Original Article
L-2-Oxothiazolidine-4-carboxylic acid mitigates the thromboembolic effects and systemic toxicity induced by sub-acute exposure to cadmium in mice

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Abstract: Exposure to cadmium (Cd) as a result of its environmental pervasiveness poses deleterious health effects, which has been attributed to oxidative stress. Therefore, this study aims to examine the sub-acute (1 mg Cd/kg/day, i.p., for 3 weeks) systemic and thrombotic influence of Cd exposure in vivo, and to assess the protective effects of the antioxidant L-2-Oxothiazolidine-4-Carboxylic acid (OTC, 80 mg/kg/day, i.p.). Cd compared with control group significantly reduced both the time for the first platelet aggregate and until blood flow stopped in pial microvessels. OTC significantly increased both the time for the first aggregate and until flow stopped. Cd induced a significant increase in total WBC, CK, AST, and LDH. The antioxidants SOD and catalase activities significantly increased in mice treated with Cd. OTC, through its antioxidant actions, appeared to effectively protect Cd induced thrombosis in mice. Hence, OTC represents a potential protective candidate from the detrimental effect of Cd toxicity.

Keywords: Cadmium, thrombosis, liver, kidney, OTC, toxicology

Introduction
With the expansion of the industrial revolution, humans can be inadvertently exposed to cadmium (Cd), which is a ubiquitous heavy metal widely used in many industries. As a result of its emissions to the air, water and soil, Cd is often present in individuals, particularly in urban areas. Cd is one of the key components abundantly present in nickel cadmium (Ni-Cd) battery wastes. Other sources of Cd exposure however, include cigarette smoke, ingestion of polluted vegetables, and ambient air in urban-industrialized areas [1-5]. Chronic exposure to Cd through the aforementioned contaminated sources have increased and become a universal concern. Once it has been absorbed by the human body, the biological half-life of cadmium is beyond 10 years [6]. It has been reported that Cd exposure in the population is associated with inducing toxicity in lungs, skeletal muscles by causing edematous emphysema, osteoporosis and osteomalacia, diabetes, cancer, blood pressure, reproduction, brain edema and hemorrhage and blood-brain barrier disruption [7-9].

As a consequence of environmental and industrial pollution, on inhalation or ingestion, Cd has been found to pose a potential threat and affects many systems in human and animals. Carcinogenic damage was observed in the lungs in Cd exposed workers [5].

Cd may also contribute towards endothelial damage either directly by interacting with the endothelium or by inducing the generation of reactive oxygen species in endothelial and inflammatory cells [5]. Cd can inhibit protein synthesis completely to facilitate apoptotic cell death [10-12]. Moreover, Cd induces cell death in vivo and in vitro at varied concentrations from 1 to 300 μM [13]. Cd has been described as a pro-necrotic metal ion [14, 15].
Cd is known to increase oxidative stress by being a catalyst in the formation of reactive oxygen species, increasing lipid peroxidation, and depleting glutathione and protein-bound sulfhydryl groups [16]. Cd has a high affinity for sulfhydryl (-SH) groups, inactivating numerous enzymatic reactions, amino acids, and sulfur-containing antioxidants including N-acetylcysteine, alpha-lipoic acid and glutathione with subsequent decreased oxidant defense and increased oxidative stress [17]. Long-term exposure to Cd increases lipid peroxidation and causes inhibition of SOD activity indicating oxidative damage in the liver, kidney and testes [9, 17]. Studies on Cd induced intoxication which caused severe platelet aggregation (PA) and thrombosis in testes and epididymis [18] have been demonstrated. The tendency to increase the leukocyte adhesion in male subjects has also been reported [19]. We have recently demonstrated that acute (1 h) exposure to Cd cause systemic and thromboembolic events in mice [20]. However, the sub-acute effects of Cd on cerebral microvessels thrombosis and systemic toxicity, and the effect of cysteine pro-drug L-2-Oxothiazolidine-4-Carboxylic acid (OTC) th eron has not been reported so far.

Consequently, the aim of the present study is to investigate the effect of Cd on cerebral microvessels thrombosis, systemic inflammation and oxidative stress, and its effect on liver. Also, to evaluate the ability of antioxidants OTC to ameliorate the detrimental effect of Cd toxicity.

Methods

Animals and treatment

This project was reviewed and approved by the Institutional Review Board of the United Arab Emirates University, College of Medicine and Health Sciences, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee. Adult male mice, of TO strain, were selected. The mice weighed 30-35 g. Each group consisted of six to ten animals from a colony maintained at our animal house facility. Animals were group-housed in a temperature maintained (22±2°C) and a light/dark cycle (12/12 h) environment. They had access to standard pelleted diet and water ad libidum.

Except for their intended treatment all animals were handled in the same manner. All animal treatment procedures met both the NIH and Institutional Animal Ethics Committee guidelines.

Cd, in the form of cadmium chloride hemipenta hydrate (Sigma Chemicals) was dissolved in normal saline. Daily dose of 1 mg/kg body weight injection was administered intraperitoneally (ip), for 3 weeks. OTC pretreatment (80 mg/kg/day, i.p.) or saline was started one hour before Cd administration. Control mice received normal saline only.

Experimental cerebral thrombosis model

In vivo cerebral microvessel thrombogenesis was assessed as previously described [20, 21]. Mice were anaesthetized with urethane (25%, 0.1 ml/10 g b.w., ip) and the trachea was intubated. Cerebral microvessels were exposed after performing craniotomy on the anaesthetized mice on the left side of the head, with the aid of a microdrill and the dura was carefully removed. Average body temperature fell to 33.2±0.6°C after anesthesia and surgery. The body temperature was stabilized at 37°C with the help of an infra-red lamp and monitored with a rectal thermoprobe (Physitemp Model RET-3). The preparation was kept moist with artificial cerebrospinal fluid (ACSF). Composition of the ACSF used was (mM): NaCl 124, KCl 5, NaH₂PO₄ 3, CaCl₂ 2.5, MgSO₄ 2.4, NaHCO₃ 23, and glucose 10; pH 7.3. Only untraumatized preparations were used. Those mice showing trauma to either microvessels or underlying brain tissue (10%) were discarded. The closed circuit set up used consisted of: an fluorescent microscope (Olympus, BH-2), a color video camera (JVC, TK-890E), a VHS-VCR (JVC, BR-S600E) and a television monitor (JVC, TM-1500PS). All microscopic observations utilized a fiber-optic light source and were made using a 4× objective lens and a 10× eye piece. Total magnification of the observed field, from the microscope stage to the television monitor was ×250. Sodium fluorescein (2%, 0.1 ml/10 g, iv.) was injected via the tail vein. Sodium fluorescein was allowed to circulate in the body for 30 seconds before high intensity mercury light was switched on. The combination of light and fluorescent dye produced a free radical which injured the endothelium of the microvessel’s
OTC mitigates cadmium toxicity

Figure 1. Effect of control, cadmium (Cd), L-2-Oxothiazolidine-4-Carboxylic acid (OTC) and concurrent Cd with OTC exposure on cerebro vascular thrombosis in which the start of the aggregation in the venule and arteriole (A) and the complete flow stop after adequate aggregation in venule and arteriole (B) in saline treated control, Cd, OTC and concurrent Cd with OTC treated animals. Data in bar graph shown are mean ± SEM, number of samples (n=10); ***P<0.001, **P<0.01, *P<0.05.

Blood collection and complete blood count analysis

Another group of animals was subjected to Cd, OTC, Cd + OTC or saline treatment at the dose mentioned earlier. One hour after the treatment, the animals were anesthetized i.p. with sodium pentobarbital (45 mg/kg), and then blood was drawn from the inferior vena cava in EDTA (4%). Part of the blood was used for a white blood cell (WBC) counts using an ABX VET ABC Hematology Analyzer with a mouse card (ABX Diagnostics, Montpellier, France). The remaining blood was centrifuged for 15 min at 4°C at 900× g, and the plasma obtained was stored at -80°C pending analysis.

Liver function and lactate dehydrogenase (LDH) and creatine kinase (CK) estimation

Liver function tests included alanine aminotransferase (ALT) and aspartate aminotransferase (AST) estimation in plasma. The ALT and AST were measured using standard laboratory methods using Lx20 multiple automated analyzer (Beckman Coulter, CA, USA) with their estimation kit. The LDH was measured using commercial kits (Sigma Chemical, St. Louis, MO, USA) which determined the conversion of lactate to pyruvate in the presence of LDH with equivalent lessening of NAD. The formation of NADH from the above reaction can show a dif-
OTC mitigates cadmium toxicity

The effects of Cd treatment on the time required for platelet aggregation (PA) and for full occlusion in cerebral microvessels are shown in Figure 1. In our results on the animals treated with Cd, the onset time for PA in both venules and arteriole showed very significant quickening (P<0.001) when compared to control group. The onset time of aggregation in arteriole was found to be 60% whereas in venule a 20% quicker response was observed when compared to control group (Figure 1). This indicates an increased susceptibility to thrombosis in cadmium exposed animals.

Subsequently, the total occlusion in the arterioles but not the venules showed a significant shortening in time (P<0.001) when compared to the control group (Figure 1). OTC treatments significantly increased the time for the first aggregate and the time for full occlusion either by itself or in conjunction with Cd treatment (Figure 1).

Haematological and biochemical findings

Acute exposure of mice with Cd significantly increased WBC level (Figure 2). Treatment of OTC in combination with Cd alleviated the harmful effects of Cd on WBC which reached the control value. There was a significant increase in LDH activity following Cd exposure. The pre-treatment with OTC reversed the effects of Cd (Figure 2). Figure 3 depicts the significant increase in serum AST, and the insignificant increase in ALT. Treatment of OTC alone or in combination with Cd alleviated the harmful effects of Cd (Figure 3).

There were significant increases in the values and CK (Figure 3) in mice treated with Cd. These values returned to nearly normal values with OTC treatment alone or in combination with Cd.

CAT and SOD estimation

Our estimation of SOD and CAT in the plasma in Cd treated animals showed significant increase at the dose tested (Figure 4). Treatment of OTC in combination with Cd alleviated the effects of Cd on SOD, and CAT levels that were returned to normal levels (Figure 4).

Discussion

In our present study, we have showed that acute intraperitoneal daily injection of Cd for 3 weeks, compared to control group, in mice showed significant quickening of platelet agg-

Figure 2. Effect of control, Cd, OTC and concurrent Cd with OTC exposure on white blood cell counts (A), LDH (B) and in saline treated control Cd, OTC and concurrent Cd with OTC treated animals. (n=6); ***P<0.001, **P<0.01.
OTC mitigates cadmium toxicity

The 1 mg/kg dose we have used in the present experiments were well below the LD50 and the minimum toxic dose for Cd [23]. The intraperitoneal route of Cd exposure was selected for its effectiveness [24]. The toxic level of Cd affects haemopoietic system and liver [25]. There is still much to discover about the mechanisms of action, the biomarkers of critical effects and strategies to protect the health risks induced by Cd. In our thromboembolic studies, the Cd treatment reduced the time required for both the appearance of the first aggregate and the time for full occlusion in pial arterioles. In-vitro study on Cd stimulates adenylate cyclase and inhibits phosphodiesterase (PDE) activity with a decrease in the rate of PA [26]. Circulating platelets do not normally adhere to the endothelial cells of the vascular wall or aggregate, except when activated or when damage to the endothelium is attained. When such damage occurs, the aggregates are induced which will lead to formation of thrombosis. The development of thrombi in the circulatory system would result in reduced blood delivery and consequently, in tissue hypoxia/ischemia [27]. Francavilla et al. [18] reported the quickening of the thrombosis induced by Cd in testes and epididemis in rats. In concurrence with this previous study, our revealed a quickening of PA activity in pial cerebral vasculature during in-vivo treatment of Cd in mice. The same authors also demonstrated the variability of in-vitro and in-vivo treated Cd on PA activity to induce thrombosis. Likewise, our results also support the previous report by Järup et al. [5] who found a correlation between the endothelial damage that was either directly induced by Cd on endothelium or was indirectly induced by releasing the reactive oxygen species in endothelial and inflammatory cells. Wolf and Baynes [28] reported that 3-5 uM of Cd exposure caused endothelial dysfunction and increased LDH leakage leading to profound cytotoxicity. They also reported that lower level of Cd caused glutathione (an important cytoplasmic antioxidant), [29, 30] induction, a compensatory protective response, which may explain the increase in the level of SOD and CAT antioxidants enzymes that were observed in the current study. Furthermore, Fagerberg et al. [31] have reported that Cd exposure was associated with prevalence and growth of atherosclerotic plaques in the carotid arteries in old women. These direct or indirect effects are mediating the quickening of thrombosis, as found in our studies. This was well supported
OTC mitigates cadmium toxicity

by our other estimations of SOD, WBC, and LDH and discussed in this paper which shows significant change in all their levels. Clearly these parameters are the indicators of oxidative stress and/or systemic inflammatory responses by tissues. As Cd primarily accumulates in the liver, it becomes more important target organ for damage [32]. Reliability of the hepatocellular function can be attained by quantifying the enzymes ALT and AST. Changes in the levels of these enzymes reveal the variations in the cell membrane functions including the alteration in the permeability which leads to cell damage [13]. Among these two enzymes, measurement of AST is considered as a good marker to indicate the liver damage particularly in animal species [33]. In our experiments we have demonstrated the acute administration of Cd significantly elevate the levels of AST indicating the severe damage to liver. Recently, rats were treated with 10 mg/kg subcutaneous for 20 days, liver depicted multifocal leukocytic infiltrate, and cellular vacuolization in the centerolobular zone [34]. Emerging evidence indicates that oxidative stress is related to regulation of multiple signaling pathways including transcription factors [35]. An imbalance between reactive oxygen species (ROS) and the antioxidant defense system leads to oxidative stress, which is closely linked to the pathogenesis. Increased levels of ROS cause direct tissue injury and promote inflammatory responses. The response of a cell to excessive ROS involves activation of multiple signaling pathways, which can cause transcriptional changes and consequently exhibit a variety of activities [35]. Antioxidant enzymes and proteins are crucial for maintaining the reducing environment of the cell and preventing the oxidative damage. Because glutathione (GSH) synthesized from cysteine is a vital protective antioxidant against oxidative stress. A thiazolidine derivative, L-2-oxothiazolidine-4-carboxylic acid (OTC) is a pro drug of cysteine that raises the concentrations of cysteine and GSH [36]. OTC raises the plasma concentrations of cysteine, which is the rate-limiting amino acid for intracellular GSH synthesis [37]. GSH is synthesized from cysteine and is a vital intra- and extracellular protective antioxidant against oxidative stress [37] subsequently potentiate the natural antioxidative cellular defense mechanisms. OTC is reported to be more effective than N-acetylcysteine in replenishing intracellular glutathione stores when GSH is depleted [38]. Recently, it has been demonstrated that OTC which can reduce the increased ROS generation is able to reduce inflammation and hyper-reactivity in animal models of hepatotoxicity [38]. Moreover, it has been shown that OTC prevents the pulmonary inflammation and thrombotic complication induced by diesel exhaust particle in mice [21] and cisplatin induced nephrotoxicity in rats [39]. Our present results have revealed that sub-acute Cd treatment has led to ROS generation which subsequently triggered an increase in CAT and SOD activities as a compensatory protection. The increased levels of these molecules are significantly reduced by administration of OTC. The administration of OTC resulted in significant increase in the time of arterial 1st aggregate and the time of arterial flow stop demonstrating a protective effect against thrombosis. OTC treatment significantly reduced the level of WBC and LDH compared with the Cd treated group. Additionally, OTC reduced the increase of AST, ALT and CK enzymes activity

Figure 4. Effect of control, Cd, OTC and concurrent Cd with OTC treatment on SOD and CAT levels in the control, Cd, OTC and concurrent Cd with OTC treated animals. Data in bar graph shown are mean ± SEM, number of samples (n=6). **P<0.01, *P<0.05.
after Cd exposure. These results indicate that the antioxidant OTC is able to mitigate the systemic toxicity induced by Cd. Moreover, OTC exposure has significantly reduced the effect of Cd on CAT and SOD activities which lend support to its capacity as a potent antioxidant.

In conclusion, Cd induced acceleration in thromboembolic formation leading to quick occlusion of the vascular tissues are in concurrence with the changes in the levels of liver enzymes, increase in the leucocyte levels. The results summarized above show the high implication of Cd against the PA and liver dysfunction and the protective effects of OTC thereon. Further studies are essential to determine the mechanisms behind these observations.

Disclosure of conflict of interest

None.

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OTC mitigates cadmium toxicity