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Cerium oxide nanoparticles attenuate monocrotaline induced right ventricular hypertrophy following pulmonary arterial hypertension

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ABSTRACT

Cerium oxide (CeO_2) nanoparticles have been posited to exhibit potent anti-oxidant activity which may allow for the use of these materials in biomedical applications. Herein, we investigate whether CeO_2 nanoparticle administration can diminish right ventricular (RV) hypertrophy following four weeks of monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH). Male Sprague Dawley rats were randomly divided into three groups: control, MCT only (60 mg/kg), or MCT + CeO_2 nanoparticle treatment (60 mg/kg; 0.1 mg/kg). Compared to the control group, the RV weight to body weight ratio was 45% and 22% higher in the MCT and MCT + CeO_2 groups, respectively ($p < 0.05$). Doppler echocardiography demonstrated that CeO_2 nanoparticle treatment attenuated monocrotaline-induced changes in pulmonary flow and RV wall thickness. Paralleling these changes in cardiac function, CeO_2 nanoparticle treatment also diminished MCT-induced increases in right ventricular (RV) cardiomyocyte cross sectional area, β-myosin heavy chain, fibronectin expression, protein nitrosylation, protein carbonylation and cardiac superoxide levels. These changes with treatment were accompanied by a decrease in the ratio of Bax/Bcl2, diminished caspase-3 activation and reduction in serum inflammatory markers. Taken together, these data suggest that CeO_2 nanoparticle administration may attenuate the hypertrophic response of the heart following PAH.

1. Introduction

Pulmonary arterial hypertension (PAH) is associated with the development of right ventricular (RV) hypertrophy which if allowed to proceed unchecked can lead to heart failure and death [1]. PAH is defined as an increase in mean pulmonary arterial pressure (PAPm) > 25 mm Hg at rest, or >30 mm Hg during exercise [2]. Although the pathogenesis of PAH is not fully understood, it is thought that disease progression is due, at least in part, to increases in nitric oxide, endothelin and prostanoids such as that seen frequently in patients with portal hypertension, congenital heart disease, and AIDS [3]. The treatment of PAH is largely symptomatic in nature and is centered around the administration of calcium channel blockers, diuretics, prostanoids, endothelin antagonists and lifestyle modifications. If improperly managed, the median life expectancy of patients with idiopathic PAH is approximately 2.8 years, with survival rates of 68%, 48%, and 34%, at 1-year, 3-year and 5-years, respectively [4]. Even with modern medical therapy, patients with PAH oftentimes experience disease progression and eventual referral for organ transplantation [5]. Despite advances in

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medical care the life expectancy of patients with PAH remains unacceptably low.

Cerium is the most abundant rare earth element that exhibits the ability to cycle between the Ce^{+3} (fully reduced) or Ce^{+4} (fully oxidized) state [6]. Recent data has suggested that CeO_2 nanoparticles can act as superoxide dismutase (SOD) and catalase mimetic [7,8], free radical scavenger [6,9] and that they can protect H_9C_2 cardiomyocytes from cigarette smoke extract-induced oxidative damage [10]. *In vivo* studies using the monocyte chemo-attractant protein-1 (MCP-1) transgenic mouse model of oxidative stress-induced cardiac hypertrophy have shown that CeO_2 nanoparticle administration can reduce the development of cardiac dysfunction and remodeling [11]. Similarly, other work has shown that CeO_2 nanoparticles attenuate carbon tetrachloride-induced oxidative stress [12], and that they can accelerate the decay of peroxynitrite [13]. Consistent with these findings, other data has suggested that the anti-oxidant activity of CeO_2 nanoparticles can protect liver against monocrotaline-induced hepatic toxicity [14]. Whether CeO_2 nanoparticle administration can attenuate the development of the RV hypertrophy seen during PAH is currently unclear.

Monocrotaline is a toxic pyrrolizidine alkaloid that is metabolized in the liver to monocrotaline pyrrole (MCTP) which can selectively injure lung endothelial cells causing the infiltration of monocytes, and thickening of the pulmonary arterioles that precede the development of PAH [15,16]. The molecular mechanisms responsible for these MCT-induced changes are presently unclear; however recent reports have suggested that increases in oxidative stress and apoptosis, like that observed in the clinical development of PAH, are likely to play a role in the pathological remodeling of the heart [17]. The purpose of this study was to investigate whether the administration of CeO_2 nanoparticles can prevent the progression of RV hypertrophy following monocrotaline-induced PAH. We hypothesized that CeO_2 nanoparticles would attenuate MCT-induced increases in oxidative stress and systemic cytokine levels and that these reductions would be associated with diminished cardiac hypertrophy. Our data suggest that CeO_2 nanoparticle treatment may be effective in reducing the hypertrophic response seen following PAH in male Sprague Dawley rats.

2. Materials and methods

2.1. Animal model and experimental design

Animal care and surgical procedures were conducted in accordance with the guidelines provided by Marshall University Institutional Animal Care and Use Committee (IACUC), and Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Seven week old (175–200 gm) male Sprague Dawley rats were purchased from Hilltop laboratories (Scottdale, PA) and housed two per cage under a 12:12H dark–light cycle and maintained at 22 ± 2 °C. Rats were provided food and water *ad libitum* and allowed to acclimatize for at least 2 weeks before initiating the study. Periodic body weight and feed intake measurements were taken throughout the duration of the study. Rats were randomly assigned to one of three different groups: Control ($n = 6$), MCT only ($n = 6$), or MCT + CeO_2 nanoparticle treatment ($n = 6$). PAH was induced by a single injection of MCT (60 mg/kg S.C.) (Sigma–Aldrich, St. Louis, MO). Animals injected with MCT were given either CeO_2 nanoparticles (0.1 mg/kg *via* tail vein) (Nanoactive™ CeO_2 nanoparticles, NanoScale materials Inc., Manhattan, KS) or vehicle (deionized water) at the time of MCT administration and twice a week for 1st and 2nd week of the study. Animals were sacrificed after 28 days and the hearts and lungs were collected for further analysis.

2.2. Characterization of CeO_2 nanoparticles

The hydrodynamic particle size distribution was estimated using an LB-550 dynamic light scattering (DLS) particle size analyzer (Horiba scientific, Edison, NJ). Briefly, CeO_2 nanoparticles were sonicated in deionized water for 2 min to ensure equal dispersion. Particle size measurement was performed as outlined by the manufacturer in triplicate. Transmission electron microscopy was performed using JEOL JEM-2010 transmission electron microscope to determine the size of naked nanoparticles. Chemical characterization of the prepared CeO_2 nanoparticles was performed using a JEOL JSM-6320F Field Emission Scanning Electron Microscope (FESEM). Prior to analysis, nanoparticles were placed on a double-sided conductive carbon tape and attached to an aluminum stub. Noran Voyager EDX software was used to determine atomic and weight percentage of present elements according to their energy lines. X-ray photoelectron spectroscopy was performed to determine the relative amounts of Ce^{+3} and Ce^{+4} in the nanoparticles using a Kratos 165 XPS. A Scintag XDS 2000 powder diffractometer was used for the X-ray diffraction studies.

2.3. Echocardiography

Echocardiography was performed prior to the start of the study and at day 28 of the study as outlined previously [18]. Rats were anesthetized with an intraperitoneal injection of ketamine and xylazine cocktail mixture (50 mg/kg; 4:1) and the ventral thorax area shaved. ECG analysis was performed during the echocardiography procedure to monitor heart rate. Echocardiography measurements were obtained with a Philips Sonos 5500 echocardiogram system using 12-MHZ transducer. M-mode and 2-D modalities were used to measure RV and left ventricular (LV) wall thickness at the end of diastole using the right parasternal long axis view with the ultrasonic beam positioned perpendicular to the ventricular wall. Papillary muscles were used as reference point for measurements and to assure proper position in subsequent studies. Pulmonary artery flow was measured using pulsed wave Doppler at the pulmonary valve level while pulmonary artery diameter was measured at the level of pulmonary out flow tract during systole using the parasternal short axis view. An apical four-chamber view was employed to measure end-diastolic right ventricular area. Pulsed-wave Doppler was used to measure pulmonary artery acceleration time and flow.

2.4. Tissue processing and histological analysis

Animals were anesthetized with ketamine-xylazine (4:1) cocktail mixture (50 mg/kg, i.p) and supplemented as necessary to achieve loss of reflexive response. After a mid-ventral laparotomy, the heart was removed and placed in Krebs–Ringer bicarbonate buffer (KRB) containing 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.2 mM $NaHCO_3$, and 10 mM D-glucose (pH 7.4) equilibrated with 5% CO_2/95% O_2 and maintained at 37 °C. The right ventricle was separated from the left ventricle and septum, weighed and immediately flash frozen in liquid nitrogen before being stored at −80 °C for subsequent use.

RV tissues were sectioned (8 μm) using a Leica CM1950 cryostat and collected on poly-L-lysine coated slides. Sections were stained for dystrophin immunoreactivity and then evaluated for cardiomyocyte cross sectional area (CSA) as outlined elsewhere [19]. Briefly, sections were incubated for 30 min in a blocking solution (5% bovine serum albumin [BSA] in phosphate-buffered saline [PBS] containing 0.05% Tween 20 (PBS-T), pH 7.5) and then incubated with specific anti sera diluted in PBS-T (anti-dystrophin 1:100, rat-igG 1:100) for 1 h at 37°C. After washing with PBS-T, sections were incubated with FITC labeled secondary antibody (1:200) for 1 h at 37 °C. Muscle cross sections were visualized by epi-fluorescence using an Olympus BX51 microscope (Olympus, Center Valley, PA), fitted with 20× and 40× objectives.

Picrosirius red staining was used to assess the collagen content deposition in RV sections as detailed elsewhere [20]. Briefly, RV frozen tissue sections were fixed with 95% reagent alcohol for 1 min, washed three times with running tap water, and then stained with hematoxylin and picrosirius red. After dehydration and mounting, collagen content was visualized at 20× using an Olympus BX51 microscope (Olympus, Center Valley, PA).

The fluorescent superoxide marker dihydroethidium was used to evaluate superoxide levels as outlined elsewhere [21]. Briefly, RV frozen tissue sections were washed with phosphate-buffered saline (PBS) for 5 min and then incubated with 200 μl of 10 μM of dihydroethidium (Molecular Probes, Eugene, OR) for 1 h at room temperature. After washing with PBS (3 × 5 min), fluorescence was visualized under a Texas red filter using an Olympus BX-51 microscope (Olympus America, Melville, NY, USA) equipped with an Olympus WH 20× wide field objective. Images were recorded digitally using a CCD camera and the integrated optical density determined using the Alpha view image analysis software (Cell Biosciences, Inc., Santa Clara, CA).

2.5. SDS PAGE and immunoblotting

Frozen right ventricles were homogenized in T-PER buffer (2 mL/g tissue; Pierce, Rockford, IL, USA) containing protease and phosphatase inhibitors (P8340 and P5726, Sigma–Aldrich, St. Louis, MO, USA). Samples were incubated on ice for 30 min and the supernatant was collected by centrifugation at 12,000 × *g* for 10 min at 4 °C. The concentration of protein in the supernatant was determined *via* the 660 nm assay (Piercenet, Rockford, IL). Samples were mixed with Laemmli sample buffer (Sigma–Aldrich, St. Louis, MO, USA) and heated for 5 min at 95°C. Forty micrograms of total protein were separated on 10% PAGEr Gold Precast gels (Lonza, Rockland, ME) and then transferred to nitrocellulose membranes using standard methods as detailed elsewhere [21,22]. Membranes were blocked with 5% milk in Tris Buffered Saline (TBS) containing 0.05% Tween-20 (TBST) for 1 h and then probed with primary antibodies for the detection of β-myosin heavy chain (Sigma Aldrich, St. Louis, MO), fibronectin (Abcam, Cambridge, MA), p-ERK1/2, ERK1/2, p-JNK, JNK, Bax, Bcl2, caspase-3, cleaved caspase-3, GAPDH (Cell Signaling Technology, Inc., Beverly, MA) and 3-nitrotyrosine (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After an overnight incubation at 4°C in primary antibody buffer (5% BSA in TBST, primary antibody diluted 1:1000) membranes were washed with TBST, and then