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Vitamin C exerts novel protective effects against cadmium toxicity in mouse spermatozoa by inducing the dephosphorylation of dihydrolipoamide dehydrogenase

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ABSTRACT

Cadmium (Cd) has been reported to inhibit mouse sperm motility by inducing the tyrosine phosphorylation of dihydrolipoamide dehydrogenase (DLD). This study aimed to assess the potential effects of vitamin C (Vc) on ameliorating Cd-induced tyrosine phosphorylation of DLD and the specific underlying mechanism. Vc induced the dephosphorylation of DLD or inhibited the tyrosine phosphorylation of DLD. Accordingly, DLD activity, nicotinamide adenine dinucleotide hydrogen (NADH) levels, ATP levels and motility parameters were all restored to normal levels by Vc. Moreover, the effects of Vc on ameliorating these indicators had striking similarities to the effects of ethylenediaminetetraacetic acid (EDTA). In addition, neither the antioxidant melatonin nor the universal oxidant H_2O_2 influenced the tyrosine phosphorylation of DLD. Hence, the protective effects of Vc on the tyrosine phosphorylation of DLD might be attributed to its binding to Cd ions outside or inside sperm, and were not due to its antioxidant properties.
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1. Introduction

Humans and other mammalian animals are generally exposed to Cd through multiple sources, including contaminated water and food, the occupational environment, smoke and dust. Once ingested into the body, Cd causes oxidative damage in various tissues, which in turn results in cell death and necrosis within the tissue [1–3] and even leads to male infertility [4]. Oxidative stress is known to cause oxidative damage by generating excessive reactive oxygen species (ROS) and hence has been regarded as a key contributing factor in approximately half of infertile men [5]. Moreover, ROS have been reported to be detrimental to sperm by inhibiting motility [6] and decreasing the acrosome reaction rate [7]. Meanwhile, during sperm capacitation, ROS were reported to be the main regulators of protein tyrosine phosphorylation [8–10]. As shown in our recent study, both in vivo and in vitro exposure of mouse sperm to Cd induces tyrosine phosphorylation and subsequent inactivation of DLD, thus disrupting intracellular energy metabolism in epididymal spermatozoa [11]. However, the relationship between Cd-induced excessive ROS production and the tyrosine phosphorylation of DLD has not been clarified; in particular, studies have not reported whether the tyrosine phosphorylation-induced inactivation of DLD can be prevented or restored.

Cd easily disrupts the antioxidant defense system by reducing catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), and glutathione reductase (GSR) activities and increasing thiobarbituric acid reactive substances (TBARS) levels [12,13]. However, this damage is prevented by enzymatic and non-enzymatic products known as antioxidants. Currently, the mechanisms of the antioxidants' actions are primarily to prevent ROS production, block radicals that are formed, and remove the damaged biomolecules through repair processes or by removing pro-oxidative transition metal ions [14,15]. For example, Vc is a popular potent antioxidant that eliminates ROS through very rapid electron transfer and thus prevents lipid peroxidation and removes cytotoxic free radicals [16,17]. Although the adverse effects of Cd and the beneficial effects of the antioxidant Vc on the mammalian reproductive systems have been extensively studied in previous studies, no studies have reported with the effects of Vc treatment on Cd toxicity by regulating the tyrosine phosphorylation of DLD and the subsequent disruption of intracellular energy metabolism.

Posttranslational modification of proteins by phosphorylation acts as a ubiquitous regulation mechanism in cells [18,19] and is an important covalent modification method for some metabolic

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enzymes. For example, the activities of pyruvate dehydrogenase complex (PDHC) and the branched-chain α-ketoacid dehydrogenase complex (BCKDC) are regulated by posttranslational phosphorylation/dephosphorylation in response to hormonal stimuli and a variety of dietary conditions [19–21]. However, researchers have not determined whether the inactivation of DLD by tyrosine phosphorylation is reversible and whether the dephosphorylation of DLD regulates its activity.

Regarding the effect of the antioxidant treatment on reducing Cd toxicity, researchers have speculated that the protection provided by antioxidants may also partially be ascribed to their metal chelating activity [22]. Furthermore, Vc has been used as a chelating agent for iron and lead ions [23–26]. A previous animal study compared the effectiveness of parenterally administered EDTA with orally administered Vc in treating the toxic effects of the heavy metal lead and found that Vc had chelating properties similar to EDTA [27]. Therefore, the present study was performed to investigate the effects of Vc on preventing Cd-induced tyrosine phosphorylation of DLD and inhibition of energy metabolism. Moreover, tyrosine phosphorylation of DLD was considered an important biomarker to indicate the initiation of Cd toxicity, and EDTA was used as a reference standard for the chelators. Therefore, we aimed to detect the potential novel mechanisms by which the Vc treatment prevented Cd toxicity in mouse epididymal sperm.

2. Materials and methods

2.1. Reagents and antibodies

Cadmium chloride (Cd^{2+}, $CdCl_2$) was obtained from National Medicines (China). L-Ascorbic acid (Vc, Vitamin C) was purchased from Sangon Biotech (Shanghai, China). Bovine serum albumin (BSA) and PVDF membranes were obtained from Millipore (Billerica, MA). Molecular weight markers, acrylamide (40%) and β-mercaptoethanol were purchased from Bio-Rad (USA). The monoclonal anti-phosphotyrosine (anti-PY) antibody was obtained from BD Biosciences (San Jose, CA). The anti-β-tubulin antibody was purchased from Abmart (Shanghai, China). The HRP-conjugated anti-mouse IgG secondary antibody was purchased from Cell Signaling Technology (Danvers, MA). The chemiluminescence detection kit (ECL) was obtained from GE Healthcare (Waukesha, WI). Other chemical products were acquired from Sigma-Aldrich (St. Louis, MO).

2.2. Media

The basal medium was modified Krebs-Ringer medium (Whitten's HEPES-buffered medium) containing the following compounds: NaCl (100 mM), KCl (4.4 mM), KH_2PO_4 (1.2 mM), $MgSO_4$ (1.2 mM), glucose (5.4 mM), calcium lactate (2.4 mM), HEPES (20 mM) and sodium pyruvate (Pv-Na) (0.8 mM) [28]. In all cases, the pH was adjusted to 7.3 and the medium was incubated in a 37 °C bath water prior to use.

2.3. Mouse sperm preparation and culture

Caudal epididymal spermatozoa were collected from sexually mature male *Kunming* mice that had been sacrificed by CO_2 inhalation in accordance with institutional guidelines for ethics in animal experimentation (Rule number 86/609/EEC-24/11/86). Each epididymis was added in 1 mL of basal medium. During a 10 min incubation in 37 °C bath water, sperm were released into the medium. Then, epididymis fractions were cast and the suspension containing motile sperm was adjusted to a final density of $1–2 \times 10^7$ cells/ml with freshly prepared warm basal medium before dilution in the experimentally designed medium.

We designed eight experimental groups to comprehensively study the protective effects of Vc on Cd toxicity in mouse sperm and to investigate the potential mechanism. Group I: Mouse spermatozoa were treated with 10 μM Cd for 1 h prior to treatment with 20 mM Vc or 20 μM EDTA for different time periods (10, 30, 60, and 90 min). Group II: Spermatozoa were treated with 10 μM Cd for 1 h prior to treatment with 10 μM melatonin (MLT) for different time periods (10, 30, 60, 90 min). Group III: Sperm were simultaneously cultured with 10 μM Cd and different concentrations of melatonin (0, 0.1, 0.5, 1, 2, 4, 6, 8, or 10 μM) for 2 h. Group IV: Sperm were incubated with different concentrations of H_2O_2 (0, 5, 15, 50, 100 μM) in the absence or presence of 10 μM Cd for 2 h in vitro. Group V: Sperm were pretreated with 20 mM Vc or 20 μM EDTA for 1 h, and then treated with 10 μM Cd for an additional 1 h. Group VI: Sperm were diluted in basal medium containing 20 mM Vc or 15 μM EDTA in the absence or presence of 10 μM Cd. Group VII: Sperm were co-administered10 μM Cd and different concentrations of Vc (0.5, 2, 4, 6, 10, 15, 20 or 30 mM) for 2 h. Group VIII: Sperm were simultaneously cultured with different concentrations of EDTA (0, 2, 4, 6, 8, 10, 15, 20 or 30 μM) and 10 μM Cd for 2 h. Sperm were incubated in a 37 °C water bath and the tubes were mixed every 15 min to prevent precipitation during the incubation. Changes in the tyrosine phosphorylation of DLD were evaluated in all groups. Meanwhile, in group I, the changes in DLD activity, NADH and ATP levels, and sperm motility parameters were detected concurrently. EDTA was used as a standard chelator to compare the effects of Vc with the EDTA-treated group.

2.4. Protein extraction and western blotting

Immediately after the incubation, sperm were collected by centrifugation (12500*g*, 6 min), washed with 800 μL of ice-cold phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl, and DDH_2O to a total of 1 L) and centrifuged at 12500 *g* for 6 min. This process was repeated three more times. Sperm were then resuspended in 13 μL of 5 × sample buffer (1.67 mL of DDH_2O, 5.83 mL of 0.5 mM Tris-base, pH 6.8, 2.5 mL of glycerol, 833 mg of SDS, 1 mg of bromphenol blue, for a total 10 mL), boiled for 4 min at 100 °C and centrifuged at 13500*g* for 10 min. Supernatants were treated with 10% β-mercaptoethanol and then boiled again for another 3 min. Protein samples were either used instantly or stored at −80 °Cuntil use.

Protein samples were electrophoresed on10% SDS-PAGE minigels and eletrophoretically transferred to PVDF membranes at 90 V for 2 h on ice. PVDF membranes were blocked with 1% BSA in T-TBS (30 mM Trisbase, 0.8% (w/v) NaCl, and 0.1% Tween 20, pH 7.5) for 1–2 h at room temperature. Western blotting was conducted as previously described [29,30] with an anti-PY antibody diluted to a1:10,000 final concentration and the corresponding secondary antibody diluted to a final concentration of 1:10,000. Bands on the membranes were detected with an enhanced chemiluminescence ECL plus kit (GE Healthcare) and a ChemiScope 3300mini integrated chemiluminescence imaging system (Clinx, China). When necessary, PVDF membranes were stripped with stripping buffer for 1 h and subsequently re-immunoblotted with an anti-β-tubulin antibody.

2.5. Detection of DLD dehydrogenase activity and NADH levels

Mitochondria were isolated from mouse epididymal sperm by Percoll gradient centrifugation using a previously reported method, with slight modifications [31,32]. Protein concentrations were measured using the Bradford assay [33]. The dehydrogenase activity of DLD was determined by measuring the DLD-catalyzed oxidation of dihydrolipoamide at the expense of NAD^+, as previously described [31]. The final volume of reaction mixture was

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