SCIENTIFIC REPORTS

SUBJECT AREAS:
PATTERN FORMATION
ANGIOGENESIS
CELL SIGNALLING
CELL MIGRATION

Received
18 June 2012

Accepted
28 August 2012

Published
20 September 2012

Correspondence and requests for materials should be addressed to S.C. (soovro@yahoo.ca)

* Authors have contributed equally.

Nitric oxide rescues thalidomide mediated teratogenicity

Jamila H. Siamwala*, Vimal Veeriah*, M. Krishna Priya, Saranya Rajendran, Uttara Saran, Swaraj Sinha, Shunmugam Nagarajan, Pradeep T. & Suvro Chatterjee

Vascular Biology Lab, AU-KBC Research Centre, Anna University, MIT Campus, Chennai, India.

Thalidomide, a sedative drug given to pregnant women, unfortunately caused limb deformities in thousands of babies. Recently the drug was revived because of its therapeutic potential; however the search is still ongoing for an antidote against thalidomide induced limb deformities. In the current study we found that nitric oxide (NO) rescues thalidomide affected chick (*Gallus gallus*) and zebrafish (*Danio rerio*) embryos. This study confirms that NO reduced the number of thalidomide mediated limb deformities by 94% and 80% in chick and zebrafish embryos respectively. NO prevents limb deformities by promoting angiogenesis, reducing oxidative stress and inactivating caspase-3 dependent apoptosis. We conclude that NO secures angiogenesis in the thalidomide treated embryos to protect them from deformities.

Thalidomide (a-(N-phthalimido) glutaramide), a drug developed by Chemie Grunenthal in the 1950's caused a lot of excitement in the medical world as it was a so called "perfect drug" for treating morning sickness in pregnant women[1]. Thalidomide was found to be easily absorbable, without any hangover and apparently safe even after an overdose, making it an alternative to the administration of barbiturates[1]. However the drug was banned by WHO (World Health Organization) after 6000–10,000 babies were affected by this drug[2,3]. Later the serendipitous discovery of anti-angiogenic[4] and immunomodulatory[5] potential of thalidomide and its analogues led to the resurgence of this drug for the treatment of cancer, leprosy, unresponsive dermatological conditions, graft-versus-host disease, Kaposi sarcoma, lichen planus, melanoma, prurigo nodularis, pyoderma gangrenosum and uraemic pruritus[6,7]. The multitude of benefits of using thalidomide and its analogues warrants novel therapeutic approaches to prevent thalidomide side effects so that thalidomide can be used in diseases with limited treatment options. This is plausible only if the mechanism of thalidomide actions is fully understood. Recent works suggest that thalidomide causes limb defects by inducing oxidative stress and apoptosis and inhibiting angiogenesis[8–12].

Nitric oxide (NO) a gaseous molecule regulates endothelial cell migration, proliferation, extracellular matrix degradation and angiogenesis[13,14]. Recent studies indicate that thalidomide modulates NO dynamics by inhibition of interferon gamma in mouse vascular endothelial cells[15]. In our previous study we have shown that thalidomide inhibits NO effects by blocking NO-cGMP signaling in endothelial cells[16,17]. Since thalidomide interferes with angiogenesis process and NO signaling in endothelium we propose that giving back NO to the system would annul thalidomide mediated limb deformities. In this study we corroborate that NO recovers thalidomide induced teratogenicity in 94% and 80% of avian and zebrafish embryos. NO counters thalidomide effects in embryo by reinforcing vasculogenesis and blocking apoptosis through modulation of redox in the endothelium. The findings holds promise for the development of drugs or combination approach that will retain thalidomide efficacy in treating clinical conditions at the same time avoiding thalidomide mediated teratogenicity.

Results

Establishment of thalidomide model. Kemper (1962) pioneered the suitability of using chick embryos for producing typical thalidomide deformities provided it was introduced into the egg at a particular period of embryonic development[18,19]. Salzgeber & Salaun (1963) further established that thalidomide produces same kind of malformations in chick as in human and rabbit embryos[20]. The dose giving the maximum deformities was 40 μg/kg body weight which corresponded to previous publications and similar to therapeutic dose used in humans (700–2000 μg/kg)[8]. Based on the previous mode of application of thalidomide, we opened the eggs through the air sac by drilling the egg shell and piercing the shell membrane with a sterile hypodermic needle and thalidomide or DMSO was added in close proximity to the embryo. At Hamburger-Hamilton (HH) stage 32 all developed embryos were dissected and scored for limb deformities. The ectromelian deformities observed were

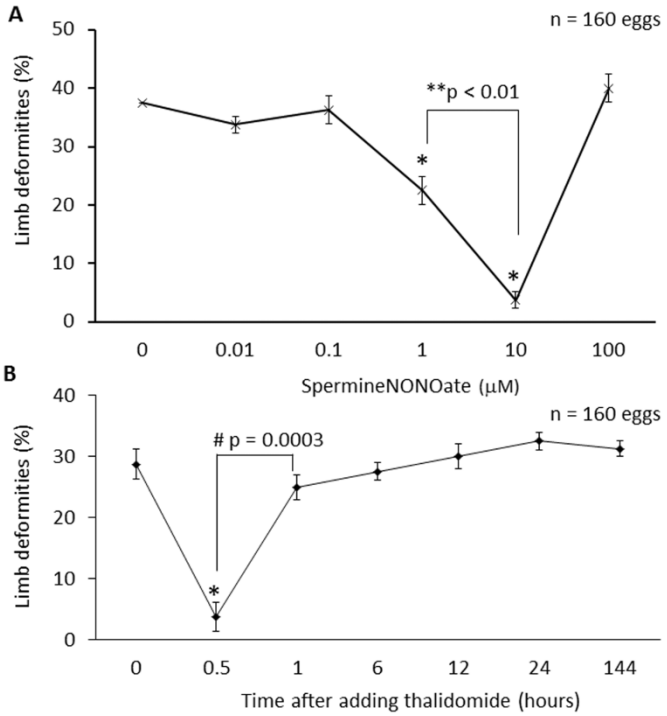

Figure 1 | Nitric oxide recovers the thalidomide teratogenic effects. (A) Chicken embryos (n=160 eggs) were treated with 40 μg of thalidomide at HH stage 8 followed by treatment with various concentrations of spNO (0.001 μM – 100 μM). An addition of 10 μM spNO after 30 min of thalidomide treatment could neutralizes the thalidomide teratogenic effect up to 94 %. **p<0.01. (B) In another set of experiments, embryos were treated with 40 μg of thalidomide followed by addition of spNO at different times (0, 0.5, 1, 6, 12, 24 and 144 h) after adding thalidomide. **p<0.05 vs vehicle control; #p<0.01 vs 1 μM.

found to be dependent on the temporal addition of thalidomide with the HH7-8 stage being the most sensitive to thalidomide assault (~30%) (n=160 eggs) (Fig. 1B). The teratogenicity gradually reduced as the embryo aged, indicating that older embryos could resist the thalidomide effect (See Supplementary Fig. S1 online). The HH7-8 period also corresponded to the blood island formation which was the primary event in vasculogenesis and angiogenesis[21]. Manipulating the system at the stage of blood island formation resulted in the drastic deformities manifested by the absence of limbs or absence of digits or abnormal digits (Fig. 2). Thalidomide effects are known to be species specific with avian and rabbit models being most sensitive while rodents being refractory to thalidomide induced deformities[12]. Avian and zebrafish models were therefore used which show 80 and 90% sequence homology to humans. The deformities induced by thalidomide were consistent in both the white (*Gallus domesticus*) and brown leghorn species of *Gallus gallus* (Fig. 2C). Zebrafish models were also used since they are transparent and their development can be easily monitored. Zebrafish assays were performed in static system where 100 zebrafish eggs from spawning fish were placed in a well of 24 well petriplate. The zebrafish embryos were given thalidomide (2 mg/animal) at 12 hpf and the zebrafish development was monitored for 2–3 days. The large dose is usually administered to ensure sufficient teratogenic amount to avoid the problems of regurgitation. Also there is a possibility that the ingested drug remains unabsorbed through the gastrointestinal tract and is excreted after one or two days of treatment. The eggs were observed to hatching and hatchability of the eggs was measured. The larval mortality and teratogenicity was also recorded. Since thalidomide primarily affects limb development, thalidomide treated zebrafish larvae appeared finless with delayed development (Fig. 2B). The DMSO or PBS control eggs did not show any abnormality or less than 1% death rate. Thalidomide induced limb and fin deformities were further visualized using alcian-blue, a cartilage stain (Fig. 2A, B).

Nitric oxide recovers thalidomide induced deformities. Thalidomide primarily affects the limbs during embryonic development. Limb bud formation is unique, requiring a complex interaction of angiogenesis and vasculogenesis[22]. The vessels in the limbs are immature compared to mature blood vessels in other parts of the body[9]. Thalidomide derivative, CPS49 destroy newly formed blood vessels, stunts the already formed vessels by preventing filopodial extensions from the endothelial tip cells[9]. In highly angiogenic tissues such as limb buds, the loss of newly formed blood vessel is highly devastating and results in limb defects. A work by Knobloch *et al.* (2007) demonstrated recovery for thalidomide induced deformities using Dkk antagonists. However the efficiency and description of the recovery was not elaborated in their studies[10]. A series of recent studies indicated that thalidomide interferes with NO signaling[23–25]. We adopted a strategy to relieve the blockage of the blood vessel and induce neovascularization by restoring the depleted NO levels in the system. Particularly endothelial nitric oxide synthase (eNOS) −/− mice had impaired angiogenesis which did not improve even after Vascular Endothelial Growth Factor (VEGF) administration[26]. NO therefore plays a pivotal role in events leading to angiogenesis. We observed that Spermine NONOate (spNO), with a half-life of 39 min, is the most efficient and stable NO donor (Unpublished data). In all the experiments, spNO, an NO donor was added externally at HH7-8 stages after 30 min of thalidomide treatment. The best dose for spNO recovery was identified as 10 μM (Fig. 1A) which corresponded to 12 μM nitrites[27]. In 900 eggs tested, thalidomide induced 50% overall mortality. spNO could reduce the overall mortality to 1% in thalidomide treated embryos. Among the embryos that survived thalidomide toxicity, spNO treated eggs showed 94% reduction in limb deformities (Fig. 1). In HH7-8 stage embryos, the intensity of NO based recovery was gradually reduced as the embryo aged. There was no significant recovery of thalidomide treated eggs after stage HH7-8. spNO mediated recovery was conserved across white leghorn, brown leghorn and zebrafish species. Addition of spNO resulted in 96% and 94% recovery in white leghorn and brown leghorn embryos (Fig. 2C) respectively. In case of zebrafish embryos, DMSO and spNO treated eggs (2 cell stage) hatched normally with well-developed pectoral fins. Among the thalidomide treated fish eggs, 45% zebrafish embryos hatched normally with well- developed pectoral fins, 35% embryos were finless, 15% embryos died before hatching and remaining were not affected by thalidomide (Fig. 2). Among the thalidomide and spNO treated fish, 9% embryos were found to be finless and 3% embryos were dead before hatching. The embryos dissected for analysis at HH32 were further stained with alcian blue. Thalidomide (Thal) affected limbs showed digit malformation (only two digits were present instead of four) whereas limbs of control, spNO and, Thal + spNO embryos appeared normal (Fig. 2A). Similarly 72 hpf zebrafish embryos were stained by alcian blue stain. DMSO control, spNO and thalidomide + spNO embryos showed normal fin formation, whereas embryo treated with thalidomide alone did not have pectoral fins (Fig. 2B).

To further verify the NO specificity, another NO donor, DEA NONOate was used along with a sulphoNONOate backbone control. In white leghorn eggs, spNO could recover deformities up to 98% whereas DEANONOate could recover up to 80% (See Supplementary Fig. S3 online). This discrepancy could be attributed to NO release pattern of NO donors. The next question was if NO recovery was global to the known teratogens; mercuric chloride and ethanol. Treatments of the embryos with mercuric chloride (50 μM) and ethanol (5%) induced deformities in 54% and 57% embryos respectively. When spNO was added to ethanol and mercury treated

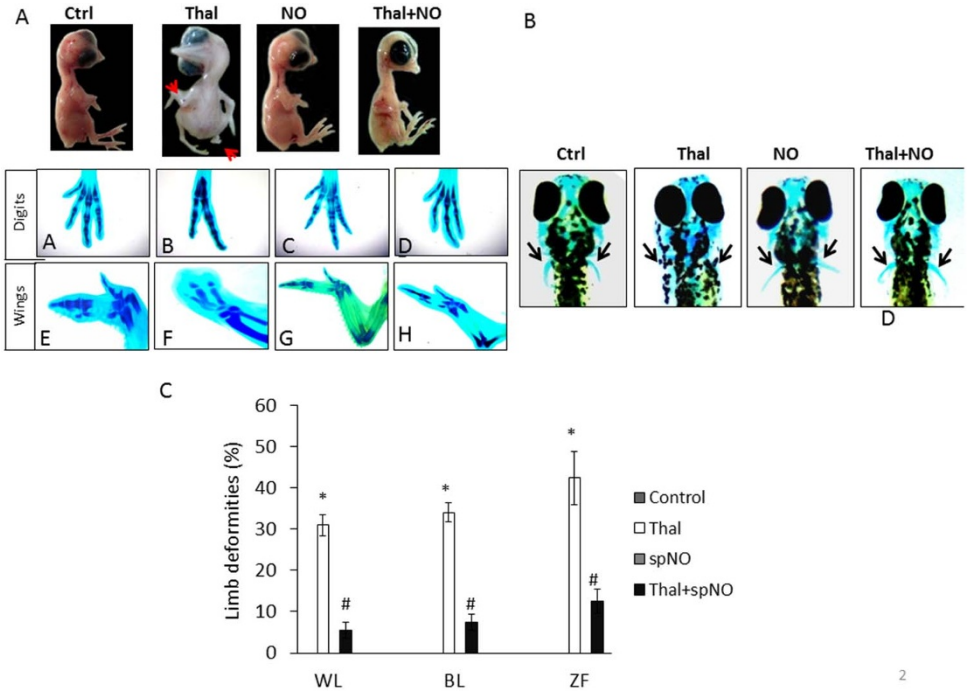

Figure 2 | Nitric oxide mediated recovery is not species specific. White leghorn (WL), Brown leghorn (BL) (HH 8) and Zebrafish (ZF) embryos (10 hpf) were treated with 40 μg thalidomide in case of chick embryos (n=50 eggs) or 2 mg/ml thalidomide in case of zebrafish embryos (n=20 fishes). SpNO (10 μM) was added after 30 min as described previously. In case of zebrafish spNO (10 μM) was added along with thalidomide in the water. Analysis of white and brown leghorn embryos at HH32 and zebrafish embryos at 72 hpf showed limb deformities and pectoral fin deformities respectively. In case of zebrafish (n=20 fishes), the deformities were scored as presence or absence of the pectoral fins. (A) Plates are representative of whole embryos with limb deformities in thalidomide, spNO and thal+spNO. (A–D) represents deformities in the number of digits in the presence or absence of treatments. (E–H) represents wing deformities as visualized using a cartilage specific, alcian blue stain. (B) Representative images of treated or untreated zebrafish embryos after 72 hpf stained with alcian blue. The arrows indicate the presence or absence of pectoral fins in the zebrafish embryo. (C) Bar graphs represent the percentage of limb deformities present in control, thal, spNO and Thal+spNO treated White Leghorn (WL), Brown Leghorn (BL) and Zebra Fish (ZF) embryos. *p <0.01 vs control; #p< 0.01 vs Thal.

embryos, there was no significant recovery of teratogenicity indicating spNO specifically rescues the thalidomide phenotype by inducing angiogenesis (See Supplementary Fig. S4 online). Ethanol and mercury chloride associated teratogenicity is attributed to increase in superoxide anion generation, lipid peroxidation, excessive cell death and dysmorphogenesis suggesting free radical damage to be the main cause of teratogenicity[28,29]. In contrast, spNO was able to recover all the abnormalities caused by the two thalidomide analogues; Pthalimide (96%) and Lenalinamide (94%) (See supplementary Fig. S5 online). The analogues of thalidomide were used to delimit the sensitivity of limb deformities to thalidomide. Lenalidomide is a 4-amino glutamyl analogue of thalidomide that lacks neurologic side effects of sedation and neuropathy is extensively used in the treatment of inflammation and malignancies[30]. Phthalimide is structurally related to thalidomide and is extensively used to treat inflammatory conditions[31]. NO based recovery of thalidomide analogues associated deformities is presumably due to common mode of action which restores angiogenesis (See supplementary Fig. S5 online).

Further to determine the role of eNOS in NO mediated recovery, Zebrafish eggs (72 hpf) (n=20) were transfected with eNOS GFP or chronically active eNOS plasmid (S1179D). Mutation of serine residue to aspartate increases NO production constitutively in the absence of agonist. We hypothesized that eNOS S1179D electroporated fish will be protected against thalidomide mediated teratogenicity. A mutant plasmid (S1179A) which did not express eNOS was also used in the experiment. eNOS GFP expression after 80 hpf was monitored under a fluorescence microscope, and the number of embryos with fins and finless were calculated. Transfection of eNOS GFP or S1179D was confirmed by isolating total RNA from the fish embryos, converting it into cDNA followed by reverse transcriptase PCR using plasmid specific primers (Fig. 3I). In another experiment, the zebrafish embryos transfected with S1179D were treated with a NO specific fluorescence dye (DAR-4M-AM) and number of fish with fins and finless were calculated. The S1179D overexpressed fish which showed DAR-4M-AM positive NO fluorescence had fins under thalidomide treatment (Fig. 3I). Exogenously added NO as well endogenous NO was able to rescue thalidomide affected embryos but role of endogenous NO in rescuing thalidomide treated phenotypes was not yet known. We used three methods of NO detection (DAR-4M-AM, DAR fluorimetry and NO electrode) to estimate the levels of endogenous NO in control, thalidomide, spNO and thal+spNO treated chick embryos. We also used NOS inhibitor L-NAME to block endogenous NO production and scored for the pectoral fins formation in zebrafish (Fig. 3J). Results from all these experiments confirm that thalidomide reduces NO levels, thal+spNO treated embryos with thalidomide restores the endogenous NO levels that links to thalidomide induced limb deformities.

Nitric oxide rescues angiogenesis. The work of D'Amato (1994) reported thalidomide causes teratogenicity by inhibiting angiogenesis[8,9]. In our experiments, thalidomide and spNO were added as described previously at HH7-8 stage. After 11 days of embryonic development the aorta was removed and about 1 mm thickness rings were cut and placed on matrigel. The aortic rings treated in this way were referred to as pre-treated aortic rings. Since thalidomide has short half-life and loses its activity within 4 h, aortic rings were removed from 11th day embryo and then treated with thalidomide and spNO for 4 h. The aortic rings treated in this way were referred to as post-treated aortic rings. The pre-treated aortic rings showed 40% reduction in the number of tubes and post-treated aorta showed 50% reduction in

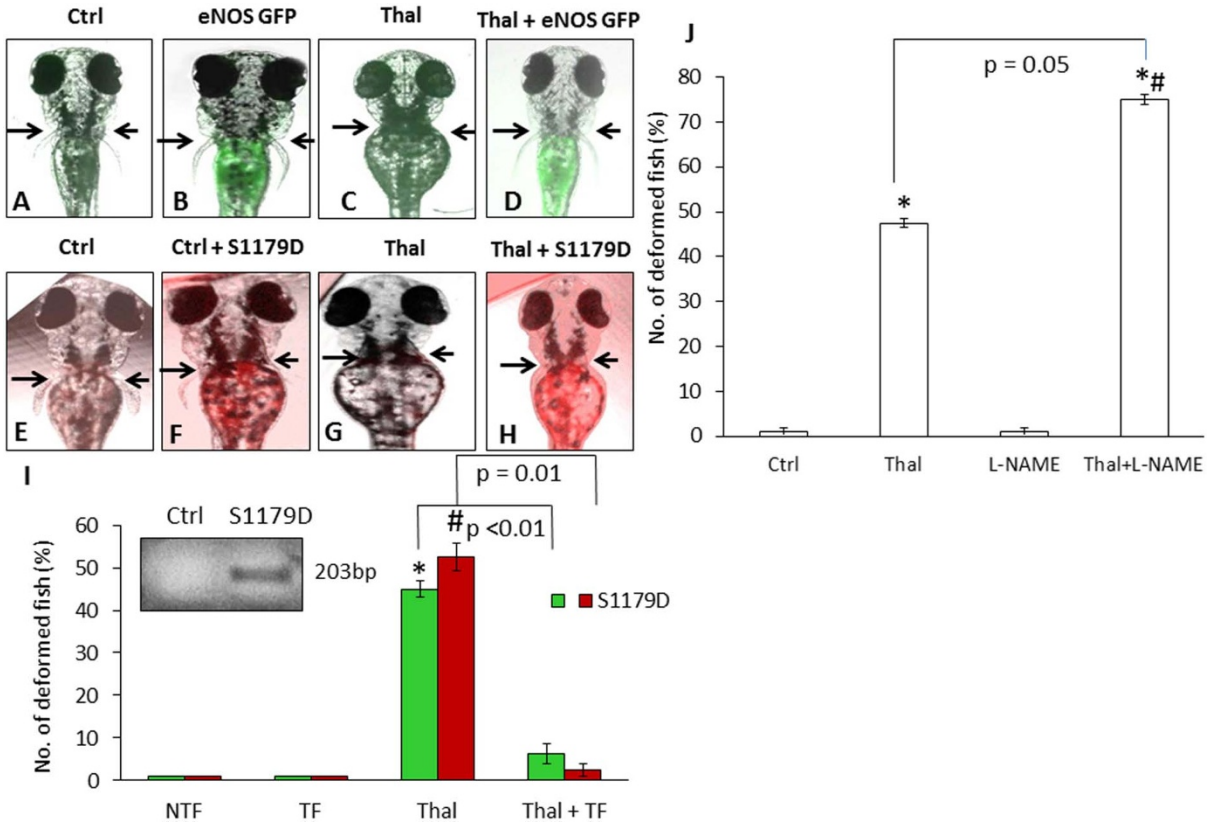

Figure 3 | Nitric oxide overexpression rescues thalidomide affected embryos. (A–D) Zebrafish eggs (2 cell stages) were electroporated with eNOS GFP plasmid or S1179D. The live embryos (10 hpf) (n=20 fishes) were grown in presence and absence of 2 mg/ml thalidomide containing water. After 80 hpf the GFP expression was observed under the fluorescence microscope. The pectoral fins abnormalities are presented in scrambled vector control, GFP alone and eNOS GFP electroporated fishes (E–H). Zebrafish eggs electroporated with S1179D were treated with NO specific dye, DAR-4M-AM. The NO production in thalidomide treated or untreated fishes were visualized with the fluorescence microscope. Arrows indicate the pectoral fins. (I) The graphs represent relative intensities of GFP expression and NO production calculated from the images using histogram analysis of image J software. NTF = Non transfected fish; TF = Transfected fish; Thal = Thalidomide treated fish; Thal+Tf = Thalidomide treated transfected fish. *p<0.01 vs eNOS GFP; #p=0.01 vs S1179D. Inset shows eNOS S1179D expression in transfected zebrafish embryos confirmed using reverse transcriptase PCR. (J) 2 cell stage zebrafish eggs were treated with eNOS inhibitor, L-NAME (10 μM). After 72 hpf the fish embryos were scored for pectoral fin abnormalities. *p<0.05 vs Thal.

the number of tubes (Fig. 4B). Addition of spNO along with thalidomide restored the tube number (Fig. 4B). This data corroborated with intensity of NO production measured with DAR-4M-AM. The numbers of sprouts were more in NO positive tubes. The highest numbers of tubes were observed in spNO treated aortic rings. Thalidomide treatment reduced the number of tubes and decreased NO level in the aortic rings. In thal + spNO treated aortic rings, the tubes were restored along with an increase in NO production which further fortifies the concept that thalidomide affects angiogenesis primarily by targeting NO production. Thalidomide specifically targets early vasculogenesis and limb development occurs later contrary to the report by Ito et al.(2010)[32]. Restoring the NO levels, rescues the defective angiogenesis in thalidomide treated aorta (Fig. 4). Therefore angiogenesis precedes apoptosis in limb cells as observed by Therapontos (2009)[9]. At the molecular level, there was a significant reduction in expression of angiogenesis markers such as VEGFR2 and Ang 1 in thalidomide treated endothelial cells, and not in spNO or Thalidomide + spNO treated endothelial cells as detected using Reverse Transcriptase PCR (RT-PCR) (Fig. 4D). From our study it is apparent that thalidomide blocks early vasculogenesis and angiogenesis while NO restores early vasculogenesis and angiogenesis, thereby protecting embryo from thalidomide effects.

Nitric oxide prevents thalidomide induced endothelial apoptosis. Our study demonstrated that thalidomide induces apoptosis in limb buds and spNO restricted thalidomide induced apoptosis in limb buds as visualized by Annexin V and propidium iodide staining (Fig. 5B,C). This was also confirmed in cultured limb cells isolated from limbs of the embryo at stage HH17-18 (Fig. 5A). Thalidomide also induced apoptosis in endothelial cells, and addition of NO to thalidomide treated cells could reduce apoptotic death of the cells. NO is a known potent inhibitor of apoptosis in endothelial cells[33] besides human B lymphocytes, splenocytes, eosinophils, ovarian follicles and cardiac myocytes[34] NO inhibited thalidomide induced apoptosis in aortic rings isolated from chick embryo (Fig. 5A). Specifically NO inhibits apoptosis through an S-nitrosylation based inhibition of the catalytic activity of caspase-3 enzymes or a cGMP dependent mechanism upstream of caspase-3 activation[35]. Our study links thalidomide induced apoptosis and caspase-3 activation (Fig. 5, Fig 6A and See Supplementary Fig. S6 online). To determine if NO could modify protease activity, endothelial cells were incubated with thalidomide for 4 h, followed by colorimetric assay based on the cleavage of caspase-3 inhibitor peptide, AcDEVD-pNA. NO was able to recover caspase-3 dependent apoptosis as evidenced by the caspase-3 expression and caspase-3 activity assay (Fig. 6A and See supplementary Fig. S6 online). Further localization of cleaved caspase-3 in de-paraffinized sections of formalin-fixed limb bud showed 60% of apoptotic cells in thalidomide treated limb bud, but 40% in thal + spNO treated limb bud (See supplementary Fig. S6 online). Thalidomide induces apoptosis, leading to regression of

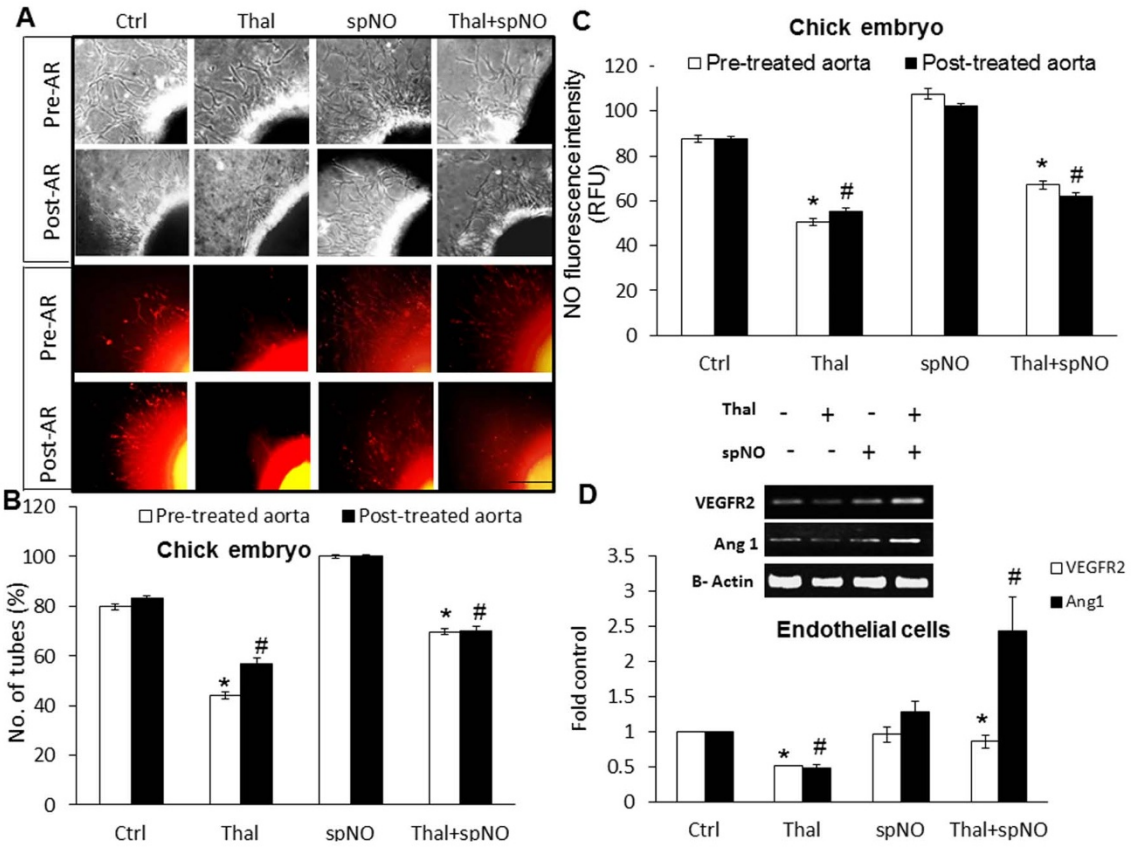

Figure 4 | Nitric oxide recovers angiogenesis. (A) Representative bright field and DAR images of pre- and post-treated aortic rings. (B) The bright field images were used to calculate the number of tubes after various treatments (n=5). *p>0.01 vs control; # p>0.01 vs Thal. (C) Fluorescence intensity of aortic rings calculated following incubation with DAR-4M-AM. *p>0.01 vs control; # p>0.01 vs Thal. (D) Embryos were treated with 40 μg thalidomide followed by treatment with spNO (10 μM). Total RNA was harvested from HH17-18 embryo and evaluated for angiogenic genes expression profile. cDNA was primed with avian specific VEGFR2 and Ang1 primers. As an internal control, β-actin mRNA was measured in parallel. Expression of VEGFR2 and Ang1 was analyzed for all treatments. Densitometric analysis of blots relative to β-actin expression. *p <0.05 vs control; #p<0.01 vs Thal.

angiogenesis by blocking migration of endothelial cells (Fig. 5D). In our study NO restored endothelial cell migration under thalidomide treatment specifically by suppressing caspase-3 activation (Fig. 5D). NO based recovery of apoptosis was seen in both aorta and limb cells, however there was 68% and 30% apoptotic cells in aorta and limb cells treated with thalidomide for 4 h, thereby exemplifying the precedence of angiogenesis over limb apoptosis (Fig. 5). The results hint that endothelial cells are more vulnerable to thalidomide than limb cells. Our study confirms that HH stage 7–8 is most vulnerable to thalidomide assaults and HH stage 7–8 corresponds to blood island formation that will eventually fuse to form the capillary network and promote early vasculogenesis. We postulate that NO recovers apoptotic endothelial cell by reducing caspase-3 expression and activity in endothelial cells (Fig. 6A). Thereby NO restores vasculogenesis in thalidomide treated embryos.

Nitric oxide and catalase reduces limb and wing deformities. Other reports suggest that thalidomide affects the limb outgrowth by inducing oxidative stress in the limb mesenchyme and inhibiting angiogenesis[12]. Of all the reactive oxygen species evaluated namely superoxide, peroxynitrite and hydrogen peroxide, the levels of hydrogen peroxide was maximum (Fig. 6B–D). Further hydrogen peroxide expression was higher in aortic rings pre-treated and post-treated with thalidomide respectively (Fig. 6C). The levels of H_2O_2 were reduced in spNO and thal+spNO treated aortic rings (Fig. 6C). An evolutionarily conserved group of transcription factors called FOXO have been associated with resistance to oxidative stress[36]. The three mammalian orthologs of FOXO are FOXO1, FOXO3a and FOXO4 of which FOXO3 and FOXO1 have been shown to regulate the catalase protein[37]. In our study we observed the modulation of FOXO1, FOXO2 and FOXO3 in the presence of thalidomide and recovery of that with spNO (Fig. 6A). Moreover catalase added externally to avian embryo and zebrafish embryo was able to prevent apoptosis in avian limb bud and reduced the number of thalidomide induced deformity in zebrafish embryos (Fig. 7). We conclude that NO recovers thalidomide effects by inducing angiogenesis and blocking H_2O_2 linked apoptosis in avian and zebrafish embryos.

Discussions

Thalidomide is implicated in different signaling pathways, which collectively define the growth and development of the embryo. Therefore, the fundamental query is which of the biological events to target to rescue the embryo from thalidomide effects? Recently the target for thalidomide binding was found to be cerebron or CRBN[32]. Further, the work of Jurgen et al. (2007) demonstrated that perturbing of BMP/Dkk1/Wnt signaling is central to the teratogenic effects of thalidomide and blocking of Dkk1 or GSK3ß counteracts thalidomide-induced limb truncations and microphthalmia when specific inhibitors were applied at HH stages 17–19 to thalidomide-treated embryos[10]. However, inhibitor application at later stages (HH stages 22–24) could not prevent limb deformities that support our observation that NO application at very early stage at HH 7–8 was maximally effective. The present work demonstrates clearly that thalidomide blocks vasculogenesis and induces apoptosis in endothelium and limb tissues. NO may act like a sink, absorbing the excess oxygen

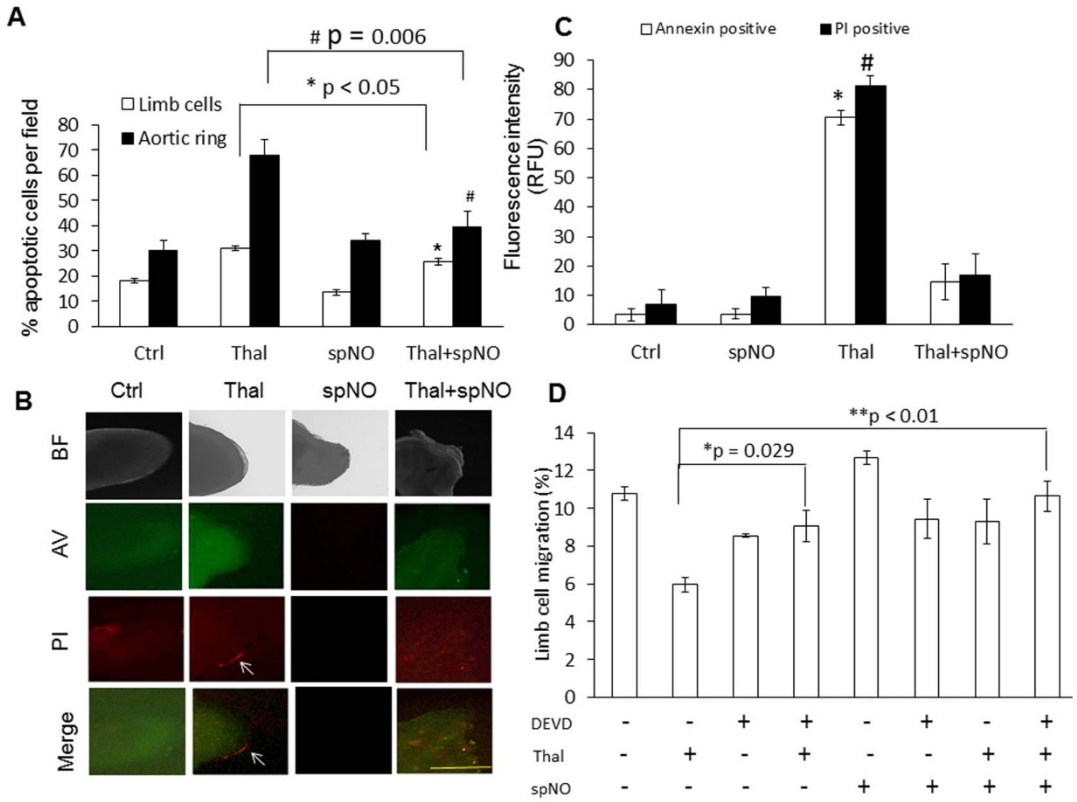

Figure 5 | Nitric oxide inhibits thalidomide induced caspase-3 dependant apoptosis. (A–C) Endothelial cells or limb cells or chick aorta or limb buds dissected from a 6th day old (HH17-18) embryo were treated with thalidomide (40 μM) for 4 h spNO (10 μM) was added 5 min after thalidomide treatment. Annexin V and propidium iodide was used to check apoptosis. Apoptotic cells per field (n=100 fields) were counted from the images taken using a fluorescence microscope. *p<0.05 vs control; #p<0.01 vs Thal. (D) Migration of the limb cells was assayed using scrape wound healing assay. Confluent limb cells were treated with caspase-3 inhibitor DEVD (10 nM) or thalidomide or spNO. A wound was created using a sterile pipette tip and difference in area of the wound was measured before and after 4 h. *p = 0.029 vs Thal; *p<0.01.

radicals produced by thalidomide thereby preventing oxidative damage and limb deformities.

Our study does not rule out other possible mechanisms of NO mediated recovery of limb deformities. For instance effects of NO on BMP and FGF8 expression in the apical ectodermal ridge (AER) and other controls of developmental programs are still not clear. Oxidative stress due to thalidomide is known to down regulate essential limb growth signaling by targeting a redox sensitive transcription factor, NF kappaB[12]. Aberrant NF kappaB activity in turn attenuates FGF8 and FGF10 expression resulting in limb deformities[12]. Cross-talk between NO pathway and NF kappaB pathway remains yet to be investigated. NO carries out its functions by two major signaling pathways; activating sGC (soluble guanylate cyclase)-cGMP and sGC-cGMP independent pathways such as direct proteins modifications in the cells[38]. Further work has to be done to dissect the NO downstream signaling in protecting embryo health under thalidomide exposure. A work by Wang et al. (2009) has shown that NO donating thalidomide analogues could retain immune-modulation property and anti-cancer activities of thalidomide in a more balanced manner[39]. Similarly NO donating thalidomide analogues could also be developed to protect vulnerable embryos and fetus from thalidomide and its analogs. We also anticipate further works in this direction would enable us to use NO and its derivatives as therapeutic or preventive measures for other thalidomide like teratogens as well.

Methods

Treatment of chicken embryo. Fertilized white and brown leghorn chicken eggs were purchased from government poultry station, Potheri, Chennai and incubated in sterile humidified incubator at 37°C. The chick embryos were staged according to Hamilton and Hamburger (HH) stages of chick embryo as described elsewhere[40]. All experimental manipulations in chick embryos were performed between HH stage 4-HH stage 37.

Drug administration. Thalidomide (99% purity) (Sigma-Aldrich Chemical Co, St.Louis, MO) was dissolved in 200 μl DMSO and the volume made up to 1 ml using 1X PBS (1 mg/ml) as described previously[10]. 40 μl of 1 mg/ml was applied to each chick embryo through a hole in the air sac made using a sterile needle. The drug was administered as a single dose at HH stage 7- HH stage 8 for all the experiments except those mentioned otherwise. Thalidomide analogs; Pthalimide, Lenalidomide (40 μg/ml) and teratogens; mercuric chloride (50 μM) and ethanol (5%) were also applied to chick embryos at HH stage 8 through the air sac. Similarly Spermine NONOate (spNO) (Cayman Chemicals, Michigan) was added after 30 min of treatments with thalidomide or thalidomide analogs or teratogens. All treated embryos were dissected at 11th day and the embryos were examined to determine the drugs effect on development and the images were taken by using canon 10 X optical zoom camera. All the procedures performed were approved by the institutional bioethics committee.

Treatment of zebrafish embryo. Zebrafish pairs (male and female) were bought from Ganesh aqua firm, Chennai. Zebrafish were maintained in our lab according to standard conditions[41]. Fishes were bred at 28.5°C on a 14 h light/10 h dark cycle and naturally allowed to mate. Fertilized zebrafish eggs were collected from the bottom of the fish tank at the 1–2 cell stage or were allowed to develop in normal water and staged according to hour post fertilization (hpf)[42].

Drug administration. Thalidomide preparation and treatment to zebrafish embryos was performed as described previously[32]. Briefly, 10 hpf dechorionated zebrafish embryos were grown in the presence or absence of 2 mg/ml thalidomide, 10 μM spNO, 200 μM L-NAME or 30 Units of catalase containing water up to 72 hpf. The water was replaced every 12 h with freshly prepared thalidomide and spNO.

Plasmid and primers. pcDNA-eNOS S1179A (Plasmid #22485), pcDNA-eNOS S1179D (Plasmid #22484), pcDNA-eNOS GFP (Plasmid #22444), constructs were purchased from Addgene deposited by Prof.William Sessa's Lab. The total RNA was extracted from pooled 10 zebrafish embryos using a spin column RNA extraction kit (Medox, Chennai). The total RNA was converted into cDNA using a Mulv Reverse

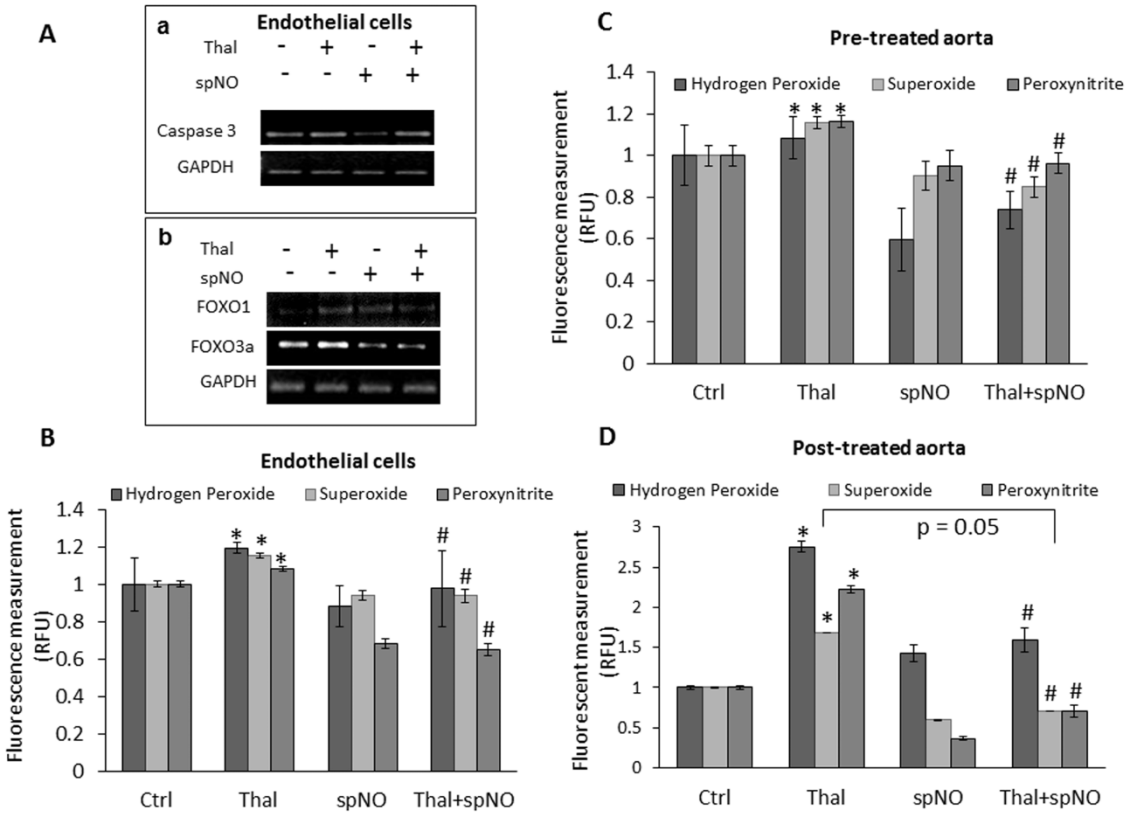

Figure 6 | Nitric oxide restricted the production of thalidomide induced reactive oxygen species (ROS). Endothelial cells were treated as described before. Total RNA was by isolated from the cells and reverse transcribed to cDNA using reverse transcriptase enzyme cDNA was amplified with sense and anti sense primers of caspase-3, FOXO1, FOXO3a and GAPDH. (B–D) Eahy926 cells or chick aorta were pre-treated and post treated with thalidomide, spNO or the combination for 4 h. Reactive oxygen species such as hydrogen peroxide, superoxide and peroxynitrite levels were estimated using amplex red, nitroblue tetrazolium and HPF assays respectively. Each of the assays were performed independently and normalized with respect to control values. *p<0.01 vs control; #p<0.01 vs Thal.

transcriptase enzyme (Fermentas, Maryland). The primers that were used to check the plasmid expression in control and treated zebrafish are given in table 1.

Cell lines and cell culture conditions. *Limb cells.* Limb cells were isolated from pooled wing and hind limbs of stages HH23-24 embryos as described elsewhere[10]. The cells were cultured in DMEM media supplemented with 10% FBS, 1% penicillin-streptomycin and 2% L-Glutamine and maintained in 5% CO_2 humidified incubator at 37°C.

Endothelial cell. Eahy926, an immortalized endothelial hybrid cell line was obtained from Tissue Culture Facility (TCF) at the UNC Lineberger Comprehensive Care Center, The University of North Carolina at Chapel Hill. The cells were cultured in DMEM media supplemented with 10% FBS, 1% penicillin-streptomycin and maintained in 5% CO_2 humidified incubator at 37°C .

Alcian blue staining. Alcian blue cartilage staining of chick and zebrafish embryos was performed as described earlier[32]. Briefly, at HH stage 33 chick embryos were removed from the egg shell and yolk removed completely by repeated washes with 1X PBS. Zebrafish embryos (72 hpf) were also removed from the medium and washed repeatedly with water. Chick and zebrafish embryos were fixed in Bouin's solution for 2 h at room temperature. Then 6 to 8 (70% ethanol + 0.1% NH_4OH) washes given to the embryos for 24 h followed by 5% acetic acid washes twice for 1 h. Then embryos were stained 0.05% Alcian blue 8GX in 5% acetic acid for 2 h. The embryos were washed again twice for 1 h with 5% acetic acid followed by clearing in 100% methanol twice for 1 h. Finally the embryos were transferred to 2 : 1 benzyl benzoate: benzyl alcohol solution. Images were taken by Olympus camera attached to the stereo microscope.

Electroporation. Electroporation was carried out in one cell stage zebrafish embryos as described previously[41]. Briefly, electroporation cuvettes were filled with media containing 30–40 viable 1 hpf zebrafish eggs. 5 µl of plasmid DNA (10 µg) such as, e-NOS GFP, S1179D or S1179A were added into individual cuvette in tissue culture hood. Electric pulses were given at 360 W to each cuvette using an electroporator (Electro Cell Manipulator, ECM 600). After electroporation, only viable embryos were separated and grown in freshly prepared medium. At 12 hpf onwards these embryos were grown in presence and absence of 2 mg/ml thalidomide containing medium up to 84 hpf. Every 12 h new medium was replaced with freshly prepared thalidomide. eNOS GFP expression was monitored in a fluorescence microscope at 84 hpf.

Chick aortic ring assay. Aortic rings were made from aortic arch of 11th day old chick embryos[43]. Chick embryos were removed from the eggs under sterile conditions,. The aortic arches were cut from the heart using a sterile glass cover slip and washed several times in 1X PBS containing 1% penicillin/streptomycin. 10 µL of matrigel was placed on the coverslips and allowed to solidify for 30 min at 37°C in a CO_2 incubator. Aortic arches were cut into small rings of similar size and placed on the top of the matrigel. The rings were treated and the endothelial tubes were analyzed after 36 h of incubation. The endothelial cell sprouting was confirmed using CD34 antibody. Number of tubes was counted manually as a double-blind study. Images were taken in 20X magnification using Olympus microscope.

(i) Pre-treatment. Two different chick aortic ring experiments, pre-treated and post-treated, were performed to analyze the effect of thalidomide on tubes from aortic ring. For pre-treatment, a small hole was created at stage HH7-8 of the embryo using a sterile needle and treated with thalidomide (40 µg/ml), spNO (10 µM), thal+spNO. After treatment, the egg shell was covered using a sterile paper. Finally, the eggs were broken on 11th day and the assay was performed as described earlier.

(ii) Post- treatment. Untreated 11th day eggs were broken and chick aorta were dissected from the embryo. Aortic rings were placed in matrigel and all treatments were provided in the media for 4 h. The images and the results were analyzed after 36 h. All the experiments were performed under sterile conditions. For both pre- and post-treatment, aortic ring without treatment is referred to as control. Five different chick embryos were used for the experiment. (NOTE: Pre-treatment was given in the air sac at HH 7–8 as described previously, whereas post-treatment was provided in the aortic ring placed in the media).

Nitric oxide fluorescence imaging using DAR-4M-AM.

(i) Chick Aortic rings
(ii) Chick Limb bud
(iii) Electroporated zebrafish

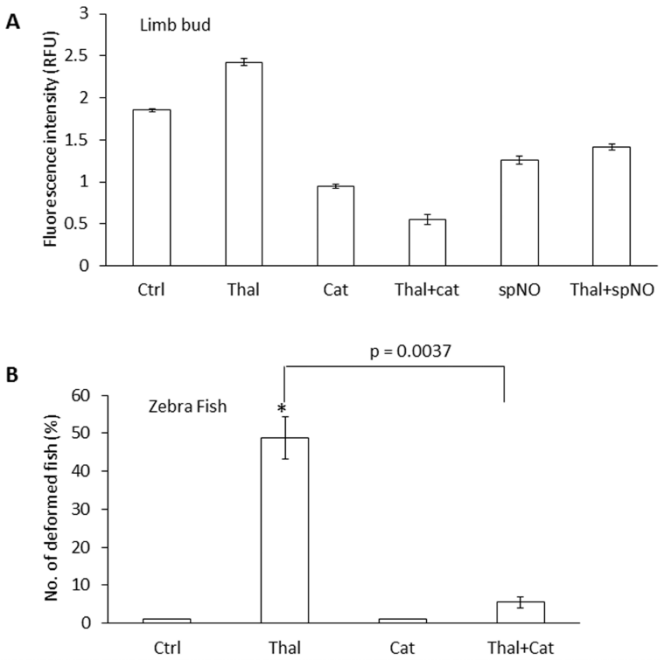

Figure 7 | Catalase protects avian and zebrafish embryos from thalidomide teratogenicity. (A) Chick limb buds from chick embryo (n=50 eggs) were removed at stage HH17-18 aseptically and treated with thalidomide or spNO or catalase (30 Units/ml) for 4 h. The limb buds were then stained with Annexin V and propidium iodide to check apoptosis. *p<0.01vs Thal. (B) 10 hpf zebrafish (n=20 fish embryos) embryos were treated with thalidomide (2 mg/ml) or catalase (30 Units/ml) up to 72 hpf. #p=0.0037 vs Thal.

The chick aortic rings (n=12) were prepared as described earlier. After 36 h of incubation and sprouting of tubes, the chick aortic rings were incubated with the nitric oxide specific fluorescence indicator DAR-4M-AM (diaminorhodamine) (2 μM) for 10 min. Then the rings were gently washed using 1 X PBS and images were taken in 20 X magnification using fluorescence microscope. Similarly the chick limb buds were isolated from 6th day embryo treated with thalidomide or spNO or thal+spNO and placed in a PBS buffer containing DAR-4M-AM for 20 min. The zebrafish eggs were collected as described previously and electroporated with or without S1179D. The aortic ring tissue or limb bud or electroporated zebrafish was then images using a Olympus XL70 fluorescence microscope at 40 X magnification. The fluorescence intensity of the DAR images was calculated using Adobe Photoshop of version 7.0.

DAF-Fluorimetry. Chick limb buds were isolated from 6th day embryo treated with thalidomide or spNO or thal+spNO and placed in a PBS buffer containing DAF-FM (10 μM) for 15 min. The buffer was then read in a 96 well plate in a fluorimetry at an excitation/emission wavelength of 488/519 nm respectively.

NO electrode. Chick embryos were treated with thalidomide or spNO or thal+spNO at HH7-8 as described previously. NO released by the embryo was measured using a sensitive NO electrode (WPI). NO electrode was calibrated overnight in 1 X PBS (pH 7.4) till the current stabilized. NO electrode (2 mm) was placed about 1 mm above the embryo and NO measurement recorded for 20 min.

Semi-quantitative reverse transcriptase PCR. Total RNA from six day incubated chick embryos and 4 h incubated endothelial cells (EaHy926) following treatments were prepared using a Total RNA Isolation Kit (Medox Inc). The total RNA was quantified and normalized according to the band intensities. Approximately 200 ng of total RNA was used for reverse transcription and gene-specific amplification using the primer pairs shown in Table S1. β-actin was used as internal control. The annealing conditions and the product size are summarized in the Table 1. The products were resolved on a 1% agarose gel at 80 V using an agarose gel electrophoresis system.

Annexin- Propidium iodide staining. Limb buds were dissected from the chicken embryo on HH18 stage and placed in HEPES buffer (pH 7.4). Aortic rings were prepared from the chicken aorta as described previously. Endothelial cells (Eahy926) or limb bud or aortic rings were treated with thalidomide (40 μg) or spNO (10μM) or thal +spNO for 4 h. After treatment they were washed with 1X PBS (x 3 times). A binding buffer was added for 30 min followed by staining with Annexin V or propidium iodide using the manufacture's protocol (ABCAM). Fluorescent images were captured in blue and green filters respectively using Olympus XL70 fluorescent microscope. The intensities were quantified using the histogram analysis of adobe photoshop version 7.0.

Caspase-3 activity. Endothelial cells (EaHy926) were treated as described before. Cell lysates were prepared with lysis buffer (50 mM HEPES, 5 mM Triton X 100, 5 mM DTT, pH 7.5). Cell lysate was incubated with a p-Nitroaniline (2 mM) conjugated DEVD substrate specific for caspase-3 for 1 h. Reactions were then measured for substrate cleavage by absorbance at 405 nm.

Immunohistochemistry. Chick limbs were dissected out from control and treated (Thalidomide, spNO, Thal + spNO) embryos. The limbs were embedded in paraffin blocks were sectioned at 5 μM for immunohistochemical analysis. Briefly, the sections were pre-treated with 10 mM sodium citrate (pH 6.0) for 10 min at 95°C. The sections were then incubated with 3% hydrogen peroxide in Tris-Buffered Saline (TBS) for 10 min at room temperature to block endogenous peroxidase followed by incubation with blocking buffer (0.5% BSA, 0.3% triton X-100, 1% horse serum, 1% DMSO in 1 X PBS) for 1 h at room temperature to inhibit non-specific binding. The sections were then incubated overnight at 4°C with caspase-3 antibody (Cell signaling) in a moist chamber. The next day, sections were washed thrice with TBS-Tween20 (10 min at room temperature per wash) before being incubated with goat anti-rabbit IgG secondary antibody (Bangalore Genei, India) for 2 h at room temperature in a moist chamber. The sections were then washed. 3,3′-diaminobenzidine (DAB) was prepared and used as per manufactures instructions (Bangalore genei, India) on the sections, which were then counterstained using haematoxylin. Images were taken with the help of a bright field microscope at 200X and analyzed for caspase-3 positively stained cells.

ROS estimation in chick aortic arches and in endothelial cells. Three different ROS parameters, includes hydrogen peroxide, superoxide and peroxynitrite, were performed in both chick aorta and in endothelial cells. For all three experiments, chick

Table 1 | Semi-quantitative RT-PCR primers

Gene of Interest	Product size	Annealing Temperature	Primer Sequence
eNOS S1179D	203bp	63.4°C	Sense- 5′ GAGGATCGTTTCGCATGATT 3′ Anti-Sense – 5′ CTCGTCCTGCAGTTCATTC 3′
VEGFR2	218bp	65.4°C	Sense- 5′ CACCATGGTCTGTTCCAGTG 3′ Anti-Sense – 5′ CCATGGCTGCAGTCTCTGTA 3′
Angiopoetin1	546bp	58°C	Sense- 5′ TGGAACATGTGATGGAAAAC 3′ Anti-Sense –5′CCTTAGAACAGAGGGTAATTAGAG 3′
Caspase-3	262bp	55°C	Sense-5′ CAA ACT TTT TCA GAG GGG ATC G 3′ Anti-Sense –5′GCA TAC TGT TTC AGC ATG GCA 3′
FOXO1	325bp	59°C	Sense- 5′ GCAGATCTACGAGTGGATGGTC 3′ Anti-Sense –5′AAACTGTGATCCAGGGCTGTC 3′
FOXO3a	438bp	59°C	Sense-5′ CTTCAAGGATAAGGGCGACAG 3′ Anti-Sense –5′TCGTCCTGGACTTCATCCAAC 3′
β-Actin	162bp	60.5°C	Sense- 5′ TCTGACTGACCGCGTTACTC 3′ Anti-Sense – 5′ CCATCACACCCTGATGTCTG 3′
GAPDH	510bp	58°C	Sense- 5′ TCAACGGATTTGGTCGTATT 3′ Anti-Sense –5′ CTGTGGTCATGAGTCCTTCC 3′

aorta and endothelial cells were treated with spNO, thalidomide (with and without spNO) for 4 h. ROS analysis was performed in pre-treated, post-treated chick aorta and endothelial cells. Cells or tissue without treatment were considered as control.

(i) Chick Aorta. Briefly, chick aortic arches were surgically removed and washed several times in 1X PBS. The chick aortic arches were incubated with spNO, thalidomide (with and without spNO) for 4 h in case of pre-treated aorta or added at HH37 stage of the embryo in case of post treated aorta as mentioned in detail under chick aortic ring assay. After treatment, the chick aortas were smashed using sterile homogenizer and the ROS fluorescence probes were added to the lysate. No additional treatment was given to pre-treated chick aortas. Aortic arches from three different chick embryos were used for the experiments.

(ii) Endothelial cells. Eahy926 cells were grown overnight in 12-well plates. The next day, cells were treated with the above said treatments and incubated for 4 h followed by the ROS assays as follows.

H_2O_2 detection using amplex red. Amplex red (10 μM) and HRP (0.5 U) was added to both post-treated chick aorta and endothelial cells and further incubated for 15 min. The supernatant measurements were recorded using fluorimetry of 563/587 nm excitation/emission.

Peroxynitrite measurement using HPF. Peroxynitrite measurement was analysed using a peroxynitrite specific fluorescent probe HPF (10 μM). Both the treated chick aortas and endothelial cells were incubated with HPF for 30 min. The supernatant readings were measured using fluorimetry of 495/520 nm (excitation/emission).

Determination of superoxide using NBT. After treatment (as mentioned earlier), both the chick aorta and the endothelial cells were incubated with NBT (1 mg/ml) for 2 h. Then the purple crystals were dissolved using DMSO and the supernatant readings were measured using colorimetry of 540 nm.

Statistical analyses. All the experiments were performed in quadruplets (n=4) unless mentioned otherwise. The data are presented as mean ± SE of 4 independent experiments. The data were analyzed using one way ANOVA, student *t*-test, Mann-Whitney U and Turkey post hoc test as appropriate. P values less than or equal to 0.05 were used as a criterion for a statistically significant difference.

1. Gordon, J. N. & Goggin, P. M. Thalidomide and its derivatives: emerging from the wilderness. *Postgrad Med J* **79**(929), 127–132 (2003).
2. Mc Bride. Thalidomide and congenital abnormalities. *Lancet* **ii**, 1358 (1961).
3. Lenz, W. Fragenaus der Praxis: kindliche Missbildungennach Medikament Einnahmewahrend der Graviditat? *Dtsch Med Wochenschr.* **86**, 2555–2556 (1961).
4. Further, S. J. Observation with thalidomide in lepra reactions. *Lepr Rev*, **36**, 183–187 (1965).
5. Sampaio, E. P. *et al.* Thalidomide selectively inhibits tumor necrosis factor alpha production by stimulated human monocytes. *J Exp Med* **173**(3), 699–703 (1991).
6. Wu, J. J. *et al.* Thalidomide: dermatological indications, mechanisms of action and side-effects. *Br J Dermatol* **153**(2), 254–273 (2005).
7. Locker, D. Superstine, E, Sulman, F.G. The mechanism of the push and pull principle VIII: endocrine effects of thalidomide and its analogues. *Arch. Int. Pharmacodyn. Ther*, **194**, 39–55 (1971).
8. D'Amato, R. J., Loughnan, M. S., Flynn, E. & Folkman, J. Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci U S A* **91**(9), 4082–4085 (1994).
9. Therapontos, C. *et al.* Thalidomide induces limb defects by preventing angiogenic outgrowth during early limb formation. *Proc Natl Acad Sci U S A* **106**(21), 8573–8579 (2009).
10. Knobloch, J., Shaughnessy, J. D., Jr. & Ruther, U. Thalidomide induces limb deformities by perturbing the Bmp/Dkk1/Wnt signaling pathway. *FASEB J* **21**, 1410–1421 (2007).
11. Lee, C. J., Goncalves, L. L. & Wells, P. G. Embryopathic effects of thalidomide and its hydrolysis products in rabbit embryo culture: evidence for a prostaglandin H synthase (PHS)-dependent, reactive oxygen species (ROS)-mediated mechanism. *FASEB J* **25**, 2468–2483 (2011).
12. Hansen, J. M. & Harris, C. A novel hypothesis for thalidomide-induced limb teratogenesis: redox misregulation of the NF-kappaB pathway. *Antioxid Redox Signal* **6**, 1–14 (2004).
13. Cooke, J. P. & Losordo, D. W. Nitric oxide and angiogenesis. *Circulation* **105**(18), 2133–2135 (2002).
14. Nath, A. K. *et al.* Nitric oxide modulates murine yolk sac vasculogenesis and rescues glucose induced vasculopathy. *Development* **131**(10), 2485–2496 (2004).
15. Badamtseren, B. *et al.* Thalidomide inhibits interferon-gamma-mediated nitric oxide production in mouse vascular endothelial cells. *Cell Immunol* **270**, 19–24 (2011).
16. Tamilarasan, K. P. *et al.* Thalidomide attenuates nitric oxide mediated angiogenesis by blocking migration of endothelial cells. *BMC Cell Biol* **7**, 17 (2006).
17. Majumder, S. *et al.* Thalidomide attenuates nitric oxide-driven angiogenesis by interacting with soluble guanylyl cyclase. *Br J Pharmacol* **158**, 1720–1734 (2009).
18. Kemper, F. Thalidomide and development of the chick embryo. *Arzneimittelforschung* **12**, 640–641 (1962a)
19. Jurand, A. Early changes in limb buds of chick embryos after thalidomide treatment. *J Embryol Exp Morphol* **16**(2), 289–300 (1966).
20. Salzgeber, B. & Salaun, J. Malformations of extremities obtained in the chick embryo after treatment by thalidomide. *C.r. hebd. Seanc. Acad. Sci.* **256**, 2719–2722 (1963).
21. Weng, W., Sukowati, E. W. & Sheng, G. On hemangioblasts in chicken. *PLoS One* **2**(11), e1228 (2007).
22. Seifert, R., Zhao, B. & Christ, B. Cytokinetic studies on the aortic endothelium and limb bud vascularization in avian embryos. *Anat Embryol (Berl)* **186**, 601–610 (1992).
23. Tweedie, D. Frankola, K. A., Luo, Y. Li. & Greig, N. H. Thalidomide Analogues Suppress Lipopolysaccharide-Induced Synthesis of TNF-alpha and Nitrite, an Intermediate of Nitric Oxide, in a Cellular Model of Inflammation. *Open Biochem J* **5**, 37–44 (2011).
24. Rafiee, P., Stein, J. D., Nelson, V. M. *et al.* Thalidomide inhibits inflammatory and angiogenic activation of human intestinal microvascular endothelial cells (HIMEC). *Am J Physiol Gastrointest Liver Physiol* **298**, G167–G176 (2010).
25. Noman, A. S. *et al.* Thalidomide inhibits lipopolysaccharide-induced nitric oxide production and prevents lipopolysaccharide-mediated lethality in mice. *FEMS Immunol Med Microbiol* **56**, 204–211 (2009).
26. Murohara, T. *et al.* Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* **101**(11), 2567–2578 (1998).
27. Homer, K. & Wanstall, J. In vitro comparison of two NONOates (novel nitric oxide donors) on rat pulmonary arteries. *Eur J Pharmacol* **356**, 49–57 (1998).
28. Kotch, L. E., Chen, S. Y. & Sulik, K. K. Ethanol-induced teratogenesis: free radical damage as a possible mechanism. *Teratology* **52**, 128–136 (1995).
29. Crespo-Lopez, M. E. *et al.* Mercury and human genotoxicity: critical considerations and possible molecular mechanisms. *Pharmacol Res* **60**, 212–220 (2009).
30. Kotla, V. *et al.* Mechanism of action of lenalidomide in hematological malignancies. *J Hematol Oncol* 2–36 (2009).
31. Lima, L. M. *et al.* Synthesis and anti-inflammatory activity of phthalimide derivatives, designed as new thalidomide analogues. *Bioorg Med Chem* **10**, 3067–3073 (2002).
32. Ito, T. *et al.* Identification of a primary target of thalidomide teratogenicity. *Science* **327**(5971), 1345–1350 (2010).
33. Dimmeler, S., Haendeler, J., Nehls, M. & Zeiher, A. M. Suppression of apoptosis by nitric oxide via inhibition of interleukin-1beta-converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J Exp Med* **185**, 601–608 (1997).
34. Figueroa, S. *et al.* SNAP, a NO donor, induces cellular protection only when cortical neurons are submitted to some aggression process. *Brain Res* **1034**(1–2), 25–33 (2005).
35. Kim, Y. M., Talanian, R. V. & Billiar, T. R. Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J Biol Chem* **272**, 31138–31148 (1997).
36. Giannakou, M. E. *et al.* Long-lived Drosophila with overexpressed dFOXO in adult fat body. *Science* **305**(5682), 361 (2004).
37. Venkatesan, B. *et al.* Downregulation of catalase by reactive oxygen species via PI 3 kinase/Akt signaling in mesangial cells. *J Cell Physiol* **211**, 457–467 (2007).
38. Radomski, M. W., Palmer, R. M. & Moncada, S. The role of nitric oxide and cGMP in platelet adhesion to vascular endothelium. *Biochem Biophys Res Commun* **148**, 1482–1489 (1987).
39. Wang, T. *et al.* Synthesis and biological evaluation of nitric oxide-donating thalidomide analogues as anticancer agents. *Chem Biodivers* **6**, 466–474 (2009).
40. Hamburger, V. & Hamilton, H. L. A series of normal stages in the development of the chick embryo. *J Morphol* **88**, 49–92 (1951).
41. Weld, M. W. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (danio rerio), University of Oregon Press, Eugene, OR, (2000).
42. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of embryonic development of the zebrafish . *Dev Dyn* **203**, 253–310 (1995).
43. Muthukkaruppan, V. R., Shinners, B. L., Lewis, R., Park, S-J., Baechler, B. J. & Auerbach, R. The chick embryo aortic arch assay: a new, rapid, quantifiable in vitro method for testing the efficacy of angiogenic and anti-angiogenic factors in a three-dimensional, serum free organ culture system. *Proc Am Assoc Cancer Res.* **41**, 65 (2000).

Acknowledgements

This research work was funded by K.B. Chandrashekar research foundation.

Author contribution

JHS prepared the manuscript, performed the experiments and analyzed the data, VV, KPM, SR, US, SS, SN and PT performed the experiments, SC supervised, designed the experiments and approved the manuscript. All authors reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Siamwala, J.H. *et al.* Nitric oxide rescues thalidomide mediated teratogenicity. *Sci. Rep.* **2**, 679; DOI:10.1038/srep00679 (2012).