ABSTRACT

Although Cadmium (Cd) is a well-known heavy metal pollutant and teratogen, the mechanism behind Cd-mediated teratogenicity remains unknown. Previously, we have reported of the protective role of Nitric oxide (NO), a key signaling molecule in the embryonic developmental process, against Thalidomide-induced teratogenicity. The objective of this study was to obtain a mechanistic insight of the antiteratogenic potential of NO against Cd-mediated teratogenicity. To achieve this goal, we first studied the effect of Cd on the vasculature of developing embryos and then we investigated whether Cd mediated its effects by interfering with the redox regulation of NO signaling in the early development milieu. We used a chick embryonic model to determine the time and dose-dependent effects of Cd and NO recovery against Cd assault. The effects of Cd and NO recovery were assessed using various angiogenic assays. Redox and NO levels were also measured. Results demonstrated that exposure to Cd at early stage of development caused multiple birth defects in the chick embryos. Exposure to Cd suppressed endogenous NO levels and cGMP signaling, inhibiting angioblast activation and subsequently impairing yolk sac vascular development. Furthermore, Cd-induced superoxide and lipid peroxidation mediated activation of proapoptotic markers p21 and p53 in the developing embryo. Cd also caused the down-regulation of FOXO1, and up-regulation of FOXO3a and Caspase 3-mediated apoptosis. Addition of exogenous NO through a NO donor was able to blunt Cd-mediated effects and restore normal vascular and embryonic development. In conclusion, Cd-mediated teratogenicity occurs as a result of impaired NO-cGMP signaling, increased oxidative stress, and the activation of apoptotic pathways. Subsequent addition of exogenous NO through NO donor negated Cd-mediated effects and protected the developing embryo.

Key words: cadmium; embryopathy; nitric oxide; redox stress; nitric oxide donor

Exposure to Cadmium (Cd) occurs mainly through food and the inhalation of tobacco smoke (Adams et al., 2011; Jarup and Akesson, 2009). Various studies have suggested that exposure to Cd leads to the development of adverse health problems in humans such as Itai-itai disease and cancer (Afridi et al., 2008; Menke et al., 2009; Satarug et al., 2011; Thomas et al., 2009). Studies have also demonstrated an increasing correlation between maternal Cd exposure and restricted fetal growth (Llanos and Ronco, 2009; Nishijo et al., 2004; Shirai et al., 2010). However to date, the exact mechanism behind the teratogenic effects of Cd remains unknown. Insufficient zinc (Zn) transfer resulting in retardation of intrauterine growth has been suggested as a potential mechanism for Cd-mediated teratogenicity (Kippler et al., 2010). Other possible mechanisms include oxidative stress, disruption of the ubiquitin proteasome system which may lead to changes in the activity of various regulatory proteins involved in cell cycle, DNA repair, oncogenesis, and apoptosis (Salomons et al., 2010; Stewart et al., 2003). Cd is well...
known to increase the redox stress of a system (Buha et al., 2012; Doi et al., 2011; Kipler et al., 2012a). Superoxides generated by Cd have been reported to induce apoptosis in human promonocytic cells (Galan et al., 2001). Furthermore, studies have shown that antioxidants such as vitamin E, glycine (Gly), and N-acetylcysteine (NAC) can provide protection against Cd toxicity (Doi et al., 2011; Karbownik et al., 2001; Panagiota-Castro et al., 2007). Interestingly, early studies reported that Cd remained accumulated in the placenta as opposed to being passed onto the fetus (Beri et al., 1992; Loiacono et al., 1992). Enhanced uptake of iron during gestation induces the DMT1 pathway which in turn augments its reported teratogenic effects.

Another important redox player that has been implicated in Cd toxicity is nitric oxide (NO). We have previously reported that Cd attenuates NO production of the endothelium causing endothelial dysfunctions (Kolluru et al., 2006; Majumder et al., 2008, 2009; Nagarajan et al., 2013). NO is also known to play a significant role in embryonic development. Murine embryos were found to be abnormal when cultured with pharmacological inhibitors of NO synthase (Nath et al., 2004).

Although a number of studies have probed into the mechanistic insight of Cd-associated embryopathy, the exact mechanism behind Cd toxicity is still unknown. The present study offers evidence that the supplementation of NO rescues Cd-induced embryopathy. The study also deciphers how Cd impinges the mechanistic interplay between the redox and NO signaling in the developing embryo.

### MATERIALS AND METHODS

#### Materials

- NO donors—Spermine NONOate (SP), Proli NONOate (Proli), MAHMA NONOate (MAHMA), and DETA NONOate (DETA) were purchased from Cayman Chemical. Diethylenamine NONOate (DEAN) and guanosine-3050-monophosphate 8-bromo-sodium salt (8-Br) were obtained from EMD Biosciences, Inc, California. DAR-4-M-AM (diaminorhodamine), 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), and catalase (CAT) from bovine liver were obtained from Sigma Chemical Co, St Louis. 2′,7′-Dichlorofluorescein diacetate (DCF-DA), Amplex Red, and Dihydorhoonadime 123 (DHR) were purchased from Molecular Grade and were obtained commercially.

#### Drug Administration to the Chick Embryos

Fertilized brown leghorn chicken eggs were purchased from the Poultry Research Station, Potheri, Chennai and were incubated at 37°C in a sterile humidified incubator. The chick embryos were staged based on the previously described Hamilton and Hamburger (HH) stages of chick development (Hamburger et al., 1951). Experiments were performed on chick embryos between the HH stages of 1–38. All treatments were administered as a single dose to the embryos through a hole made with a sterile needle in the air sac of each egg. 50 eggs were used for each treatment and time point. All procedures performed were approved by the institutional bioethics committee.

Cd chloride was dissolved in 1× PBS to obtain final concentrations of 0.1, 0.2, 0.4, 0.8, 1, 2, 4, 8, 10, 20, 40, and 60 μM. NO donor Spermine NONOate (SP) was also dissolved in 1× PBS to obtain final concentrations of 1, 2, 4, 8, 10, 20, 40, and 120 μM. For standardization of the dosage, 50 μl of each concentration of Cd and SP, respectively, were administered to HH-8-staged (26–29 h) embryos. 10 μM concentrations of Cd and SP, respectively, were selected as optimal concentrations and were used for all subsequent experiments unless specified differently. Although we inject 50 μl of 10 μM of Cd into the air sacs, when normalized against the total volume of the egg (60 μl), the embryos were finally exposed to a low concentration of Cd (90 ng/50 μl). We have previously shown that this concentration of Cd effectively interfered and inhibited endothelial functioning (Majumder et al., 2008). For standardization of the time point, HH-1-12-staged embryos were treated with 10 μM Cd, HH-8 stage was determined to be the most sensitive to Cd assault. For Cd + SP combination treatment, SP was administered 30 min following treatment with Cd. Similarly other treatments (ie, NO donors, cGMP analog, and antioxidants) were also administered as a single concentration (10 μM) at the HH-8 stage, 30 min following Cd treatment. Control embryos were treated with 50 μl of 1× PBS as vehicle control.

#### Morphological Analysis

Vehicle control-, Cd-, SP-, and Cd + SP-treated HH-26 (5th day) and HH-37 (11th day)-staged embryos were dissected out to examine the morphological effect of the respective treatments on embryo development. Images were taken using an Olympus camera (Olympus India Pvt Ltd, New Delhi, India) attached with a stereo microscope. HH-26 staged embryos were used to catalog the various deformities induced by Cd (detailed in Table 1). Additionally a digital chick heartbeat monitor was used to measure the heartbeat per min (bpm) of these embryos according to manufacturer instructions (Avian Biotech International, UK). The HH-37-staged embryos were weighed (wet weights) and measured (crown-rump length) to observe the differences between the growth rates of the embryos, that is normal versus abnormal.

#### Table 1. Comprehensive Evaluation of the Morphological Effects of Cd and NO Rescue on Chick Embryos

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cd</th>
<th>SP</th>
<th>Cd + SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal embryos</td>
<td>96.7%</td>
<td>27.3%</td>
<td>97.3%</td>
<td>90.7%</td>
</tr>
<tr>
<td>Dead embryos</td>
<td>3.3%</td>
<td>12%</td>
<td>2.7%</td>
<td>4%</td>
</tr>
<tr>
<td>Abnormal embryos</td>
<td>0%</td>
<td>60.7%</td>
<td>0%</td>
<td>5.3%</td>
</tr>
<tr>
<td>(a) Retard growth embryos</td>
<td>0%</td>
<td>28%</td>
<td>0%</td>
<td>2.7%</td>
</tr>
<tr>
<td>Length of the embryos(cm)</td>
<td>4.5 ± 0.5</td>
<td>2.5 ± 0.5</td>
<td>5 ± 0.5</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td>(g) Heart rate of the embryos (bpm)</td>
<td>3.2 ± 0.4</td>
<td>2.3 ± 0.6</td>
<td>3.4 ± 0.7</td>
<td>3 ± 0.4</td>
</tr>
<tr>
<td>(b) Omphalocoele</td>
<td>0%</td>
<td>6.7%</td>
<td>0%</td>
<td>1.3%</td>
</tr>
<tr>
<td>(c) Encephaly</td>
<td>0%</td>
<td>5.3%</td>
<td>0%</td>
<td>0.7%</td>
</tr>
<tr>
<td>(d) Micromelia</td>
<td>0%</td>
<td>5.3%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>(e) Polydactylly</td>
<td>0%</td>
<td>3.3%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>(f) Microphthalmia</td>
<td>0%</td>
<td>4%</td>
<td>0%</td>
<td>0.7%</td>
</tr>
<tr>
<td>(g) Hemorrhage</td>
<td>0%</td>
<td>4.7%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>(h) Twisted neck</td>
<td>0%</td>
<td>2%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>(i) Heart rate of the embryos (bpm)</td>
<td>280 ± 12</td>
<td>230 ± 16</td>
<td>280 ± 7</td>
<td>280 ± 5</td>
</tr>
</tbody>
</table>

**Notes:** n = 50 eggs per treatment group. Values represent the percentage mean for each group, obtained from 3 independent experiments ± SEM. *P < 0.003, Cd versus control; *P < 0.01, Cd + SP versus Cd (1-way ANOVA and LSD).
retarded growth. The embryos at this stage can be visibly checked to see whether their external organs (beak, eyes, and limbs) have developed normally, embryos only having reduced height and weight were identified as retarded growth.

**Alcian Blue Staining**

Alcian blue staining was used to analyze the digit deformities (Polydactality) following treatment (Table 1). Limbs isolated from vehicle control-, Cd-, SP-, and Cd + SP-treated HH-37-staged embryos were stained with Alcian blue stain as described previously (Siamwala et al., 2012). Briefly the limbs were fixed in Bouin’s solution for 2 h at room temperature. The limbs were then washed 6–8 times (3–4 h incubation per wash) with wash buffer (70% ethanol and 0.1% NH₄OH) over 24 h. The limbs were then washed twice for 1 h each with 5% acetic acid, before being stained with 0.05% Alcian blue 8GX in 5% acetic acid for 2 h. Following staining, the limbs were washed twice for 1 h each with 5% acetic acid and 100% methanol, respectively. The limbs were stored in benzyl benzoate:benzyl alcohol solution (2:1) and then washed twice with 1% PBS before being incubated with 0.2% Trypan Blue (0.4 mg/ml) for 15 min. The cells having blue colored nuclei were counted using an inverted bright-field microscope. The number of viable cells was counted based on the dye exclusion property of viable cells (Nagarajan et al., 2013).

**MTT Proliferation Assay**

Isolated angioblast cells were grown to 70% confluence in 12-well plates and treated with vehicle control, Cd, SP, 8-Br, and their combinations for 2 h. After treatment, the cells were washed twice with 1× PBS before being incubated with 0.2 μg/ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) for 2 h. The purple colored product formed was then dissolved with isopropyl alcohol and the optical density was measured at 570 nm using Varian Cary 4000 UV-Vis spectrophotometer (Majumder et al., 2008).

**Cell Viability Assay**

Angioblast cells isolated from HH-8-staged embryos were treated for 2 h with vehicle control, Cd, SP, and Cd + SP. Treatment media were removed and the cells were incubated with Trypan Blue (0.4 mg/ml) for 15 min. The cells were then washed twice with 1× PBS for 10 min. Cells having blue colored nuclei were counted using an inverted bright-field microscope. The number of viable cells was counted based on the dye exclusion property of viable cells (Nagarajan et al., 2013).

**Tube Formation Assay**

Chick aortic arches were carefully dissected from the hearts of HH-37-staged embryos under aseptic conditions. The arches were cut into small rings having the same size and washed several times with 1× PBS. Each ring was then placed in a 12-well plate precoated with matrigel and were treated with vehicle control, Cd, SP, 8-Br, CAT, and their combinations. Endothelia cell sprouting was confirmed using CD34 antibody. Images were taken after 36 h using an Olympus microscope at ×20 magnification. The number of tubes was counted manually as a double blind study (Siamwala et al., 2012).

**Angioblast cell tube formation assay**. Embryos were pretreated with vehicle control, Cd, SP, 8-Br, and their combinations for 2 h before isolating out the angioblast cells as per the protocol described above. The cells were seeded onto a matrigel-coated 12-well plates and incubated at 37°C in a CO₂ incubator for 36 h. The cells were imaged using an Olympus camera attached to microscope and the number of tubes formed was counted manually.

**Benzidine staining.** Benzidine staining for blood island and primary plexus was performed on HH-10- and HH-13-staged embryos (Niemisto et al., 2005). Briefly, the EEM was removed from yolk sac and treated with freshly prepared benzidine solution for 15 min. The membranes were then washed with 100% methanol and imaged immediately using an Olympus camera attached stereo microscope. The total number of plexus formations per image was counted manually.

**Assessment of hemoglobin levels of blood island formation.** The hemoglobin levels of HH-10-staged EEM were measured to determine the extent of blood island formation. Briefly, The EEM were carefully isolated out, homogenized, and centrifuged for 10 min at 2500 rpm at 4°C. The supernatant was read at 540 nm in a colorimeter. The data were normalized against the weight of the tissue.

**Assessment of Angiogenesis Parameters**

Vascular maturity Assay. HH-15 (50–55 h)-staged embryos pretreated with vehicle control, Cd, SP, and Cd + SP were gently opened and their contents transferred carefully onto sterile petri-dishes. 2 and 4× images were taken using an Olympus camera attached to a stereo microscope at 2 h intervals for 8 h. Changes in blood vessel length, size, and junction were then analyzed with the help of the Angioquant software as described elsewhere (Niemisto et al., 2005).
Yolk sac vascular pattern analysis. HH-17 (52–64 h)-staged embryos pretreated with Vehicle control, Cd, SP, and Cd + SP were gently opened and their contents transferred carefully onto sterile petri dishes. 2 and 4× images of the 4 major areas of the yolk sac vasculosa—the left omphalomesenteric vessels (LROM), right omphalomesenteric vessels (RROM), posterior vitelline veins (PFV), and anterior vitelline veins (AVV) were taken using an Olympus camera attached to a stereomicroscope at 2 h intervals for 8 h. The Angioquant software was then used to quantitate the changes in the density of blood vessel length, size, and junction as described elsewhere (Niemisto et al., 2005).

**Estimation of NO**
DAR-4 M-AM imaging of chick aortic rings. Chick aortic rings were cultured as described above and the endothelial sprouting around the rings were treated with vehicle control, Cd, SP, and Cd + SP for 30 min. After treatment, the rings were gently washed with 1× PBS and further incubated with the NO-specific fluorescence probe DAR-4 M-AM for 10 min. The rings were then washed twice with 1× PBS. Corresponding bright field and fluorescence images were taken at ×20 magnification using an Olympus fluorescence microscope. The fluorescence intensity of the sprouted tubes was calculated using Adobe Photoshop of version 7.0 (Siamwala et al., 2012).

Amperometric measurement of NO using a NO-sensitive electrode. Real-time NO production was measured using an amperometric probe (Apollo 4000 analyzer) as per the protocol described elsewhere (Majumder et al., 2009). A NO standard curve was generated by calculating the amount of NO released by different concentrations of SP (0, 1, 10, 100, and 500 nm/ml) for 15 min (Supplementary Fig. 3C). SP, which has a half-life of 39 min, is used to estimate NO concentrations by linear regression of the SP standard curve to obtain the exact amount of NO produced by the angioblast cells.

**Estimation of Reactive Oxygen Species**
Four reactive oxygen species (ROS) parameters including total ROS, superoxide, peroxynitrite, and hydrogen peroxide (H2O2) production were assessed for both whole embryo (HH-8 stage) and isolated angioblast cells following treatment (vehicle control, Cd, SP, and Cd + SP) for 30 min prior to performing the assays.

H2O2 detection using Amplex Red. Hydrogen peroxide was measured using Amplex red, a H2O2-specific fluorescence probe. Briefly, angioblast cells were incubated with 10 μM Amplex Red and 0.5 units of horseradish peroxidase for 15 min after treatments. The supernatant was then collected and read using a fluorescence spectrophotometer at excitation/emission of 570/585 nm (Muley et al., 2010).

**Measurement of peroxynitrite**. Following treatment, peroxynitrite levels were measured using DHR as described elsewhere (Majumder et al., 2010). Briefly, 10 μM DHR was added to the angioblast cells and incubated for 20 min at 37°C. The supernatant was collected and read using a fluorescence spectrophotometer at 490 nm for excitation and at 515 nm for emission.

**Measurement of superoxide and total ROS**. Nitro blue tetrazolium (NBT) assay and fluorescent probe DCF-DA assay were used to measure the superoxide levels and total ROS levels in the angioblast cells. Briefly, following treatment the angioblast cells were incubated with NBT for 2 h and supernatant was measured calorimetrically at 560 nm. For total ROS levels, the treated angioblast cells were incubated for 30 min with 5 μM DCF-DA. Then the supernatant was collected and read using a fluorescence spectrophotometer at excitation/emission of 570/585 nm (Muley et al., 2010).

**Measurement of lipid peroxidation**. Lipid peroxidation (LPO) levels of the whole embryo following treatment were evaluated by measuring TBARS as per the protocol described elsewhere (Majumder et al., 2010) albeit slightly modified. Briefly, HH-9-staged embryos were dissected and homogenized in 5 vol of ice-cold 0.05 M phosphate buffer (pH 7); 1 ml and 1.5 ml of buffer solution and trichloroacetic acid (TCA)-thiobarbituric acid (TBA)-HCl reagent were prepared in 0.85 N HCl with 13.5% wt/vol TCA and 0.33% wt/vol TBA, respectively, and then deoxygenation was carried out by bubbling with nitrogen; this was then added to 0.2 ml homogenate. The samples were then placed in sealed tubes containing nitrogen and heated for 15 min in a boiling water bath. One millilitre of TCA 70% wt/vol was slowly added after cooling and the precipitate was removed by centrifugation at 2500 × g for 10 min. The supernatant was measured at 533 nm/515 nm using a Varian Carry Eclipse Fluorescence Spectrophotometer. The level of fluorescence was taken as the index of LPO in the embryo.

**Live Cell Tracking Using DCF-DA and Mito Tracker Red**
Isolated angioblast cells were treated with vehicle control, Cd, and Cd + MnTBAP (SOD mimetic—20 μM) for 30 min. The cells were then washed with PBS before being incubated with 10 μM DCF-DA and 100 nM Mito Tracker Red for 30 min. Fluorescence images were taken under ×20 magnification using an Olympus Fluorescence Microscope (Chen et al., 2011).

**Measurement of Apoptosis using Annexin-Propidium Iodide Staining**
Apoptosis was detected in angioblast cells and aortic rings using Annexin-propidium iodide (PI) staining (Calbiochem PF032). Briefly, aortic rings were prepared as described above. After 36 h of incubation, the rings were treated with vehicle control, Cd, SP, and Cd + SP, respectively, for 30 min. After treatment, the rings were washed thrice with 1× PBS. The rings were then incubated with binding buffer, Annexin V and PI, respectively, as per the manufacturer’s protocol. Fluorescent images of Annexin and PI were captured using Olympus XLI70 fluorescent microscope. The fluorescence intensity of the images was quantified using Adobe Photoshop version 7.0. The same protocol was applied for angioblasts following treatment.

**Isolation of RNA and Semi-quantitative PCR**
Total RNA was isolated from whole embryos and isolated angioblast cells following treatment with vehicle control, Cd, SP, and Cd + SP using Tri-reagent (Invitrogen—AM9738) as per the manufacturer’s instructions. Total RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo scientific) and cDNA synthesis was performed on 200 ng of RNA using reverse transcriptase (Applied Biosystems) as per the manufacturer’s protocol. The primer sequence, annealing temperature, and product sizes for PCRs performed are summarized in Table 2. β-Actin was used as the internal control. The annealing conditions and the products were resolved on a 1% agarose gel at 80 V using an agarose gel electrophoresis system.
selected this stage as the time point for all treatments and crucial time point of blood island formation, we stratified that the HH-8 stage to be most sensitive to thalidomide (Siamwala).

A semi-quantitative RT-PCR was performed using the SIGMASTAT software package. One-way analysis of variance (ANOVA), Student’s t test, and Turkey’s post hoc tests were used to analyze all data. P < 0.05 was considered as statistically significant.

RESULTS

Establishment of the Cd Model

The chick embryo is an ideal model for studying the teratogenic properties of new compounds, their risk and hazard management processes as well as for investigating mechanistic insight of known teratogens (Flint, 1993). The advantages of using the chick embryo model over other in vivo models include its inexpensiveness, less time-consuming and that the embryos grow independent of maternal influence enabling researchers to observe the effect of teratogens directly on the embryo (Kotwani, 1998; Wierzbicki et al., 2013). To identify the most effective concentration, we treated HH-8-staged chick embryos (which corresponds to the 8.5th and 20th day of development in humans and mice, respectively) with various concentrations of Cd (0.1–60 μM). Establishment of proper circulatory system is essential for subsequent embryogenesis and organogenesis. In mouse and chick embryos, blood island formation (precursors of vasculogenesis in the embryo) occurs during the stages of E-7-8 and HH-8, respectively (Folkman and D’Amore, 1996; Nakazawa et al., 2006; Ollie, 1990). We have previously demonstrated that the HH-8 stage to be most sensitive to thalidomide assault (Siamwala et al., 2012); and considering the sensitivity and crucial time point of blood island formation, we selected this stage as the time point for all treatments (Cd and cotreatments). Morphological analysis of embryos revealed that 10 μM of Cd effectively caused deformities (58%) but not death in majority of the exposed embryos (Fig. 1A). This dose was selected and used for subsequent experiments. Furthermore, embryos from HH stages 1–12 were treated with 10 μM Cd to verify the period most sensitive to Cd assault. Results showed that the period from HH-7 to -9 stages were the most sensitive to Cd, with HH-8-staged embryos exhibiting the highest cohort of deformities (62%) (Fig. 1B).

SP Recovered Cd-Induced Deformities

HH-8-staged embryos were first exposed to Cd (10 μM) for 30 min before being treated with different doses of SP (1–120 μM). SP is routinely used in experimental biology as a pharmacological donor of NO. Recently, we have demonstrated that the NO release pattern of SP is the best suited among other NONOate-based NO donors in angiogenesis scaffolds (Loiacono et al., 1992). Although there are not many reports regarding the toxicology of SP, some studies have suggested that SP may produce nitrosamine at higher concentrations (Loiacono et al., 1992). Results showed that 10 μM of SP conferred the best protection against Cd assault with a recovery rate of approximately 94% when compared with other SP concentrations (Fig. 2A). This concentration was used for subsequent experiments. Next, we determined the best time point (0–120 min) to administer SP following initial Cd assault. Results demonstrated that addition of 10 μM SP, 30 min after Cd exposure significantly decreased the percentage of deformed embryos by 50% while allowing for a corresponding 60% increase in the percentage of normal embryos observed (Fig. 2B). Furthermore, we compared the protective effects of SP (half-life 39 min) against Cd with other known NO donors based on their half-lives including Proli (half-life 1.8 s), DEAN (half-life 2 min), MAHMA (half-life 2.7 min), and...
and SP-treated embryos, respectively (Fig. 3B and lower hemoglobin levels when compared with vehicle control that Cd-treated embryos exhibited less Benzidine spots and blood islands) and hemoglobin assays. Results demonstrated Early blood island formation occurring at HH-10 stage (Fig. 3A)

Vasculogenesis in Chick Embryo

NO Rescued Cd Impaired Blood Island Formation and Yolk Sac development.

The abnormalities seen despite SP recovery included retarded growth (3.6%), hematoma (1.2%), micromelia (0.8%), and microphthalmia (0.4%). Embryos treated with only SP did not demonstrate any deformities.

NO Protects Against Cd-induced Teratogenicity

The current study presents a comprehensive list of the most common deformities induced by Cd (Table 1). Morphological analysis revealed that compared with vehicle control-treated embryos (96.8% normal, 3.2% dead, and no deformed embryos), 10μM Cd resulted in 26.4% normal, 61.6% deformed embryos, and 12% dead embryos. The most common abnormalities exhibited included retarded growth (28%), Omphalocoele (failed form ventral body wall) (6.8%), microphthalmia (6%), exencephaly (5.6%), hemorrhage (4.8%), micromelia (4.6%), polydactyly (3.6%), and twisted neck (2%). Interestingly, addition of SP to Cd-exposed embryos recovered approximately 90.4% of the embryos from Cd-mediated effects, albeit 6% of the embryos still demonstrated abnormalities and 3.6% were found dead when compared with vehicle control and SP-treated embryos. The abnormalities seen despite SP recovery included retarded growth (3.6%), hematoma (1.2%), micromelia (0.8%), and microphthalmia (0.4%). Embryos treated with only SP did not demonstrate any deformities.

NO Rescued Cd Impaired Blood Island Formation and Yolk Sac Vasculogenesis in Chick Embryo

Early blood island formation occurring at HH-10 stage (Fig. 3A) was assessed with the help of Benzidine staining (which stains blood islands) and hemoglobin assays. Results demonstrated that Cd-treated embryos exhibited less Benzidine spots and lower hemoglobin levels when compared with vehicle control and SP-treated embryos, respectively (Fig. 3B and Supplementary Fig. 2A). Addition of SP to Cd-treated embryos blunted Cd-mediated effects and restored blood island formation. Next, analysis of the primary plexus formation of HH-12-staged embryos showed that Cd treatment significantly decreased the formation of primary plexus tubules compared with vehicle control and SP-treated embryos. Furthermore, SP restored and recovered tube formation in Cd + SP embryos (Figs. 3C and 3D). Assessment of blood vessel maturation in the yolk sac vasculosa of HH-15-staged embryos showed that the length, size, and junction of matured blood vessels in the area vasculosa region were decreased following Cd exposure when compared with vehicle control and SP-treated embryos, the density of length, size, and junction of matured blood vessels were decreased following Cd exposure (Fig. 3F and Supplementary Figs. 2D and 2E). Blood vessel maturation was found to be normal in Cd + SP-treated embryos indicating that SP was able to negate Cd-mediated effects.

Cd Inhibition of NO-c-GMP-Dependent Angioblast Cells Activation Is Rescued by Administration of Exogenous NO

Analysis of the endogenous NO levels of HH-8-staged angioblast cells revealed that Cd treatment significantly decreased NO production of these cells. Treatment of Cd-exposed cells with SP significantly recovered NO production of these cells when compared with Cd-treated cells (Figs. 4A and 4B and Supplementary Fig. 3A). Time-dependent real-time measurement of NO levels using an ultra-sensitive NO electrode showed that the NO levels of Cd-treated angioblast cells decreased steadily from time of exposure when compared with vehicle control-treated cells (Fig. 4C). Results showed that 10μM of Cd decreased NO production of angioblast cells by 50% at 18 min post-treatment (Supplementary Fig. 3B). Subsequent quantification revealed that when compared with control (0.67 pM/cell), NO production of a single angioblast cell was decreased to (0.04 pM/cell) following Cd incubation for 30 min (Fig. 4C and Supplementary Fig. 3C). As cGMP is known as a major downstream target of the NO pathway, we assessed the cGMP activity of these cells following Cd treatment. Results showed that Cd treatment decreased cGMP levels of the angioblast cells while addition of SP restored the cGMP levels (Fig. 4D). Assessment of the functional parameters of Cd-treated angioblast cells revealed that Cd exposure decreased cell migration, proliferation, and tube formation of these cells compared with vehicle control-treated cells (Figs. 4E and 4F and Supplementary Figs. 3D and 3E). Addition of SP and cGMP analog 8-Br, respectively, blunted Cd inhibition of these functional parameters indicating that SP- or 8-Br-mediated restoration of NO production protected the chick embryo from Cd-mediated effects. Next, we used chick aortic ring assays to determine the corresponding NO production and tube formation following Cd treatment. Results showed that Cd treatment decreased NO production and subsequent tube formation of the aortic rings while addition of SP to Cd-treated rings blunted Cd-mediated effects (Fig. 5). The results further emphasis the importance of maintaining NO bioavailability during embryonic development.

NO Reduced Cd-induced Oxidative Stress in Chick Embryo

Results showed that Cd significantly increased the redox levels (total ROS, superoxide, and hydrogen peroxide) of the whole embryo and isolated angioblast cells, when compared with the vehicle control and SP-treated counterparts, respectively.
Analysis of results showed that total ROS and hydrogen peroxide levels were maximally elevated by Cd in the angioblast cells (Fig. 6A). Similarly, hydrogen peroxide levels were found to be elevated in chick aortic rings following Cd treatment (Fig. 6B). In contrast, superoxide levels were found to be maximally increased in the whole embryo following Cd treatment (Fig. 6C). Addition of SP to Cd-exposed cells, embryos, and rings blunted Cd-induced effects and decreased redox levels. Results also revealed increased LPO levels in Cd-exposed embryos which were subsequently negated by addition of SP to Cd-exposed embryos (Supplementary Fig. 4A). To further corroborate these results, we tracked the mitochondrial ROS production of angioblast cells via live cell imaging following treatment. Results demonstrated that Cd induced ROS generation in the mitochondria of these cells, while addition of MnTBAP was able to negate Cd-induced mitochondrial ROS production (Supplementary Fig. 4D).

As antioxidants have been previously reported to play a protective role against Cd-mediated teratogenicity (Doi et al., 2011), we compared the protective effects of known antioxidants with that of SP. Results showed that SP conferred better protection, rescuing almost 90% of the embryos from Cd-mediated effects compared with CAT (60%), Zn (50%), NAC (45%), SOD (40%), and Gly (30%), respectively (Supplementary Fig. 4B).

NO Prevents Cd-Mediated FOXO3-Caspase 3-Dependent Apoptosis in Angioblast Cells

Cell viability assays performed on isolated angioblast cells demonstrated that Cd treatment significantly decreased the viability of these cells by 60% compared with controls. Whereas the addition of SP blunted Cd-mediated effects and restored cell viability (Supplementary Fig. 4C). Further analysis revealed that the decreased viability observed following Cd treatment may be attributed to the occurrence of apoptosis in these cells. Annexin/PI staining of the angioblast cells and chick aortic rings, respectively, showed that Cd treatment induced apoptosis in these cells (Figs. 7A, 7D, and 7E). Additionally PCR results confirmed that this apoptosis occurred via the activation of FOXO3a-Caspase 3 signaling pathway in the angioblast cells. When compared with controls, Cd treatment caused down-regulation of FOXO1 expression and subsequent up-regulation of FOXO3a and Caspase 3 expressions, respectively (Fig. 7B), resulting in apoptosis. Furthermore, PCR results from whole embryo showed that p21 and p53 gene expressions increased following
Cd treatment when compared with controls (Fig. 7C). Addition of SP to Cd-treated cells was able to protect the cells/embryo and prevent apoptosis (Fig. 7). Results also showed that antioxidant CAT was able to confer protection against Cd effects and prevent apoptosis (Supplementary Fig. 5).

**DISCUSSION**

Although the placental transport of Cd is limited, Cd exposure is a major concern for intra and post uterine development as even ultra-low doses can be lethal to the embryo. It is evident from previous reports that higher levels of Cd in cord blood were associated with decreased height, weight, and head circumference of children (Kippler et al., 2012b; Lin et al., 2011). Similarly, other studies found inverse associations between cord blood Cd concentrations and birth weight (Galicia-Garcia et al., 1997; Salpietro et al., 2002). Interestingly, morphological analysis in the current study also showed decreased head circumference and growth of chick embryos exposed to Cd. This result is relevant as it is known that hematopoietic development in mammals appears to mimic that of the chick embryo.

Previously, Butler et al. (2006) demonstrated that cord blood contained approximately 0.6–7.5 μg/L Cd with a geometric mean of 0.43 μg/L. In a recent study on the association of cord blood metal concentrations and child characteristics at birth (Garcia-Esquinas et al., 2013), it was reported that mother’s blood contains 450–610 ng/L and cord blood contains 230–310 ng/L. Based on these 2 studies we decided to maintain the similar concentrations in our model, however as we load Cd through the air...
sac, it is possible that this causes a more acute effect than Cd exposure from the cord blood. Hence, 2 important observations obtained from the present study are that low concentration of Cd (90 ng/50 ml) was the most effective teratogenic concentration and that the developmental stage HH-8 was determined to be the most sensitive to Cd assault. Results demonstrated that low concentration of Cd induced 68% embryopathy in Cd-treated groups. Previously, we have shown that ultra low levels of Cd interfere with endothelial functions and specifically angiogenesis (Majumder et al., 2008). Based on this clue, we hypothesized that Cd interferes with vasculogenesis and the present study demonstrated that the severity of Cd-mediated effects observed at the HH-8 stage coincides with the formation of blood islands in the developing embryo (Figs. 1 and 3). Blood islands are the precursors of early blood vessels in the embryo; the time of their formation is an extremely delicate and dynamic phase which ultimately defines the vasculature of area vasculosa and embryo development. Results demonstrate that Cd exposure at HH-8 stage caused significant deformations of area vasculosa vascular network of embryos (Fig. 3). These results in turn parallel our previously published work which showed that the exposure of classical teratogenic drug thalidomide to HH-8-staged embryos was detrimental inhibiting early vasculogenesis and subsequent embryo development (Siamwala et al., 2012).

A classic publication by Kleinzeller and Werner (1939) demonstrated that catalase activity rises along with the growth of chick embryos. Similarly the present study confirmed that as angioblasts and blood island cells lack catalase-based protection, H2O2 is the predominant redox player during the early phase of development (Fig. 6A). We delineate that the loading of Cd at HH-8 stage, a phase already overwhelmed with H2O2, generated more (Cd-mediated) oxidative stress in the embryo making this phase particularly vulnerable to teratogenic assault. NO is well known to play an essential role during embryonic development (Nath et al., 2004). Gentile et al. (2003) reported that VEGF-mediated phosphorylation of eNOS regulates angioblast and embryonic endothelial cell proliferation. Other studies have demonstrated that L-NAME, an inhibitor of NOS induced limb defects in mice fetus (Fantel et al., 1999; Tiboni et al., 2003).

Similarly we have previously shown that Cd interferes with endothelial functions by attenuating NO production via the inhibition of eNOS phosphorylation in the endothelium (Majumder et al., 2008). Additionally in the current study we critically quantified the absolute NO production by angioblast cells under Cd treatment. We observed that 10 μM of Cd dampens NO production by 50% at 18th min of incubation. Further we calculated that NO production of a single angioblast cell decreases from 0.55 and 0.67 pM/cell (control values) to 0.20 and 0.04 pM/cell after 20 and 30 min treatment with Cd, respectively (Fig. 4C and Supplementary Figs. 3B and 3C).

Recently, we reported that the administration of exogenous NO through NO donor, SP could protect and rescue endothelial cells from Cd-mediated effects (Nagarajan et al., 2013). Other studies have also shown that increasing NO levels could rescue...
mice embryos from teratogenic effects of copper deficiency and glucose-induced vasculopathy, respectively (Nath et al., 2004; Yang et al., 2007). Our recent report parallels these studies demonstrating that NO rescued chick embryos from thalidomide-mediated teratogenicity (Siamwala et al., 2012). The present study demonstrates that supplementation of exogenous NO via NO donor to Cd-exposed HH-8-staged embryos significantly rescued embryos from Cd-mediated embryopathy (Table 1). In extension of this work we confirmed that NO release pattern of SP (a NO donor with 39 min half-life) (Maragos et al., 1991) fits well with the dynamics of early angiogenesis, reducing Cd-induced oxidative stress and ultimately protecting the angioblast cells from undergoing apoptosis (Figs. 7 and 8). The reaction between NO and superoxide occurs in an almost diffusion-limited rate, and is 6 times greater than the removal of superoxide by copper-zinc superoxide dismutase (Cu/Zn SOD) as reported previously (Guzik et al., 2002).

Emerging evidence suggests that teratogen-induced birth defects could occur due to deregulation of apoptosis pathways during embryonic development (Mallela and Hrubec, 2012). Knobloch et al. (2007) demonstrated that thalidomide’s inhibition of redox-sensitive transcription factor NF-κB in limb buds increased expression of proapoptotic bone morphogenetic proteins and suppressed corresponding Wnt/β-catenin and Akt-dependent survival signaling. Although Siamwala et al. (2012) reported that thalidomide induced Caspase 3-dependent apoptosis in chick embryo and isolated cells from limb buds. Results of the present study demonstrate that Cd elevates oxidative stress levels and suppresses NO levels in isolated angioblasts. Schmidt et al. (2007) previously reported an up-regulation of SOD in angioblasts. Therefore, this is a plausible hypothesis that a higher activity of SOD neutralizes Cd-induced superoxide and added H$_2$O$_2$ to the ROS pool in the angioblasts. This could be further amplified by SOD activity of egg yolk since a detailed proteome analysis by Mann and Mann (2008) showed that egg yolk has SOD like enzymes. This in turn activated the Caspase 3-dependent apoptotic pathway causing erroneous vasculogenesis. Oh and Lim (2006) previously showed that Cd-mediated rapid and transient ROS generation triggered apoptosis through Caspase-dependent pathway in HepG2 cells, which was subsequently inhibited via NAC-mediated catalase upregulation. However, the question remains elusive as to how Cd induces over-expression of Caspase 3. A recent study suggested that an interaction between Cd and H-ras significantly activates H-ras expression and subsequently the activation of Caspase 3 in cancer cells (Petanidis et al., 2013). It has also been proposed that Cd signaling, which activates Caspase 3-dependent apoptosis, comprises of calpain and phospholipase C (PLC). Lawal and Ellis (2012) showed that the activity of the calcium-dependent protease calpain was elevated by Cd, whereas PLC-specific inhibitor, U73122, prevented the Cd-dependent increase in Ca(2+) levels and also abolished Cd-dependent calpain and Caspase 3 activation as well as Cd-dependent mitochondrial Bax accumulation. Recently, a study by Yiran et al. (2013) elaborated that Cd treatment elevated the mRNA levels of Bax while decreasing the mRNA and protein levels of Bcl-2 promoting Caspase-3 activation, thereby establishing a link between oxidative stress, MAPK pathways, and Caspase 3 activation.

The present study demonstrates that the supplementation of exogenous NO restored ROS-NO homeostasis, thereby preventing the activation of apoptotic pathways and protecting vasculogenesis. The usage of NO donors for rescuing or restoring embryopathy is a delicate issue particularly as NO donors are known to produce carcinogenic nitrosamine (Miller and Megson, 2007) and have been shown to induce apoptosis when administered at higher doses (Miller and Megson, 2007). In accordance with data reported in previous studies (Nath et al., 2004; Siamwala et al., 2012), the present study offers evidence...
that low doses of NO successfully protects and rescues embryos from teratogenic assaults (Fig. 7C). It is well known that heavy metals induce oxidative stress in vitro, in vivo, and clinical settings. However, the mechanism of heavy metal-induced oxidative stress remains unknown. Wang et al. (2004) demonstrated that Cd inhibits the activity of complexes II (succinate:ubiquinone oxidoreductase) and III (ubiquinol:cytochrome c oxidoreductase) of the mitochondrial electron transport chain in liver, brain, and heart samples. Subsequent kinetic and electron turnover experiments suggested that Cd binds between the semiubiquinone and cytochrome b566 of the Q0 site of cytochrome b of complex III, resulting in the accumulation of semiubiquinones at the Q0 site. The semiubiquinones readily transfers one electron to molecular oxygen to form superoxide, providing a possible mechanism for Cd-induced generation of ROS in mitochondria. In current study, on tracking the ROS production from mitochondria of the treated angioblast cells, we observed that Cd induced ROS generation from the mitochondria (Supplementary Fig. 4D). Antioxidants such as glycine (Paniagua-Castro et al., 2007) and Zn (Jihen et al., 2009) have been previously reported to rescue murine embryos from toxicity and teratogenicity by attenuating apoptotic events in the developing embryos. Additionally 2 antioxidant enzymes, CAT and superoxide dismutases (SOD2), have also been reported to inhibit Cd-induced injury and LPO in human fetal lung fibroblasts (Yang et al., 1997). Doi et al. (2011) demonstrated that up-regulation of SOD2 and CAT gene expression preventing Cd-induced omphalocele phenotype in chick embryos, suggesting both SOD2 and CAT could play important roles in preventing Cd teratogenesis. As Cd is a heavy metal, it is non-specific and affects all cell types in the micro-milieu. Therefore, we utilized soluble extra-cellular CAT to reduce the load of H2O2 from the affected micro-environment irrespective of the cell target. We followed the approach taken by Ding et al. (2007) to confer global protection to the embryos against H2O2 assault. It is well known that antioxidative defense mechanisms such as CAT, glutathione peroxidase, and the manganese-dependent SOD are up-regulated in angioblasts and cultivated endothelial progenitor cells (EPCs) (Dernbach et al., 2004; Ramalho-Santos et al., 2002). Similarly, the present study indicates that Cd-induced stress caused up-regulation of SOD activity in angioblasts (Fig. 6A). Based on these observations and results of the present study we propose that Cd induces generation of mitochondrial ROS, specifically superoxide, which instantaneously dismutated by cytosolic SOD and produces H2O2. Next, H2O2 readily crosses through plasma membrane to extra-cellular domain, and targets neighboring cells for the induction of apoptosis in them. This is the reason CAT, which is non-permeable to cell membrane, is able to scavange the H2O2 in extra-cellular fluid as it is evident from our study (Fig. 8). Data from present study also elaborate that scavenging of ROS, specifically H2O2 with the help of antioxidant CAT or via either NO donor SP or cGMP.

FIG. 7. NO rescues embryo from Cd-induced oxidative stress mediated Caspase 3-FOXO 3-dependent apoptosis. A, Apoptosis of vehicle control, Cd, SP, and Cd + SP-treated angioblast cells was detected using Annexin V and PI. B, Representative gel images depicting results of semi-quantitative PCR performed on cDNA obtained from angioblast cells for FOXO1, FOXO3, Caspase 3 expressions following treatment. C, Representative gel images of p21, and p53 expressions for whole embryo following treatment. β-Actin was used as the internal control. D, Representative images of corresponding bright field, Annexin V, and PI of chick aortic rings following treatment. E, Graphical representation of the intensity of Annexin V and PI fluorescence calculated. **P < 0.001, Cd versus control; #P < 0.001, Cd + SP versus Cd. n = 3. Values represent the mean for each group ± SEM, 1-way ANOVA and LSD.
analog 8-Br inhibited subsequent apoptosis in angioblasts, thereby protecting embryos against teratogenic effects of Cd. Interestingly in contrast to the rescue observed with SP although CAT and 8-Br were able to recover retarded growth of embryos, they were unable to recover deformed embryos.

Forkhead box O (FOXO) transcription factors are a group of transcription factors that regulate angiogenesis and oxidative stress (Essers et al., 2004; Potente et al., 2005). FOXOs are downstream
targets of the serine/threonine protein kinase B/Akt. FOXO 1 and FOXO3a are highly expressed in endothelial cells (Potente et al., 2005). Phosphorylation of FOXOs by Akt inhibits its transcriptional functions and contributes to cell survival, growth, and proliferation. Silencing of FOXO3a expression using siRNA attenuated CdCl₂-induced cellular damage and accumulation of cytoplasmic nucleosomes in HK-2 cells (Fujiki et al., 2013). Similarly, in the present study we observed decreased FOXO1 expression and increased FOXO3a expression in angioblast cells following Cd treatment. Subsequent addition of SP to Cd-treated cells reversed Cd-mediated effect on FOXO1 and FOXO3a expressions (Fig. 7B). Our studies parallel previous studies including Furuyama et al. (2004) who reported deformed yolk sac vasculogenesis and embryonic lethality in FOXO1-deficient mice. Potente et al. (2005) observed that overexpression of FOXO3a inhibited endothelial tube formation and migration, while we previously demonstrated that thalidomide mediated its teratogenic effects via FOXO3 signaling, which was subsequently attenuated by addition of NO (Siamwala et al., 2012). These observations indicates that thalidomide and Cd share similar proapoptotic signaling to mediate their respective teratogenic activities, however this warrants further research to confirm this dogma.

Based on our observations we propose that supplementation of NO to Cd exposed embryos targets 2 critical points of Cd activated ROS-apoptosis axis in the developing embryo and its vasculature namely (1) NO quenches superoxide to reduce the load of SOD-dependent H₂O₂ followed by FOXO1 down regulation, which was subsequently attenuated by addition of NO (Siamwala et al., 2012). These observations indicates that thalidomide and Cd share similar proapoptotic signaling to mediate their respective teratogenic activities, however this warrants further research to confirm this dogma.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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**REFERENCES**


