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Valproic Acid Increases Formation of Reactive Oxygen Species and Induces Apoptosis in Postimplantation Embryos: A Role for Oxidative Stress in Valproic Acid-Induced Neural Tube Defects

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Abbreviations:

8-OHdG, 8-hydroxyguanosine; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate; GD, gestational day; GSH, glutathione; IMDM, Iscove's modified Dulbecco's medium; NTD, neural tube defect; PARP, poly(ADP-ribose)polymerase; PEG, polyethylene glycol; PHS, prostaglandin H synthase; RIPA, radioimmunoprecipitation; ROS, reactive oxygen species; s.c., subcutaneous; SOD, superoxide dismutase; TBST, tris-buffered saline with 0.1% Tween; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; VPA, valproic acid.

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Abstract

Exposure to the anticonvulsant valproic acid (VPA) during the first trimester of pregnancy is associated with an increased risk of congenital malformations including heart defects, craniofacial abnormalities, skeletal and limb defects, and most frequently, neural tube defects (NTDs). The mechanisms by which VPA induces teratogenic effects are not fully understood, although previous studies support a role for oxidative stress. To investigate the effects of VPA on early development, a whole embryo culture model was used to evaluate the protective effects of antioxidants, measure intracellular reactive oxygen species (ROS) levels, and assess markers of oxidative damage and apoptosis. Furthermore, *in vivo* teratological evaluations of antioxidant protection were also completed. VPA (0.60 mM in embryo culture, 400 mg/kg *in vivo*) induced significant decreases in embryonic growth and increases in NTDs. Of the antioxidants tested, catalase provided partial protection against VPA-mediated reductions in morphological and developmental growth parameters in both whole embryo culture and *in vivo* systems. VPA exposure resulted in an increase in ROS staining in the head region, as assessed by whole mount staining with CM-H_2DCFDA. Markers of embryonic oxidative damage including 8-hydroxyguanosine, 4-hydroxynonenal adducts, and 3-nitrotyrosine were not affected by VPA treatment. Increased ROS levels were correlated with increased staining for apoptotic markers, as assessed by western blotting and immunohistochemistry. Addition of catalase to the media attenuated VPA-induced increases in ROS formation and apoptosis. These studies identify regions of the embryo susceptible to ROS and apoptosis induced by VPA, thus establishing a possible molecular pathway by which VPA exerts teratogenicity.

Introduction

Valproic acid (VPA) is a known human teratogen that causes a cluster of birth defects described by the term 'Fetal Valproate Syndrome' and includes developmental anomalies such as cleft palate, atrial septal defects, polydactyly, craniosynostosis, hypospadias, and most frequently, spina bifida (Jentink et al., 2010). Spina bifida is a neural tube defect (NTD) that results from failure of the caudal neural tube to close during organogenesis, and may lead to sensory and motor deficiencies in areas caudal to the site of the defect (Copp et al., 2003). Another NTD, exencephaly, refers to failure of the cranial neuropore to close and is presented in fetuses following maternal VPA exposure in a murine model (Ornoy, 2009). In addition to congenital malformations, VPA exposure during pregnancy is associated with decreased cognitive function 3 years after birth (Meador et al., 2009a). It is currently used in the treatment of a wide variety of neurological disorders including migraines, bipolar disorder, and epilepsy (Bowden, 2009; Evers, 2008). Although VPA use in women of childbearing age has declined slowly over the last decade (Meador et al., 2009b; Ackers et al., 2009), it remains one of the most frequently prescribed and effective antiepileptic drugs available, thus creating a clinical dilemma when less teratogenic substitutions are not available or suitable for treatment (Duncan, 2007). Despite its widespread use, the mechanisms by which VPA causes teratogenicity are not fully understood.

Oxidative stress has been implicated in the mechanisms of teratogenesis of several compounds including phenytoin, benzo[α]pyrene, hydroxyurea, and 5-bromo-2'-deoxyuridine (Winn and Wells, 1996; Winn and Wells, 1995; Yan and Hales, 2006; Sahambi and Hales, 2006). Excessive reactive oxygen species (ROS) production can cause direct damage to cellular macromolecules such as DNA, protein, and lipids and can alter normal signaling pathways through activation of redox sensitive transcription factors (Wells et al., 2009). Detoxification of ROS can be achieved through enzymatic and non-enzymatic antioxidant

mechanisms. Although the embryo is equipped with enzymatic antioxidant defense mechanisms, several studies have demonstrated that the expressions of superoxide dismustase (SOD), catalase, and glutathione peroxidase are lower during early embryonic development when compared to maternal levels (el-Hage and Singh, 1990; Zaken et al., 2000; Winn and Wells, 1999). Since early embryonic antioxidant systems are immature and considerably lower compared to adult levels, it is possible that the developing embryo may be more susceptible to ROS-initiated damage.

Indeed, several studies provide evidence suggesting a role for oxidative stress in VPA-induced teratogenesis. In a whole embryo culture model, VPA was shown to increase oxidized to reduced glutathione (GSH) ratios, and decrease total GSH content in embryo homogenates. These effects were reversed by pretreatment with vitamin C (Zhang et al., 2009). *In vivo* studies have demonstrated that vitamin E pretreatment prevents VPA-induced NTDs in mice (Al Deeb et al., 2000). Furthermore, *in vitro* studies have shown that VPA increases ROS formation and inhibits cardiomyocyte differentiation, and these VPA-induced alterations were prevented by antioxidant pretreatment (Na et al., 2003). Although these studies indirectly suggest that excessive ROS production during organogenesis may be initiating a teratogenic effect following VPA exposure, direct measurements of ROS production and oxidative damage have not been assessed in VPA-treated embryos.

The current study was designed to further characterize the contribution of oxidative stress in VPA-induced teratogenesis. A murine whole embryo culture model was used to examine whether and which antioxidants protect against VPA-induced embryotoxicity. Furthermore, we measured and localized ROS formation, oxidative damage, and apoptotic markers. Finally, a teratology study was completed to investigate the *in vivo* effects of antioxidant supplementation on VPA-induced teratogenesis.

Materials and Methods

Experimental Animals

Female virgin CD-1 mice (Charles River Laboratories Inc., St. Constant, Canada) aged 4 to 6 weeks were maintained in a temperature-controlled room with a 12 hour light/dark cycle. Standard rodent chow (Purina Rodent Chow, Ralston Purina International, Strathroy, Canada) and tap water were provided *ad libitum*. Following a week of acclimatization, mice were bred by housing three females with one male overnight. Females with a vaginal plug the following morning were separated from the colony, housed together, and designated as gestational day (GD) 1. All practices were in accordance with the guidelines of the Canadian Council on Animal Care and experimental procedures were approved by the Queen's University Animal Care Committee.

Whole Embryo Culture

On GD 9.0, pregnant dams were sacrificed by cervical dislocation and embryos were explanted by the method of New (New, 1976). Briefly, uteri were removed from dams, rinsed in phosphate buffered saline (PBS), and dissected to expose the individual implantation sites. The outer layers of Reichert's membrane, trophoblast, and parietal endoderm were removed, leaving the yolk sac, amnion, and ectoplacental cone intact. Explanted embryos at similar developmental stages (4-6 somites) were cultured individually in 1.3 mL of pregassed (5% CO_2 in air) embryo culture media consisting of 90% male rat serum (Cocalico Biologicals Inc., Reamstown, USA) and 10% Hanks' balanced salt solution (Sigma-Aldrich Canada Ltd., Oakville, Canada). Embryos were cultured for 24 hours at 37°C rotating at 30 rpm.

For the dose response study, embryos were cultured with one of the following concentrations of VPA (Sigma-Aldrich Canada Ltd., Oakville, Canada): 0, 0.075, 0.15, 0.30, 0.60, 1.2 mM. In the antioxidant studies, embryos were cultured in the presence of polyethylene

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glycol-conjugated catalase (PEG-catalase) (400 Units/mL), polyethylene glycol-conjugated superoxide dismutase (PEG-SOD) (25, 50, or 150 Units/mL), N-acetylcysteine (NAC) (0.5, 1, or 5 mM), or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (50, 100, or 300 µM), with or without 0.60 mM VPA.

At the end of the culture period, embryos were removed from media, rinsed in PBS, freed from membranes (amnion and yolk sac), and examined by an established developmental scoring system (Van Maele-Fabry et al., 1990). Embryos were assessed for viability by the presence of a heartbeat and only viable embryos were included in the results. The following developmental parameters were measured: dorsal-ventral flexure (embryo turning), anterior neuropore closure, and somite development. Morphological parameters assessed were yolk-sac diameter and crown-rump length. Yolk-sac diameter was measured at the widest position perpendicular to the ectoplacental cone. Crown-rump length was measured in embryos that had turned.

Animal Treatment and Teratological Assessment

For teratology studies, 8 litters of control dams, 7 litters of PEG-catalase treated dams, 9 litters of VPA-treated dams, and 9 litters of PEG-catalase pre-treated VPA dams were used to assess catalase protection against VPA-induced teratogenesis. PEG-catalase was dissolved in phosphate buffered saline (PBS) and injected intraperitoneally at a dose of 10 KU/kg on GD 8.5. Dams were then injected with 400 mg/kg VPA or its vehicle (0.9% saline) subcutaneously on GD 9. On GD 19, one day prior to spontaneous delivery, dams were sacrificed by cervical dislocation. Fetuses were collected, weighed, measured, and kept in an incubator at 37°C for one hour to assess for viability. Live and dead fetuses were recorded and examined for external malformations.

Whole Mount Fluorescent Staining for ROS

Embryonic ROS levels were measured in cultured embryos as previously described with the following modifications (Zhao and Reece, 2005). Embryos were removed from media after 24 hours, cleared from membranes, and incubated with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H$_2$DCFDA) (5 µM) in serum-free IMDM for 1 hour. Following incubation with CM-H$_2$DCFDA, embryos were washed with PBS and examined under a confocal microscope immediately. Scanning parameters were set up using a VPA-treated embryo and all embryos were examined with the same parameters. Quantification of fluorescent staining was determined by measuring fluorescence intensity of three area regions of the embryonic structure and then determining the mean fluorescence intensity. Image analysis was performed blinded.

Protein Extraction and Western Blotting

Two to three embryos were pooled after 24 hours of culture for each n value for Western blotting. Embryos were sonicated on ice in radioimmunoprecipitation (RIPA) buffer [50 mM Tris-HCl, 150 mM NaCl, 0.2% (w/v) NP-40, and 1.0 mM EDTA]. Protein concentrations were determined using the BioRad Protein Assay. Protein samples (20 µg) were separated on 8% or 15% polyacrylamide gels followed by transfer to PVDF membranes and then incubated with the following primary antibodies overnight: anti-4-hydroxynonenal Michael adducts (dilution 1:2000 in 2% milk in TBST) (Calbiochem, Gibbstown, USA), anti-3-nitrotyrosine (1:500 in 2% milk in TBST) (Millipore, Billerica, USA), anti-cleaved caspase-3 (1:500 in 2% milk in TBST) (Cell Signaling, Danvers, United States), and anti-cleaved PARP (1:500 in 3% milk in TBST) (Cell Signaling, Danvers, United States). Membranes were then washed and incubated with appropriate secondary antibodies and developed using an enhanced chemilumnescence

detection kit (Perkin-Elmer, Boston, United States). Differences in protein loading were controlled by stripping and reprobing the membranes with an anti-β-actin antibody (Sigma-Aldrich Ltd., Oakville, Canada).

Immunohistochemistry

Embryos were collected after culture, cleared from membranes, washed in PBS, and fixed immediately in 10% neutral buffered formalin. At least 8 embryos were collected in each treatment group. Fixed samples were processed for paraffin embedding and sectioned at 5 µm for immunohistochemical analysis. Slides were deparaffinised in xylene and rehydrated through a graded alcohol series. Antigen retrieval was achieved by 0.1 M sodium citrate at 95°C, and endogenous peroxidase was blocked with 3% hydrogen peroxide in water. Slides were probed with the same primary antibodies as used in Western blotting, as well as an anti-8-hydroxyguanosine (8-OHdG) antibody (Abcam, Cambridge, USA), and then stained using the anti-rabbit Vectastain Elite ABC kit or M.O.M. immunodetection kit (Vector Laboratories) with DAB as the substrate. Hematoxylin was used for counterstaining. Staining intensity for 8-OHdG was determined by qualitative assessment by a blinded observer using a 9-point scale where 0.5 is little staining and 4 is dark staining.

Statistical Analysis

Statistical analysis for embryo culture, CM-H$_2$DCFDA staining, Western blotting, and teratology studies were assessed by one way analysis of variance, followed by the Neumann-Keuls multiple comparison test for post-hoc analysis (GraphPad Prism 4.0, GraphPad Software, San Diego, CA). Binomial data was assessed by chi-square test. $P < 0.05$ was designated as statistically significant.

Results

Effect of VPA on Mouse Embryo Development in Whole Embryo Culture

To verify the teratogenic profile of VPA in a whole embryo culture system, embryos with 4-6 somites were extracted from dams on GD 9.0 and cultured in the presence of 0, 0.075, 0.15, 0.30, 0.60, or 1.2 mM VPA for 24 hours. A dose dependent decrease was observed in most developmental and morphological parameters assessed (Figure 1). Significant decreases in yolk-sac diameter were observed at 0.60 and 1.2 mM. In addition, crown-rump length was significantly decreased at 0.15 and 0.60 mM, although this parameter was only measured in 2 embryos that had turned at 1.2 mM. Total somite number was significantly decreased in embryos exposed to 0.60 and 1.2 mM VPA. Developmental parameters assessed were dorsal-ventral flexure (embryo turning) and anterior neuropore closure. A significant decrease in embryo turning was observed at 0.075 mM, and this decrease occurred in dose dependent manner. Treatment with 0.60 and 1.2 mM VPA lowered the number of embryos with closed anterior neuropore after the 24 hour culture period.

Embryoprotective Effects of Catalase on VPA-Induced Embryotoxicity in Whole Embryo Culture

To determine if antioxidant co-culture could protect against VPA-induced embryotoxicity, embryos were cultured in the presence of PEG-catalase (400 Units/mL), PEG-SOD (25, 50, or 150 Units/mL), NAC (0.5, 1, or 5 mM), or Trolox (50, 100, or 300 µM), with or without 0.60 mM VPA. PEG-catalase (400 Units/mL) supplementation eliminated VPA-induced decreases in yolk-sac diameter and somite number (Figure 2). In addition, embryo turning and anterior neuropore closure were restored in embryos exposed to both PEG-catalase and VPA. PEG-SOD, NAC, and Trolox did not prevent embryotoxicity induced by VPA at all doses tested (Table 1).

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Embryoprotective Effects of Catalase on VPA-Induced Teratogenesis *In Vivo*

To assess if PEG-catalase could protect against VPA-induced teratogenesis *in vivo*, pregnant mice were injected with 10 KU/kg of PEG-catalase 16 hours prior to dosing with a teratogenic dose of VPA (400 mg/kg). PEG-catalase and/or VPA treatment did not significantly alter litter size between treatment groups (mean litter sizes were 13.13 ± 0.44, 11.71 ± 0.92, 13.22 ± 0.68, and 11.67 ± 0.58 for control, PEG-catalase, VPA, and PEG-catalase/VPA-treated groups, respectively). In addition, PEG-catalase and/or VPA administration did not significantly affect resorption rates (1.93 ± 1.27, 8.38 ± 2.89, 5.41 ± 3.04, and 7.35 ± 3.75 for control, PEG-catalase, VPA, and PEG-catalase/VPA-treated groups, respectively). VPA alone caused a significant decrease in fetal viability due to increased fetal death in each litter; in addition, fetal weight and length were significantly decreased by VPA treatment. VPA also significantly increased the number of fetuses presenting with open eye and exencephaly (Figure 3). Pretreatment with 10 KU/kg PEG-catalase prevented the VPA-induced decrease in fetal weight, and decreased the percentage of fetuses presenting with NTDs. PEG-catalase pretreatment did not have an effect on fetal viability, length, or the incidence of open eye when compared to the VPA treatment group.

Catalase Prevents VPA-Induced ROS Formation

As catalase was shown to protect against VPA-induced malformations in whole embryo culture and *in vivo*, intracellular ROS levels in mouse embryos were measured by whole mount immunofluorescent staining with CM-H$_2$DCFDA in cultured embryos. A significant increase in fluorescent staining was observed in VPA-treated (0.60 mM) mouse embryo heads when compared to control embryo heads (Figure 4). This increase was attenuated by addition of

PEG-catalase (400 U/mL) to the media. Significant changes in fluorescent staining in the heart and somites were not observed.

VPA Does Not Alter Markers of Oxidative Damage in Embryos

Western blotting and immunohistochemistry were used to assess markers of oxidative damage in embryos after 24 hours of culture. VPA did not alter levels of 4-hydroxynonenal Michael adducts or 3-nitrotyrosine (markers of lipid peroxidation and protein nitration, respectively) as assessed by Western blotting (Figure 5). Immunohistochemistry conducted for 4-hydroxynonenal adducts and 3-nitrotyrosine showed diffuse cytoplasmic localization in the neuroepithelium of both control and VPA-treated embryos, as indicated by DAB (brown) staining. Staining for 8-hydroxyguanosine, a marker of oxidative DNA damage, did not reveal any apparent differences between control and VPA-treated embryos in the anterior neural tube.

Catalase Protects Against VPA-Induced Apoptosis

Western blotting and immunohistochemistry were used to assess alterations in cleaved caspase-3 and cleaved PARP levels in embryos after 24 hours of culture. VPA (0.60 mM) significantly increased the expression of both cleaved caspase-3 and cleaved PARP when compared to control embryos (Figure 6). Co-culture with PEG-catalase (400 U/mL) attenuated VPA-induced increases in cleaved caspase-3 and PARP protein expression. Immunohistochemistry showed staining for cleaved caspase-3 and cleaved PARP in discrete regions in the neuroepithelium of the anterior neural tube of control embryos. Increased substrate staining (brown) was observed along the anterior neuroepithelium as well as in the somites of VPA-treated embryos. PEG-catalase and VPA co-treated embryos showed similar staining patterns to control embryos.

Discussion

Studies have previously demonstrated that antioxidant pretreatment or supplementation protects against embryotoxicity induced by VPA (Zhang et al., 2009; Al Deeb et al., 2000). We expand upon these findings in a whole embryo culture model using several antioxidants to further distinguish the molecular mechanisms by which VPA causes teratogenesis. First, a dose-dependent decrease was observed in developmental and morphological parameters including yolk-sac diameter, somite number, embryo turning, and anterior neuropore closure in embryos culture with VPA. This increase in growth retardation and malformed embryos is consistent with previously published data examining VPA-induced embryotoxicity in a whole embryo culture system (Andrews et al., 1997). The antioxidants used in the current study were selected based on previous reports that validated their embryoprotective effects against agents and conditions that cause oxidative stress and consequent developmental abnormalities in the embryo, and also for their different antioxidative mechanisms of action (Winn and Wells, 1995; Winn and Wells, 1999; Na et al., 2003; Gareskog and Wentzel, 2007). Of the antioxidants tested, only PEG-catalase protected against the reduction in embryonic growth and anomalies caused by 0.60 mM VPA, an embryotoxic concentration of VPA that is comparable to therapeutic maternal serum levels observed in humans on long-term VPA therapy (Ornoy, 2009). Treatment with PEG-catalase restored VPA-induced decreases in yolk-sac diameter, crown-rump length, somite number, embryo turning, and anterior neuropore closure, suggesting that catalase exerts a specific protective mechanism against VPA.

To further verify the protective effects of catalase against VPA-initiated malformations, an *in vivo* teratological study was completed. Treating mice with PEG-catalase at a dose that increases embryonic catalase activity prior to a teratogenic dose of VPA resulted in protection against VPA-induced reductions in fetal weight and VPA-induced NTDs (Winn and Wells, 1999). Interestingly, catalase did not protect against the incidence of open-eye or decrease in fetal

length caused by VPA. While our results suggest that catalase has a specific effect on neural tube closure, future studies will be required to determine the significance of ROS on molecular and structural changes during neurulation. It is possible that VPA exerts specific embryonic effects via different mechanisms, and NTDs are particularly sensitive to increases in ROS (Ornoy, 2009). Nonetheless, our embryo culture and *in vivo* studies verify a role for ROS in VPA-initiated teratogenesis, and identify hydrogen peroxide as a specific mediator of NTDs.

Catalase catalyzes the decomposition of hydrogen peroxide to water and molecular oxygen. Although hydrogen peroxide itself is a weak oxidant, it can freely cross cellular membranes and in the presence of transition metals, be reduced to the highly reactive hydroxyl radical via the Fenton reaction (Fantel, 1996). Embryonic catalase activity has been shown to be approximately 3-7% of maternal levels and remains consistently low from GD 9 to GD12, indicating that organogenesis may be a period when embryos are particularly susceptible to damage by hydrogen peroxide (Abramov and Wells, 2011). Interestingly, the same study also demonstrated in embryos cultured with the ROS-initiating teratogen phenytoin that embryos with failed anterior neuropore closure and embryos that failed to turn have significantly decreased levels of catalase activity when compared to embryos that developed normally. This supports the results of our present study, suggesting that hydrogen peroxide plays a specific role in mediating some aspects of VPA-induced embryotoxicity.

To directly measure ROS formation and localize increases in ROS, whole mount immunofluorescence was performed with CM-H$_2$DCFDA, a dye sensitive to various ROS including hydrogen peroxide, hydroxyl radicals, peroxyl radicals, and peroxynitrite anions. In the present study, we observed a significant increase in fluorescent staining in the heads of mouse embryos that were exposed to 0.60 mM VPA, as compared to controls, and these increased ROS levels were attenuated by co-culture with catalase. Previous studies have demonstrated that VPA may be inducing its teratogenic effects via increased ROS formation; however, to our

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knowledge, we are the first to directly localize increased staining for ROS following VPA exposure within the whole embryo. Many teratogens have been shown to enhance ROS formation via different mechanisms, for instance, some can undergo redox cycling while others are metabolized to a reactive intermediate (Juchau et al., 1992). VPA is extensively metabolized. VPA-induced hepatoxicity has been attributed to metabolism of the parent compound to the highly reactive metabolites 4-ene VPA and E-2,4-diene VPA by CYP2C9 and CYP2C19 (Chang and Abbott, 2006). Although levels of these CYPs are low in embryos during organogenesis, other enzymes such as prostaglandin H synthases (PHS) and lipoxygenases are present in high levels in the developing embryo (Winn and Wells, 1996). These enzymes have previously been shown to bioactivate other teratogenic agents such as phenytoin and benzo[a]pyrene to reactive intermediates, thereby increasing ROS production (Winn and Wells, 1996). Thus, it is possible that VPA is metabolized by PHS and lipoxygenase to generate reactive metabolites in the embryo. Therefore, VPA-induced increases in ROS production combined with immature embryonic antioxidant defence systems may together contribute to the generation of a teratogenic effect.

As increases in ROS were observed, we predicted that markers of oxidative damage would be increased by VPA exposure and reduced by catalase supplementation. The markers of oxidative damage assessed in this study were selected based on data that previously demonstrated their contribution to teratogenesis, and to cover a broad range of macromolecular damage (Winn and Wells, 1995; Yan and Hales, 2006; Beckers-Trapp et al., 2006). Differences in 4-hydroxynonenal adduct, 3-nitrotyrosine formation, 8-hydroxyguanosine were not observed in all treatment groups. Despite observing a significant increase in ROS formation, it is possible that the levels of ROS generated by VPA were not high enough to cause direct oxidative damage to cellular macromolecules. Early organogenesis occurs in a relatively hypoxic environment, where moderate changes in ROS production can cause alterations in cellular

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proliferation, differentiation, and cell fate through changes in cell signalling (Dennery, 2007). Interestingly, ROS-mediated alterations in signaling pathways are attributed to the less reactive and membrane diffusible hydrogen peroxide, which selectively oxidizes cysteine residues on proteins, consequently altering its function and downstream signal transduction pathways (Wells et al., 2009). In addition, VPA can alter cell signaling through gene expression changes mediated through histone deacetylase inhibition (Phiel et al., 2001). VPA is a direct inhibitor of class I and II HDACs and several laboratories including ours have shown that embryonic histone acetylation levels are increased following exposure to VPA (Menegola et al., 2005; Tung and Winn, 2010). Furthermore, studies have supported a role for HDAC inhibition as a mechanism of teratogenesis as analogs of VPA that lack HDAC inhibitory activity are less teratogenic (Gurvich et al., 2005). Gene microarray studies have also demonstrated that VPA targets genes regulated by HDAC, including *Mt1* and *Mt2*, both of which are ROS-sensitive (Jergil et al., 2009). In addition, HDAC inhibitors have also been shown to increase ROS production and induce apoptosis in several cancer cell lines (Carew et al., 2008). Therefore, alterations in gene expression and/or increases in ROS formation mediated by HDAC inhibition during development may induce teratogenesis.

Our current study also demonstrated that VPA exposure resulted in increased expression of markers of apoptosis, cleaved caspase-3 and cleaved PARP, that was attenuated by catalase supplementation. Immunohistochemistry revealed that staining was particularly increased in the neuroepithelium and also in the somites, suggesting that increased cell death may be contributing to the formation of NTDs and axial skeletal malformations. Apoptosis is known to occur during and after neurulation in the neuroepithelium, although the significance of this is not fully understood. It has been postulated that apoptosis is required for bending of the neural folds at the dorsolateral hinge point and for midline epithelial remodelling once the neural folds have come into contact and fused (Copp et al., 2003). Recently, studies have shown that

apoptosis is not a requirement for neural tube closure (Massa et al., 2009). Nevertheless, excessive cell death in the cranial neuroepithelium can disrupt anterior neural tube closure by leaving the embryo with an inadequate number of cells to undergo proper closure, resulting in exencephaly. Increased apoptosis in the neuroepithelium caused by VPA-induced increases in ROS production and alterations in embryonic signalling may be the underlying causes of NTDs in this model.

In summary, we have shown that VPA causes an increase in embryonic ROS production and an increase in apoptosis that was attenuated by catalase supplementation. The incidence of embryonic and fetal defects induced by VPA was prevented by catalase in both whole embryo culture and *in vivo* models. The results of this study strongly support the hypothesis that ROS, specifically hydrogen peroxide, play an important role in mediating VPA-induced teratogenesis. As markers of oxidative damage were not altered by VPA exposure, we now postulate that VPA may be mediating teratogenesis by alterations in redox-sensitive signalling pathways. The identification of this molecular pathway furthers our understanding the mechanisms of VPA-mediated teratogenesis and will aid in development of prevention strategies.

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Authorship Contributions

Participated in research design: Tung and Winn

Conducted experiments: Tung

Performed data analysis: Tung

Wrote or contributed to the writing of the manuscript: Tung and Winn

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Footnotes

Preliminary results from these studies have been previously presented at the 45th Annual Meeting of the Society of Toxicology (U.S.A.), March 2006; at the 11th International Congress of Toxicology, June 2007; at the 48th Annual Meeting of the Teratology Society, June 2008; and at the 42nd Annual Symposium of the Society of Toxicology of Canada, December 2010. This work was supported by a research grant from the Canadian Institutes of Health Research. [MOP 86593]

Legends for Figures

Figure 1. VPA Inhibits Development in Mouse Embryo Culture. Embryos were cultured for 24 hours in the presence of increasing concentrations of VPA. The number of embryos is given in parentheses. * indicates significant difference from 0 mM VPA ($p < 0.05$).

Figure 2. Embryoprotection by Catalase in Whole Embryo Culture. Embryos were cultured for 24 hours in the presence of vehicle, PEG-catalase (400 U/mL), 0.60 mM VPA, or PEG-catalase (400 U/mL) and 0.60 mM VPA. The number of embryos is given in parentheses. * indicates significant difference from vehicle treated and PEG-catalase treated embryos ($p < 0.05$). + indicates significant difference from PEG-catalase/0.60 mM VPA treatment group ($p < 0.05$). Vehicle-treated controls were not statistically different from embryos treated with VPA and PEG-catalase.

Figure 3. Prevention of VPA-Induced Teratogenesis *In Vivo* by Catalase. PEG-catalase (10 KU/kg) was administered to pregnant dams 16 hours prior to dosing with VPA (400 mg/kg) on GD 9.0. The number of litters is given in parentheses. Results are expressed as a percentage per litter (viability, open eye, exencephaly) or mean per litter (fetal weight and length). * indicates significance from vehicle treated and PEG-catalase treated dams ($p < 0.05$). + indicates significant difference from PEG-catalase/VPA-treated litters ($p < 0.05$).

Figure 4. VPA Increases Production of Reactive Oxygen Species in Whole Embryo Culture. Whole mount staining for CM-H$_2$DCFDA after 24 hours of culture resulted in significant increases in fluorescent staining in VPA-treated (0.60 mM) embryonic heads that was attenuated by PEG-catalase (400 U/mL) co-culture (A). Fluorescent values measured in control embryos were not statistically different from embryos exposed to both PEG-catalase and VPA. Differences were not observed for CM-H$_2$DCFDA staining in hearts (B) and somites (C). (i and ii, control; iii and iv, PEG-catalase; v and vi, exencephalic embryo treated with VPA; vii and viii,

PEG-catalase and VPA). The number of embryos is given in parentheses. *indicates a significant difference from vehicle treated and PEG-catalase treated embryos (p<0.05). +indicates a significant difference from PEG-catalase/VPA treated embryos (p<0.05). Photos of embryonic heads were taken at 10x magnification.

Figure 5. VPA Does Not Alter Markers of Oxidative Damage in Whole Embryo Culture. (A) Western blotting and immunohistochemistry for 4-hydroxynonenal Michael adducts did not show any differences between control (i) and VPA-treated (0.60 mM) embryos (ii). n=4 for Western blotting. (B) Western blotting and immunohistochemistry for 3-nitrotyrosine did not reveal any differences between control (i) and VPA-treated (0.60 mM) embryos (ii). n=3 for Western blotting. (C) Immunohistochemistry for 8-hydroxyguanosine was not different between control (i) and VPA-treated (0.60 mM) embryos (ii), as assessed by qualitative assessment by a blinded observer using a 9-point scale where 0.5 is little staining and 4 is dark staining. ↑ indicates cranial neuroepithelium. Photos show microscopic sections of mouse embryonic cranial neural tubes at 200x magnification.

Figure 6. VPA Increases the Expression of Apoptotic Markers in Whole Embryo Culture. (A) Western blot analysis for cleaved caspase-3 after 24 hours of culture. VPA (0.60 mM) significantly increased cleaved caspase-3 protein levels which were attenuated by PEG-catalase (400 U/mL) co-culture. Cleaved caspase-3 expression was not statistically different in embryos treated with both PEG-catalase and VPA when compared to controls. Immunohistochemical staining for cleaved caspase-3 was detected in control embryonic head and somites (i and ii, respectively). Increased staining was observed in the neuroepithelium of exencephalic embryos treated with VPA, as well as the somites (iii and iv), however PEG-catalase co-culture prevented VPA-induced staining of cleaved caspase-3 (v and vi). (B) Western blot analysis for cleaved PARP on embryos 24 hours after culture. VPA treatment significantly increased the expression of cleaved PARP. PEG-catalase prevented this increase.

Cleaved PARP expression was not statistically different in embryos treated with both PEG-catalase and VPA when compared to controls. Similar staining patterns were observed for cleaved PARP, as compared to cleaved caspase 3. (i and ii, control embryonic head and somites; iii and iv, VPA-treated exencephalic embryonic head and somites; v and vi, PEG-catalase and VPA treated-head and somites). n=3 for all Western blotting treatment groups. *indicates a significant difference from vehicle treated and PEG-catalase treated embryos (p<0.05). +indicates a significant difference from PEG-catalase/VPA treated embryos (p<0.05). ↑ indicates cranial neuroepithelium. Embryonic head pictures were taken at 100x magnification. Photos of somites are at 200x magnification.

Tables

Table 1:

Treatment	n	Yolk sac diameter (mm)	Crown rump length (mm)	somite number	embryo turning %	anterior neuropore closure %
control	33	2.67 ± 0.34	2.29 ± 0.33	20.1 ± 2.1	100 ± 0	84.8 ± 2.1
0.60 mM VPA	62	2.36 ± 0.34*	2.24 ± 0.21	18.3 ± 1.9*	43.5 ± 3.9*	29.0 ± 3.6*
0.60 mM VPA + 400 U/mL CAT	24	2.63 ± 0.37†	2.23 ± 0.33	18.9 ± 1.8†	82.3 ± 5.2†	61.5 ± 9.5†
0.60 mM VPA + 25 U/mL SOD	11	2.46 ± 0.34	2.17 ± 0.24	18.7 ± 2.5	63.6 ± 14.5*	18.2 ± 11.6*
0.60 mM VPA + 50 U/mL SOD	10	2.53 ± 0.26	2.23 ± 0.24	19.7 ± 1.3	60.0 ± 15.5*	40.0 ± 15.5*
0.60 mM VPA + 150 U/mL SOD	13	2.42 ± 0.40	2.27 ± 0.21	18.2 ± 2.3	53.8 ± 13.8*	30.7 ± 12.8*
0.60 mM VPA + 0.5 mM NAC	12	2.29 ± 0.29	2.32 ± 0.04	18.5 ± 3.0	41.7 ± 14.2*	25 ± 12.5*
0.60 mM VPA + 1 mM NAC	11	2.16 ± 0.29	2.10 ± 0.16	17.9 ± 2.0	36.4 ± 14.5*	18.2 ± 11.6*
0.60 mM VPA + 5 mM NAC	9	2.39 ± 0.23	2.16 ± 0.17	19.3 ± 1.9	55.6 ± 16.6*	22.2 ± 13.8*
0.60 mM VPA + 50 µM Trolox	12	2.18 ± 0.55	2.35 ± 0.06	17.1 ± 3.1*	33.3 ± 13.6*	8.3 ± 8.0*
0.60 mM VPA + 100 µM Trolox	13	2.41 ± 0.37	2.22 ± 0.19	17.9 ± 2.1*	46.2 ± 13.8*	15.4 ± 10.4*
0.60 mM VPA + 300 µM Trolox	10	2.22 ± 0.37	2.04 ± 0.05	17.9 ± 2.3	50 ± 15.8*	10.0 ± 9.5*

Table 1. Effect of Antioxidant Treatment on VPA-Induced Embryotoxicity in Whole Embryo Culture. * indicates significant difference from control (vehicle) treated embryos. Results given in percentage or mean ± SEM. † denotes significant difference from 0.60 mM VPA-treated embryos. All other treatment groups were not statistically different from 0.60 mM VPA embryos in all parameters assessed.
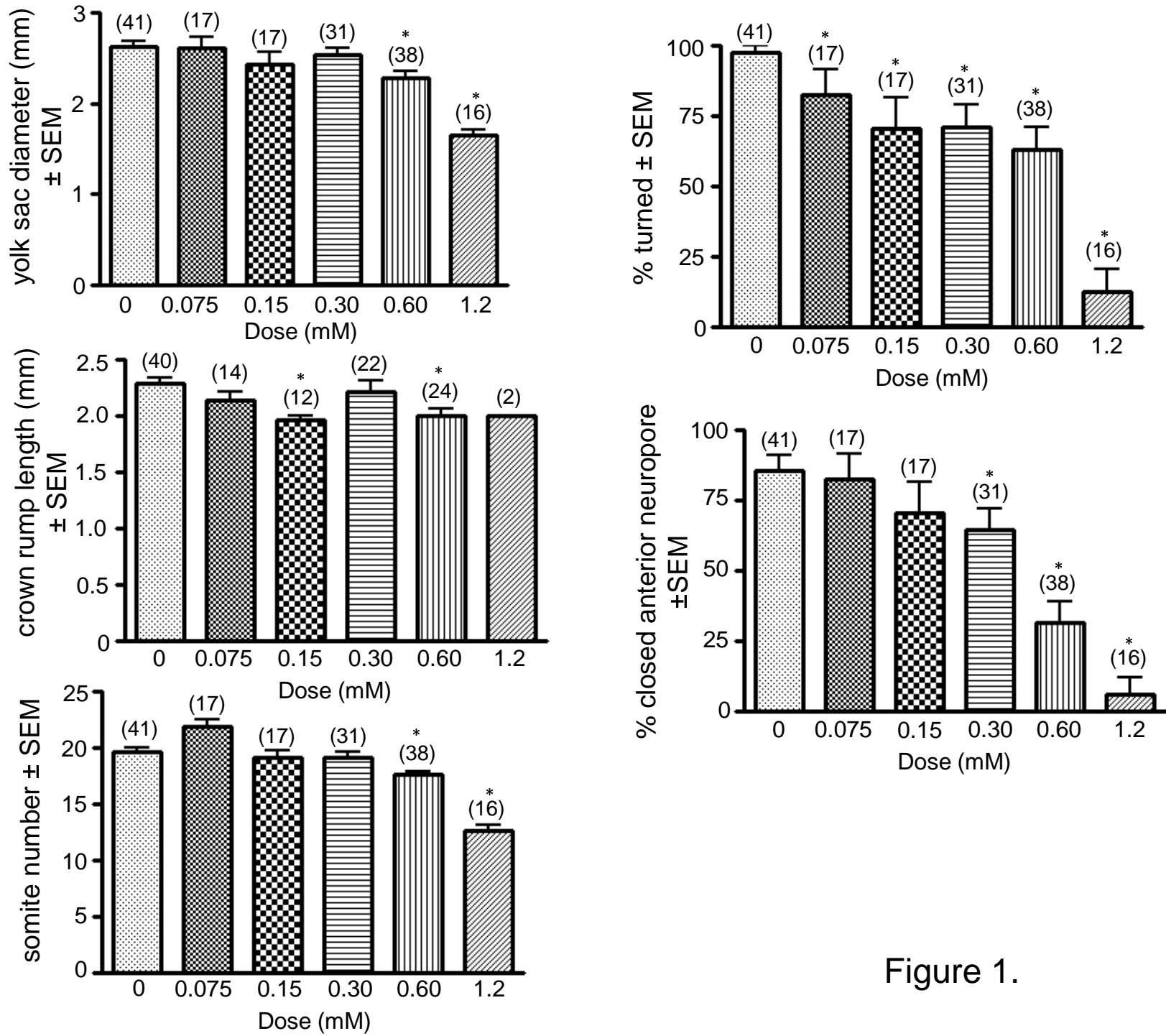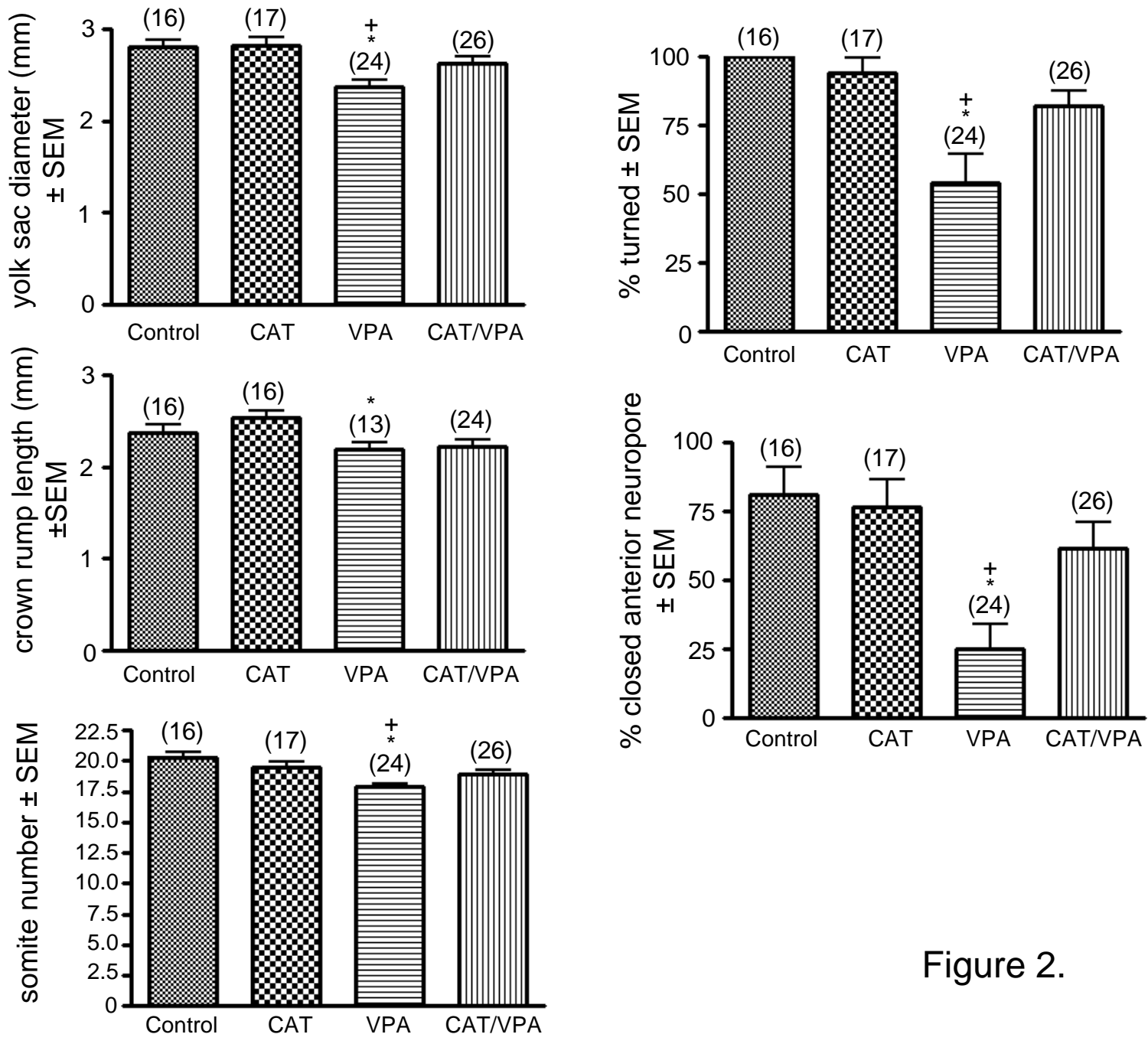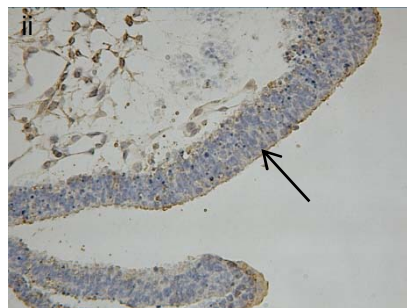
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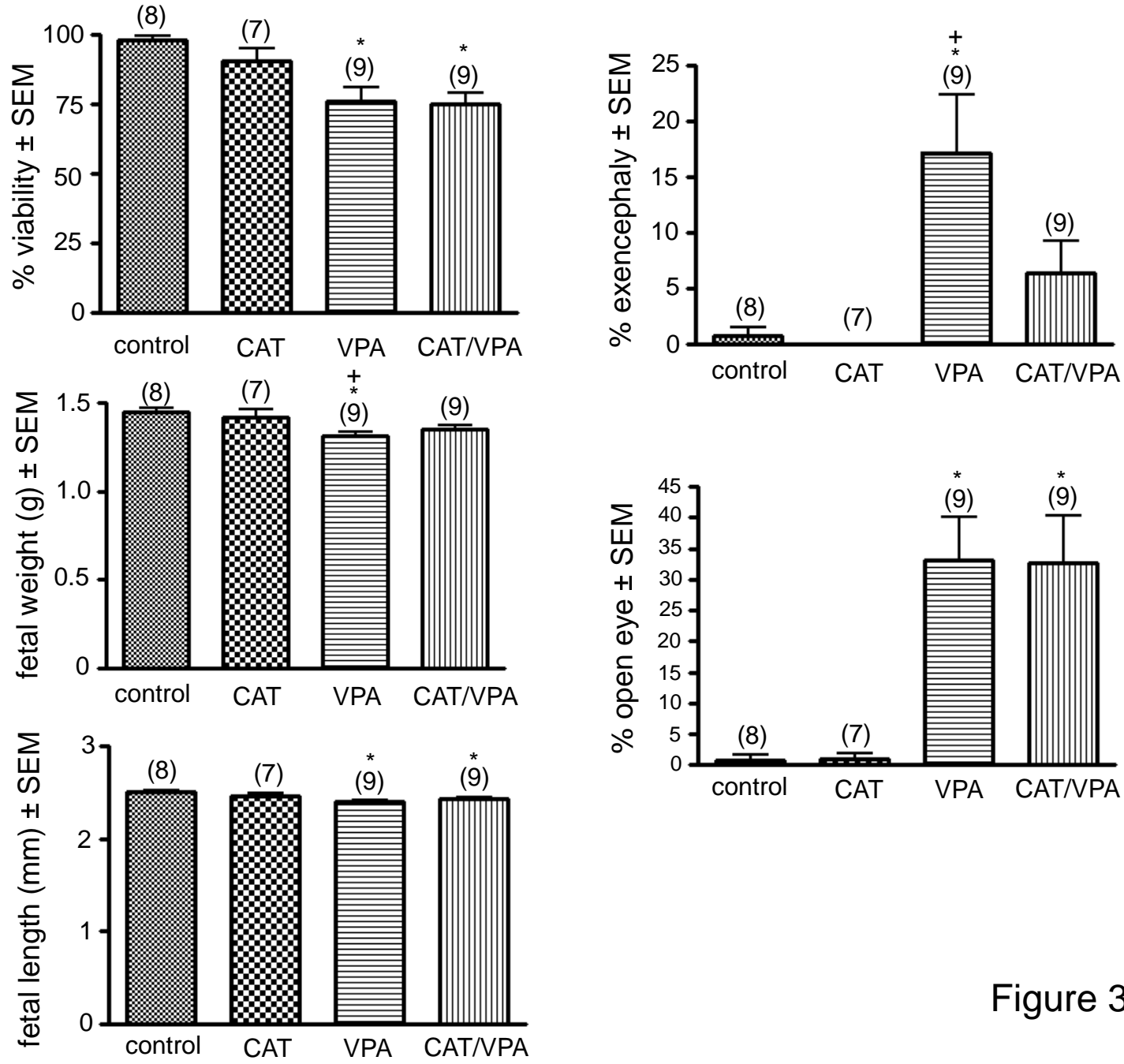
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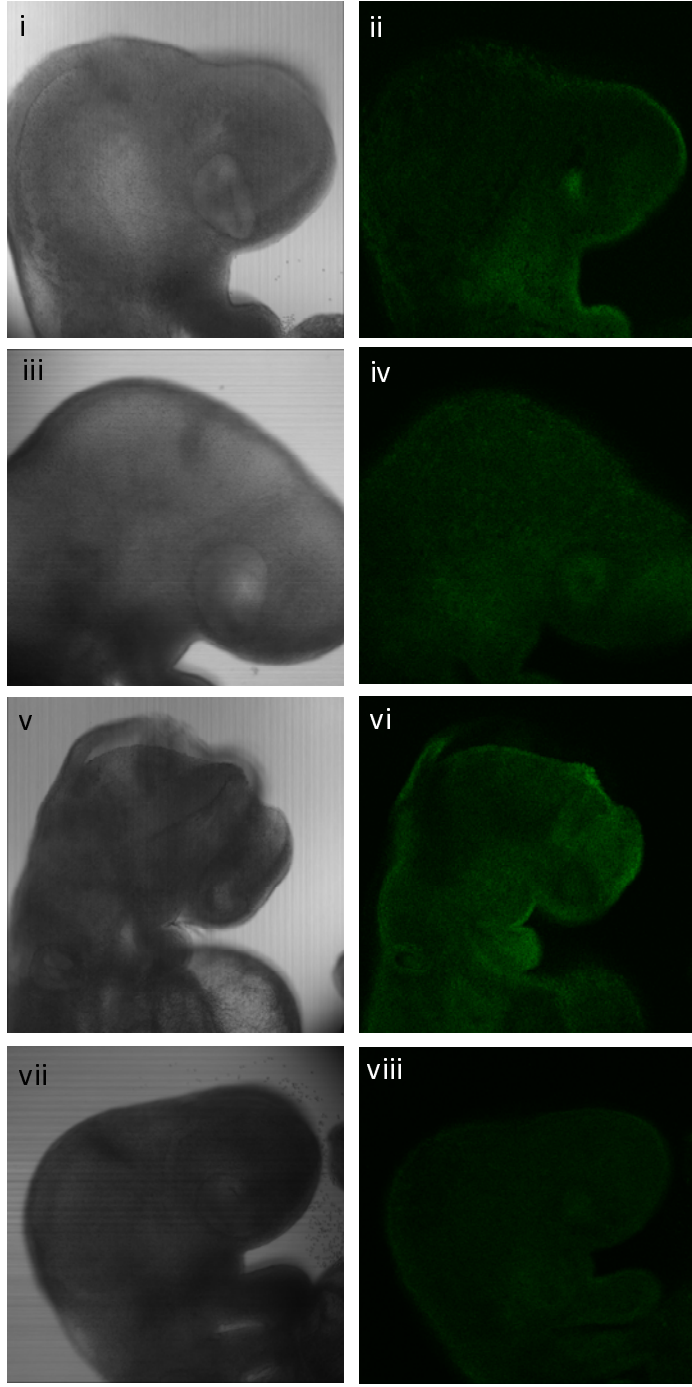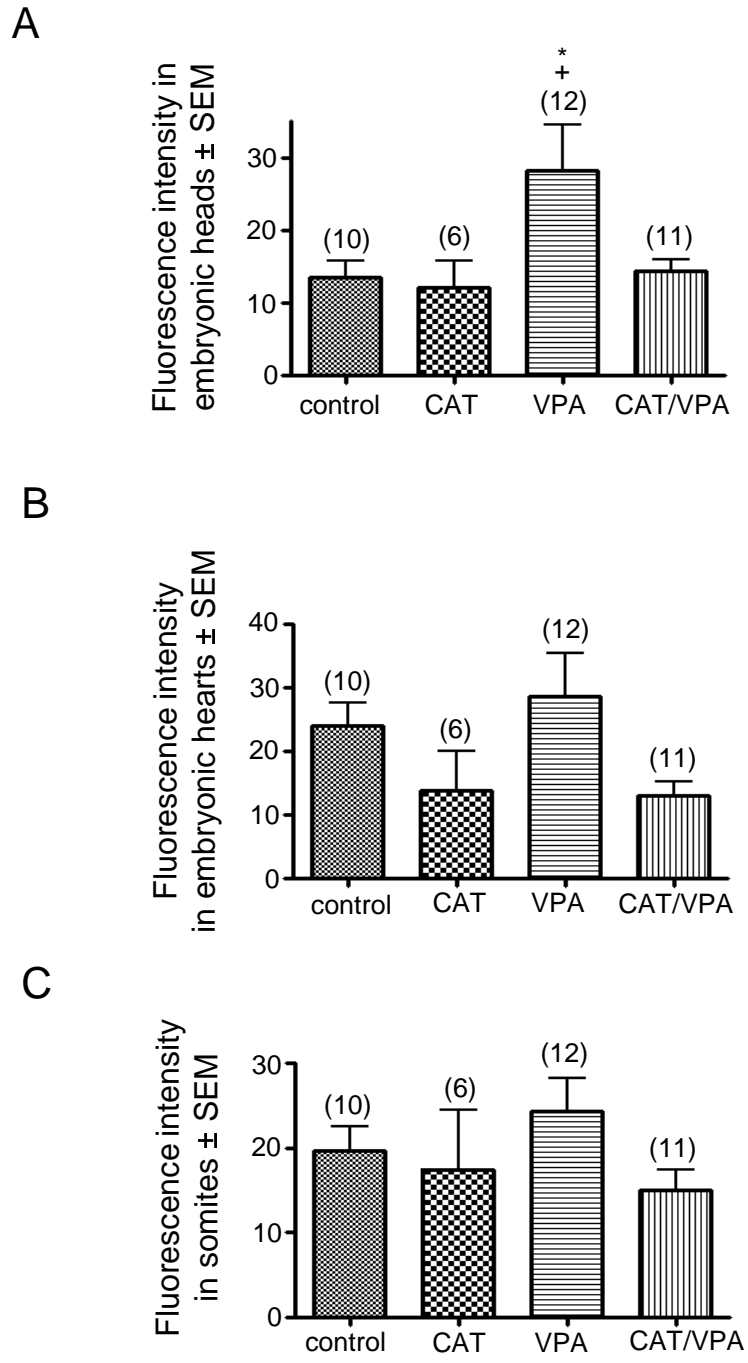
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