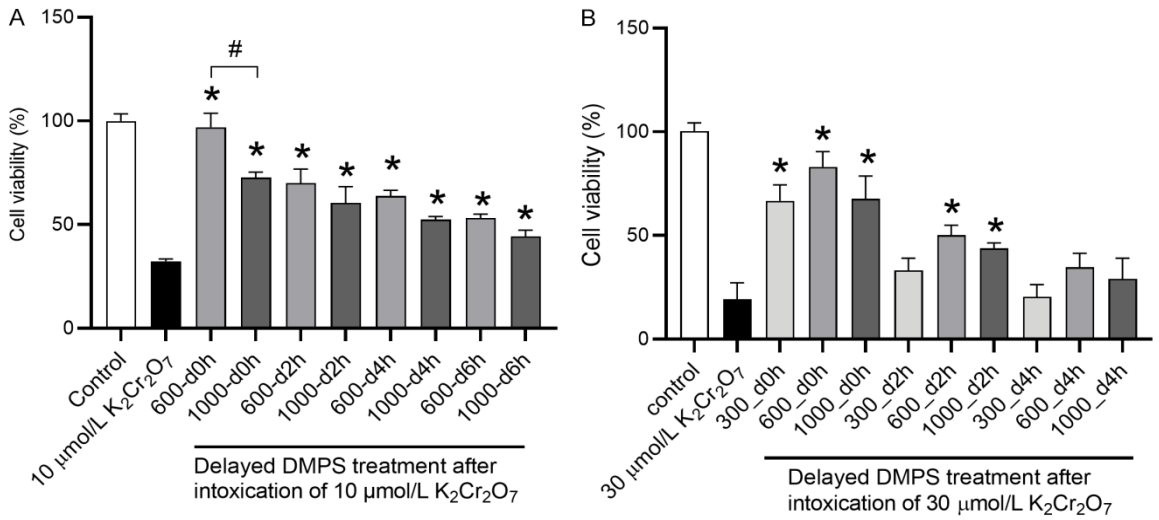

Figure 4. The viability in HK-2 cells with delayed DMPS treatment after exposure to 10 and 30 μmol/L Cr(VI). A. The viability of 10 μmol/L Cr(VI)-intoxicated HK-2 cells with delayed 600 and 1000 μg/mL DMPS treatment. * indicates *p*-value < 0.05, when compared with the group exposed to 10 μmol/L Cr(VI) alone. # indicates *p*-value < 0.05 between two groups. B. The viability of 30 μmol/L Cr(VI)-intoxicated HK-2 cells with delayed DMPS treatment. Data are presented as the mean ± SD. * indicates *p*-value < 0.05, when compared with the group exposed to 30 μmol/L Cr(VI) alone.

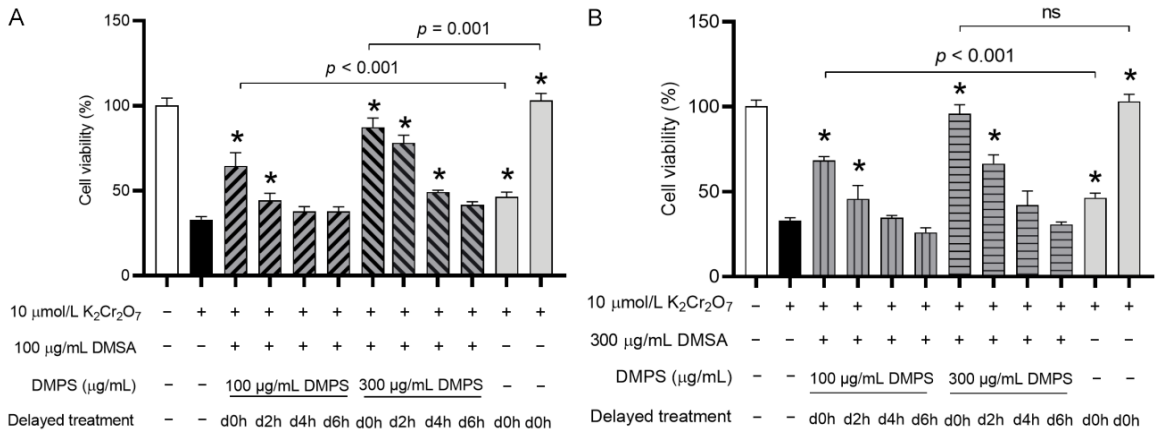

Figure 5. The protective effect of DMPS and DMSA combined in Cr(VI)-exposed HK-2 cells. A. The protective effect of 100 μg/mL DMSA combined with 100 and 300 μg/mL DMPS. B. The protective effect of 300 μg/mL DMSA combined with 100 and 300 μg/mL DMPS. Data are presented as the mean ± SD. **p*-value < 0.05, when compared with 10 μmol/L Cr(VI)-exposed group; ns, no significant difference. The right gray bar indicates the cell viability of 100 and 300 μg/mL DMPS treatment alone. The *p*-value between DMSA combined with DMPS group, and DMPS treatment alone group is shown in this Figure.

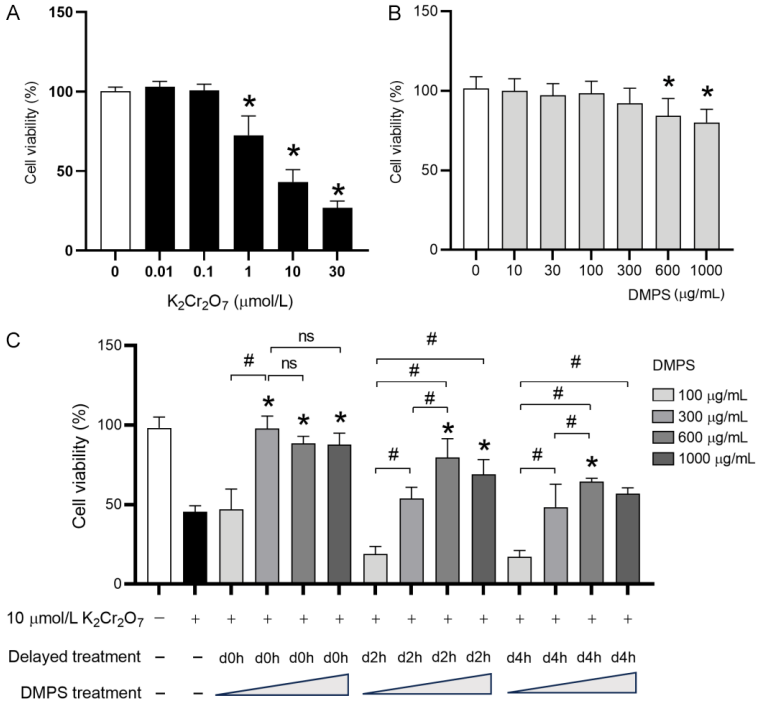

Figure 6. The protective effect of DMPS in Cr(VI)-exposed human primary renal proximal tubule epithelial cells (RPTEC). A. The viability of Cr(VI)-intoxicated RPTEC cells. B. The viability of DMPS-treated RPTEC cells. C. The protective effect of DMPS on Cr(VI)-intoxicated RPTEC. Data are presented as the mean ± SD. * indicates *p*-value < 0.05, when compared with the group with 10 μmol/L Cr(VI) intoxication; # indicates *p*-value < 0.05, when compared with each DMPS-treated group at the same time point of delayed treatment; ns, no significant difference.

epithelial cell line, to determine its efficacy compared to HK-2, an immortalized proximal tubular epithelial cell line. When RPTEC cells were exposed to $K_2Cr_2O_7$ concentrations exceeding 1 μmol/L, cell viability was notably compromised, as shown in **Figure 6A**. Additionally, DMPS treatment alone at 600 and 1000 μg/mL significantly reduced RPTEC cell viability, while concentrations below 300 μg/mL had no detectable impact (**Figure 6B**). RPTEC cells were then exposed to 10 μmol/L $K_2Cr_2O_7$ and subsequently treated with varying concentrations of DMPS (100, 300, 600 and 1000 μg/mL) at three different time points: d0h, d2h and d4h. Cell viability was assessed following these treatments. For co-administration $K_2Cr_2O_7$ and DMPS groups (d0h), DMPS concentrations of 300, 600 and 1000 μg/mL significantly improved cell viability compared to the untreated control group. For the d2h group, only the 600 and 1000 μg/mL DMPS treatments increased cell viability, while at d4h, the protective effect was observed at a concentration of 600 μg/mL, as depicted in **Figure 6C**. In

summary, the results indicated that in RPTEC cells poisoned with 10 μmol/L $K_2Cr_2O_7$, 300 μg/mL DMPS was the optimal treatment concentration at hour 0 (d0h), while 600 μg/mL DMPS was the optimal treatment concentration for delayed treatment at 2 hours (d2h) and 4 hours (d4h). These findings indicate that the optimal treatment concentrations of DMPS in RPTEC cells were consistent with those observed in HK-2 cells.

Discussion

Systemic Cr(VI) poisoning is rare in clinical cases. According to a study, a blood chromium concentration of 10 mg/L is considered lethal [27]. In contrast, a patient with a chromium blood concentration of 3.4 mg/L survived after undergoing intermittent hemodialysis treatment [28]. A concentration of 34 μmol/L $K_2Cr_2O_7$ corresponds approximately to the lethal dose of 10 mg/L Cr(VI), while 10 μmol/L $K_2Cr_2O_7$ is roughly equivalent to 2.95 mg/L. Based on this, cells in the present study were exposed to $K_2Cr_2O_7$ at concentrations representing curable (10 μmol/L) and lethal (30 μmol/L) dosages. Previous research demonstrated that exposure to 10 μmol/L of $K_2Cr_2O_7$ led to increased intracellular ROS production, induced apoptosis, and reduced cell viability in HK-2 cells [20, 29]. In the current study, less than 50% cell viability was observed in HK-2 and RPTEC cells exposed to 10 and 30 μmol/L $K_2Cr_2O_7$ for 32 hours. These concentrations were therefore deemed appropriate for examining acute and severe Cr(VI) poisoning *in vitro*.

Due to its low water solubility, BAL was administered via intramuscular injection in an oil-based solution [16]. In comparison, DMPS and DMSA exhibit higher water solubility than BAL, with DMPS being more soluble than DMSA [30]. For the treatment of heavy metal poisoning, both DMSA and DMPS are effective when administered orally, while DMPS can also be delivered intravenously [31, 32]. Although no formal medical guidelines currently recommend the use of DMPS and DMSA for Cr(VI) poisoning, a previous study reported their protective effects in a human cervical cancer cell line (HeLa cells) and a mouse model exposed to $K_2Cr_2O_7$ [33]. Specifically, treatment with DMPS and DMSA (25-100 μmol/L) reduced growth inhibition in HeLa cells exposed to 5 μmol/L $K_2Cr_2O_7$. Additionally, intraperitoneal administration of 500 mg/kg DMPS or DMSA immediately following a 40 mg/kg $K_2Cr_2O_7$ injection significantly improved survival rates in $K_2Cr_2O_7$-intoxicated mice [33]. These findings suggest that DMPS and DMSA might serve as potential antidotes for Cr(VI) poisoning. In the present study, we further investigated the potential of DMPS and DMSA to protect human renal proximal tubule epithelial cells from Cr(VI)-induced toxicity. The results demonstrate that 300 μg/mL DMPS is sufficient to provide protection when co-administered with 10 μmol/L $K_2Cr_2O_7$. Furthermore, a concentration of 600 μg/mL DMPS proved optimal for delayed treatment of 10 μmol/L $K_2Cr_2O_2$ intoxication, while both 600 μg/mL and 1000 μg/mL DMPS effectively mitigated toxicity from 30 μmol/L $K_2Cr_2O_7$ in delayed treatment scenarios. In contrast, DMSA failed to exhibit any protective effects in our cell-based experiments.

We hypothesize that the differing effects of DMSA and DMPS treatments in our cell models likely stem from their ability to cross the cell membrane. DMPS is distributed across both intracellular and extracellular compartments [34], whereas DMSA is unable to permeate the cell membrane [30]. Extracellular Cr(VI) is known to enter cells through non-specific anion transport proteins [35, 36], where it undergoes reduction by biological thiols like glutathione, leading to the production of harmful hydrogen peroxide and ROS [37]. It is established that despite structural differences, DMPS and DMSA utilize dithiol binding to interact with heavy metals through their thiol groups (-SH) [38]. It is important to note that our experiments were conducted *in vitro*, bypassing the complexities of *in vivo* kidney drug metabolism and absorption. Based on this, we propose that the key distinction might lie in the fact that DMPS can penetrate the cell membrane to eliminate intracellular Cr(VI). Supporting evidence comes from advances in novel DMSA-based therapeutics, particularly Biomimetic Nano-drug Delivery Systems (e.g., AEM-DMSA-NPs) that facilitate cellular entry of DMSA, enabling efficient removal of intracellular chromium and providing effective detoxification against chromium poisoning [39]. This indirect evidence further reinforces the critical role of chelating agents in clearing Cr(VI) from within cells for cellular detoxification. However, these considerations remain speculative and

were not confirmed within the scope of this study. Further research is necessary to validate these hypotheses.

In this study, we did not offer experimental evidence to examine the specific mechanisms through which DMPS treatment influences cell death. However, the literature indicates that exposure to hexavalent chromium can trigger several programmed cell death pathways, such as apoptosis, autophagy, and ferroptosis [20, 40, 41]. Known pharmacological effects of DMPS are primarily attributed to its ability to bind heavy metals like Cr(VI), rather than influencing the intracellular uptake or efflux of Cr(VI) [42, 43]. Drawing upon existing literature, the primary role of DMPS in safeguarding cells from death is hypothesized to lie in its ability to chelate both intracellular and extracellular Cr(VI). By reducing the amount of free intracellular Cr(VI), DMPS likely decreases peroxide production from Cr(VI) reduction, which in turn reduces apoptosis and cell death. Consequently, we further speculate that cellular phenomena associated with Cr(VI) exposure-such as decreased cell membrane potential, increased cleaved-caspase-3 expression and an altered Bax/Bcl-2 ratio-might be reversed by the addition of DMPS. Because ferroptosis markers (e.g., GPX4) are not detected, the effect of DMPS on ferroptosis is currently unknown.

Combination therapy represents an alternative strategy aimed at reducing the dosages of individual pharmacological agents [16]. While the combination of DMPS and DMSA did not exhibit synergistic effects in our cellular models, it is plausible that distinct therapeutic outcomes could emerge via *in vivo* animal studies, particularly when utilizing alternative administration routes. Moreover, combination therapies involving various chelators have been successfully employed to address other types of heavy metal toxicity. For instance, CaNa2EDTA or DMSA are commonly used for lead poisoning [44], while DMSA combined with monoisoamyl DMSA has also shown efficacy in similar contexts [45]. Additionally, integrating chelating agents with antioxidants has been demonstrated to enhance the efficacy of conventional chelation approaches. Examples include the co-administration of vitamin C with DMSA or MiADMSA for lead intoxication [46], as well as N-acetylcysteine (NAC) with DMSA for arsenic poisoning [47], both of which have shown improved protective outcomes in experimental settings. Previous studies from our group have reported optimized regimens of vitamin C and NAC in addressing hexavalent chromium-induced toxicity in HK-2 cells [29, 48]. The potential therapeutic efficacy of combining agents such as DMPS, vitamin C, and NAC warrants further evaluation in future investigations.

This study's conclusions come with certain limitations. Since it relied solely on an *in vitro* cell model, it falls short of accurately replicating the physiological conditions of Cr(VI) and drug metabolism observed *in vivo*. Additionally, the *in vitro* approach cannot evaluate aspects such as the route of administration, dosage, or the excretion of hexavalent chromium through urine. A recent study indicates that in healthy adults, a single oral dose of DMPS can result in a maximum plasma concentration of 4.3 μg/mL. Higher oral doses, such as 1500 mg, have been shown to achieve peak plasma concentrations of up to 14.7 μg/mL without causing significant adverse effects. In comparison, a single intravenous dose of 5 mg/kg produces a plasma concentration of 23.5 μg/mL. It has also been established that DMPS binds rapidly to plasma proteins and due to its short half-life, is quickly excreted in urine [49]. To bridge this gap, animal studies are necessary to better translate these findings into potential clinical applications.

At present, no comprehensive clinical guidelines exist for the use of DMPS and DMSA in treating acute hexavalent chromium poisoning. This study is the first to reveal that DMPS offers protective effects against hexavalent chromium-induced death of proximal renal tubular epithelial cells. It also demonstrates that higher doses of DMPS can effectively shield HK-2 cells and RPTECs from damage caused by elevated levels of hexavalent chromium while extending the maximum allowable delay in treatment. These results are anticipated to serve as a valuable reference for future *in vivo* experiments and clinical applications.

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Disclosure of conflict of interest

None.

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