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Reduction in Valproic Acid–Induced Neural Tube Defects by Maternal Immune Stimulation: Role of Apoptosis

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Abstract

Teratogenic deregulation of apoptosis during development is a possible mechanism for birth defects. Administration of valproic acid (VA) during first trimester of pregnancy causes neural tube defects (NTDs). Nonspecific stimulation of the mother's immune system has been shown to reduce various teratogen-induced fetal malformations including NTDs in rodents. This present study investigated the role of reduced apoptosis by maternal immune stimulation in prevention of VA-induced NTDs in CD-1 mice. Prevention of VA-induced NTDs by nonspecific maternal immune stimulation using IFN γ was employed to evaluate the role of reduced apoptosis by IFN γ in this protective mechanism. Apoptosis was quantified using flow cytometry. Terminal Transferase dUTP Nick End Labeling assay was used to localize the apoptosis. Increased apoptosis, suggesting involvement in VA teratogenicity, was observed along the neural tube in both normal and abnormal embryos from VA-exposed dams. Increased apoptosis in normal VA-exposed embryos suggests that VA may alter other cellular processes such as cell proliferation and differentiation in addition to apoptosis. Apoptotic levels in embryos with closed neural tubes from IFN γ + VA dams were similar to controls indicating resistance to VA-induced apoptosis and protection against teratogenicity of VA. In IFN γ + VA exposed embryos with open neural tubes, maternal immune stimulation failed to regulate apoptosis resulting in an NTD. Overall, these results suggest that VA alters several biological processes including apoptosis in the developing embryos to induce fetal malformations. Resistance to VA-induced apoptosis in embryos resulting from maternal immune stimulation may be involved in protective mechanism.

Keywords

apoptosis; mechanisms of teratogenesis; neural tube defects; maternal immune stimulation

INTRODUCTION

Neural tube defects (NTDs) are the second most common malformation after congenital heart defects (Christianson et al., 2006) affecting 324,000 births every year (March of Dimes global report 2006). Failure of the neural tube to close during development results in

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exencephaly, anencephaly, and spina bifida. Nonspecific stimulation of maternal immune system in mice reduces a wide variety of fetal malformations induced by various physical and chemical agents, and also diseases such as diabetes mellitus (Nomura et al., 1990; Toder et al., 1996; Holladay et al., 2000; Prater et al., 2004; Punareewattana and Holladay 2004; Laudermilch et al., 2005; Khaksary et al., 2006; Latorre et al., 2007; Hrubec et al., 2012). Maternal immune stimulation with IFN γ reduces the incidence of valproic acid (VA) induced NTDs in late gestational fetuses (Hrubec et al., 2006a). The protective mechanism of maternal immune stimulation is not well understood; however, and may include alteration to cell cycle, cell signaling, and/or apoptotic pathways. Studies have also suggested that teratogenic deregulation of apoptosis during development may be responsible for birth defect formation including NTDs (Jiang et al., 2008; Oyama et al., 2009; Singh et al., 2011). Strategies such as maternal immune stimulation or administration of folic acid or antioxidants may normalize dysregulated apoptosis, and thus prevent the birth defects. This present study investigated the role of reduced apoptosis, programmed cell death, in reduction of VA-induced NTDs by maternal immune stimulation. VA (2-propylvaleric acid) is widely used for the treatment of epileptic seizures and bipolar disorder (Kostrouchova and Kostrouch, 2007). Administration of VA during early organogenesis can induce fetal malformations including craniofacial, urogenital, and skeletal anomalies in animals and humans (Dieterich et al., 1980; Nau et al., 1991; Clayton-Smith and Donnai, 1995). Apoptotic levels were evaluated to identify changes in location and amount of apoptosis in head regions of embryos as a result of teratogen exposure. Nonspecific maternal immune stimulation using IFN γ was employed to evaluate whether reduction of apoptosis played a role in protection against VA-induced teratogenesis.

MATERIALS AND METHODS

Animals and Experimental Design

CD-1 mice were housed in conventional cages with a 12-hr day length, 70 to 73°F, and 50 $\pm$ 20% humidity. Mice were given food (Ziegler Rodent diet 18–6) and distilled water ad libitum. For breeding, two or three females mated with a male and were checked for copulation plugs every morning. When a copulation plug was found, the female was separated and midpoint of the preceding dark cycle was designated as gestation day (GD) 0. Mice were euthanized on GD 9.5 by an overdose of CO₂ and embryos were evaluated for NTDs under a stereozoom microscope (Olympus SZX7, Melville, NY) equipped with a CFW 1310 Scion camera (Scion Corporation, Frederick, MD). All procedures related to animal use were approved by and conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) at the College of Veterinary Medicine at Virginia Tech, an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility.

Female mice were randomly divided into four treatment groups (Control, VA, IFN γ , VA + IFN γ) with 8 to 10 mice in each group. VA (500 mg/kg body wt.) was administered on gestation day (GD 8.0) by IP injection. For immune stimulation, IFN γ (1000 IU; Peprotech, Rocky Hill, NJ) was administered on prebreeding days 10 and 5 by IP injection. Males and

females were paired for one heat cycle and then separated. Control mice were administered an equal volume of phosphate buffer saline (PBS) by IP injection.

Flow Cytometry Analysis

An assay to quantify apoptotic cells in early embryos was developed. The technique used differential staining of disassociated embryonic cells with Annexin V and propidium iodide (PI). Apoptotic and dead cells both stain with Annexin V-FITC since it binds to the exposed phospholipids phosphatidyl-serine of disintegrating cells. Dead cells only stain with PI since this stain enters through leaks in the cell membrane to bind DNA. Thus, single-staining apoptotic cells can be differentiated from the double-staining dead cells.

The quantitative estimation of apoptotic cells in the developing heads of GD 9.5 embryos was quantified in each treatment group using flow cytometry. Embryos were isolated from the deciduas in Dulbecco's Modified Eagle Medium. For flow cytometric analysis, embryos of each dam from VA-treated mice were pooled based on open or closed neural tube. The heads were then incubated for 1 hr at 37°C in 1 ml of Dispase II enzyme (2.4 U/ml) (Roche Diagnostics GmbH, Penzberg, Germany) to disassociate the cells. After incubation, cells were further disassociated by gently pressing the tissue with a plunger. Cells in the Dispase II solution were collected and centrifuged for 5 min at $500 \times g$. The supernatant was poured off, cells were then resuspended in 1 ml Dulbecco's Modified Eagle Medium and aliquoted into four separate tubes of 250 μ l each. Annexin V-FITC (BD Biosciences Pharmingen, San Jose, CA) and PI (Sigma-Aldrich, St. Louis, MO) dyes were added to the four tubes as follows: Negative control (no Annexin V and no PI), Annexin V control (5 μ l of Annexin V only), PI control (20 μ l of PI only), Test (5 μ l of Annexin V + 20 μ l of PI). The cells and dye were incubated in the dark for 5 min and were then evaluated by flow cytometry to quantify apoptosis. Flow cytometry acquisition was performed on a BD FACS Aria I (BD Bioscience) and data were analyzed with FlowJo Software (TreeStar, Ashland, OR). Three different gates were used to isolate singlet cells and exclude aggregate cells. The first gate was drawn on a forward scatter pulse area (FSC-A) versus side scatter pulse area (SSC-A) plot and included all events except debris. The second gate was drawn on a forward scatter pulse width (FSC-W) versus a forward scatter pulse height (FSC-H) plot to exclude most aggregated cells. The third gate was drawn on a side scatter pulse width (SSC-W) versus side scatter pulse height (SSC-H) plot to exclude remaining aggregated cells missed in the second gate. On the final plot of Annexin V-FITC versus PI, a quadrant gate was drawn using the single-color controls to determine placement. For each sample, 10,000 singlet cells were counted.

Terminal Transferase dUTP Nick End Labeling (TUNEL) Assay

Apoptotic regions in the embryonic heads were localized by TUNEL assay, which visualizes fragmented DNA, one of the hallmarks of apoptosis. Gestation day 9.5 embryo heads from the above treatment groups were fixed in 4% paraformaldehyde in phosphate buffer solution with 0.1% Tween-20 (PBS-T) overnight. Heads were then transferred to 100% methanol and stored at 20°C until the assay. The assay was performed using an In-situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN). The embryos were permeabilized in 70% methanol with PBS-T for 10 min, 100% PBS-T for 10 min, and then

1% Triton X-100 in PBS-T for 2 min on ice to allow the reagents to reach their target molecules. Non-specific activity of endogenous alkaline phosphatase was blocked using 3% bovine serum albumin in PBS-T. Positive controls were prepared by pretreating embryos with DNase to induce DNA strand breaks. Negative controls were prepared by only adding TUNEL labeling solution containing nucleotides (no TdT enzyme) to ensure the staining did not result from nonspecific binding or other reagents such as Converter-AP and substrate solution.

Statistical Analysis

One-way analysis of variance (Statistix 8; Analytical Software, Tallahassee, FL) with the mother as the treatment unit was used to determine differences between the treatment groups. When a significant difference was observed ($p < 0.05$), a Tukey's means comparison was used to determine differences between treatment groups.

RESULTS

NTDs were reduced in the embryos of dams receiving both IFN γ + VA ($21.2 \pm 4\%$) compared to those receiving VA alone ($45.3 \pm 10.4\%$) (Fig. 1). This indicated that prebreeding IFN γ administration reduced the detrimental response to VA administration. No NTDs were observed in either control group or IFN γ -only group. The percentage of apoptotic cells was higher in embryos with open neural tubes from VA-exposed mice compared to controls (PBS injected). A significant increase in apoptosis was also observed in embryos with closed neural tubes from VA-exposed dams compared to controls (Figs. 2 and 3). This indicates that VA caused apoptosis in heads of embryos with both open and closed neural tubes. There was no significant difference in percentage of apoptotic cells between embryos from IFN γ and saline control groups indicating IFN γ itself did not affect amounts of apoptosis. There was no significant difference in percentage of apoptotic cells between the IFN γ + VA treated embryos with closed neural tubes compared to saline or IFN γ controls. This indicates that maternal immune stimulation was able to prevent apoptosis in embryos with closed neural tubes. Apoptosis was significantly increased in IFN γ + VA treated embryos with open neural tubes compared to controls (Figs. 2 and 3), which indicates IFN γ failed to prevent VA-induced apoptosis in embryos with open neural tubes.

Embryos from saline-injected control mice exhibited slight apoptotic staining, as detected by TUNEL, along the midline of the closed neural tube. Darker apoptotic staining was observed along the open neural folds of the embryos from VA-treated dams. This localizes the increased apoptosis observed by flow cytometry to the midline of the neural folds. Apoptotic staining was observed along the entire neural fold in VA-exposed embryos and was most pronounced in midbrain/hindbrain region (Fig. 4). Embryos with closed neural tubes from VA-treated dams also exhibited apoptosis along the midline but to a lesser degree than in embryos with open neural tubes.

Embryos from dams treated with IFN γ exhibited slight apoptotic staining along the midline similar to saline controls. Embryos with closed neural tubes from IFN γ + VA dams also showed low apoptotic staining similar to saline controls (Fig. 5). In IFN γ + VA exposed

embryos with open neural tubes, there was increased staining compared to IFN γ + VA with closed NT. The staining was particularly prevalent in the midbrain/hindbrain region of the neural fold (Fig. 5), similar to embryos with open neural tubes from VA-exposed dams.

DISCUSSION

Neural tube closure involves cell migration, proliferation, differentiation, and apoptosis (Sabapathy et al., 1999). Disruption of these processes can cause NTDs. A certain amount of cell death occurs normally during formation of the neural tube in mice (Schluter, 1973). Neural folds elevate dorsally and medially and appose to each other to fuse. During fusion, the continuity between surface ectoderm and neuroepithelium has to be restructured by apoptosis so that the opposing neural folds can converge to form the neural tube (Copp, 2005). This tissue remodeling by apoptosis is a tightly controlled process. Alteration of this process in several knockout mouse strains demonstrated neural tube closure defects associated with apoptosis-related genes (Hsu et al., 2008; Shirane et al., 2008; Wong et al., 2008; Barrier et al., 2009). Insufficient number of cells resulting from increased apoptosis may affect morphogenetic processes that are critical to neural tube closure (Massa et al., 2009).

During embryonic development, cells proliferate rapidly and are vulnerable to teratogens. Exposure to teratogens can cause programmed cell death (Sulik et al., 1988). Many studies detected increased apoptosis after exposure to various teratogens suggesting a correlation between increased apoptosis and teratogen-induced fetal malformations (Torchinsky et al., 1995; Savion et al., 2003; Singh et al., 2005). Increased apoptosis was observed in developing embryos exposed to cyclophosphamide (Chen et al., 1994; Torchinsky et al., 1995; Savion et al., 2003; Singh et al., 2005), arsenic (Li et al., 1998), salicylic acid (Singh et al., 2009), homocysteine (Li et al., 1999) and these studies proposed a relation between increased apoptosis and teratogenesis. Exposure to retinoic acid (Sarkar and Sharma, 2002) and nicotine (Zhao et al., 2005) resulted in apoptosis in the neuroepithelium of developing mouse embryos suggesting apoptosis as a possible mechanism for teratogen-induced NTDs. Correlations among infectious diseases, apoptosis, and fetal malformations were also reported (Lee and Bowden, 2000; Chen et al., 2009).

Increased apoptosis as a mechanism for VA-induced teratogenicity has also suggested in cell culture and inferred by gene expression studies but has not been clearly elucidated in vivo. Di Renzo et al. (2010) showed a relationship between axial skeletal malformations and increased apoptosis in somites caused by VA exposure in CD-1 mice. VA has also been shown to cause apoptosis of uterine and ovarian cells in rats (Cansu et al., 2010). In embryo culture, VA caused oxidative stress and led to increased cell death (Tung and Winn, 2011). Cell culture studies have demonstrated increased oxidative stress and apoptosis following VA exposure (Defoort et al., 2006). Inhibition of histone deacetylase (HDAC) increased sensitivity to apoptotic stimuli (Pei et al., 2004; Kisseberth et al., 2008; Bai et al., 2011). VA is known to inhibit HDAC and has induced apoptosis in various cell lines (Hubaux et al., 2010; Papi et al., 2010). Jergil et al. (2009) demonstrated a dose-dependent increase in cell death in VA-treated P19 embryonal carcinoma cells. Evaluation of gene expression in these

VA-treated carcinoma cells indicated approximately 200 altered genes; 30% of which were involved in NTD formation.

Few studies showed that protective agents against teratogen-induced fetal malformations act by decreasing teratogen-induced apoptosis. Oyama et al. (2009) showed increased apoptosis in hindbrain region of mouse embryos from diabetic dams compared to controls and these apoptotic levels decreased when the diabetic dams were treated with folic acid. Jiang et al. (2008) showed an increase in apoptotic cells by TUNEL staining in cranial neural tube of embryos from diabetic mice. Apoptosis was decreased in embryos from iNOS (inducible nitric oxide synthase) treated diabetic mice suggesting iNOS may be reducing the NTDs by decreasing the apoptosis in embryos from diabetic mice (Sugimura et al., 2009). These studies indicated that the reduction of teratogen-induced apoptosis can potentially play a role in prevention of fetal malformations.

Nonspecific stimulation of the maternal immune system in mice reduces a wide variety of teratogen-induced fetal malformations (Hrubec et al., 2012). Many different immunologically active agents and stimulation regimes have proved effective in reducing fetal malformations. The mechanism(s) by which broad-spectrum activation of the immune system reduces birth defects has not been elucidated. Specific pathways responsible for the protective effects have not been established. In some cases, the immune stimulant is given hours before the teratogen exposure and in other instances immune activation occurs weeks before teratogen exposure. This implies that both acute and long-term alterations in immune status may be involved in the protective mechanism.

Initial studies by Nomura et al. (1990) that showed stimulation of maternal immune system reduces fetal malformations hypothesized that maternal immune cells cross placenta to remove cells that were detrimentally affected by teratogens, however, later studies disproved this notion. Later studies suggested several potential mechanisms by which maternal immune system can prevent teratogen-induced fetal malformations. Damage to the placental structure can expose the developing embryo to teratogens present in the maternal circulation. Maternal immune stimulation may prevent birth defects by reducing the placental damage caused by teratogens (Sharova et al., 2003; Prater et al., 2004; Laudermitch et al., 2005). VA did not cause any histological changes in placenta in mice (Hrubec et al., 2006a) suggesting placenta may not be involved in VA teratogenicity. On the other hand, many maternal cytokines can cross the placenta and reach the embryos. It is possible that increased levels of maternal cytokines resulting from immune stimulation prevent teratogenic effects on normal morphogenic processes such as cell proliferation and apoptosis in the embryo (Hrubec et al., 2012).

Studies employing different methods of immune stimulation and a variety of teratogens demonstrated that apoptosis was altered in offspring of immune-stimulated dams. Sharova et al. (2000) showed that maternal immune stimulation reduced urethane-induced cleft palate and reduced expression of genes related to apoptosis and cell proliferation (Sharova et al., 2000). Savion et al. (2003) found that maternal immune stimulation prevented cyclophosphamide-induced apoptosis in the head region of developing mouse embryos and suggested reduced apoptosis as the protective mechanism. Similarly, Toder et al. (1996)

showed a protective effect of immune stimulation on cyclophosphamide-induced limb defects. In this study, cyclophosphamide induced limb bud apoptosis; maternal immune stimulation prevented apoptosis and reduced the malformations. Heat-shock also induced apoptosis causing NTDs and eye abnormalities. Maternal immune modulation prevented the heat-shock-induced alterations in apoptosis and also reduced the incidence of these fetal malformations (Yitzhakie et al., 1999).

In our study, flow cytometric analysis of cells from VA-treated embryonic heads with open neural tubes revealed increased apoptosis compared to saline injected controls. This correlated with increased apoptotic TUNEL staining along the neural folds of the VA-exposed embryos compared to controls. These findings localize the increased apoptosis to neural folds suggesting VA-induced apoptosis may lead to NTD formation. In contrast, Kultima et al. (2004) observed a transverse band of apoptotic cells in forebrain region following VA exposure in GD 10 embryos. Kultima et al., measured apoptosis 48 hr post exposure to VA while we measured apoptosis 36 hr after VA exposure, the additional time may have allowed for removal of apoptotic cells from the neural tube area. Exuberant apoptosis in the neural folds as a result of VA exposure could provide insufficient tissue for normal fold elevation and apposition resulting in NTDs.

Increased apoptosis from VA exposure possibly resulted from a protective mechanism in the embryo that removes damaged cells and replaces them with new cells. If the ratio of damaged cells and live cells is too high, the beneficial effects of apoptosis become detrimental causing too many cells to be removed and disrupting normal organogenesis (Levy et al., 2001). In addition to induction of apoptosis, VA also inhibits cell proliferation rates (Byun et al., 2009; Voisard et al., 2010). Decreased cell proliferation rates coupled with increased apoptosis in embryos may reduce the number of viable cells in VA-exposed embryos. Low numbers of viable cells in the neural fold could result in failure of neural tube closure thus causing NTDs. This idea was supported by microarray analysis of VA-treated mouse embryonic carcinoma cell lines (Jergil et al., 2009) and in VA exposed mouse embryos (Okada et al., 2005) where both cell cycle arrest genes and apoptosis genes were altered.

Interestingly, apoptotic levels, measured by flow cytometry, were also increased in VA-exposed embryos with closed neural tubes, indicating VA enhanced apoptosis but did not result in NTDs in these embryos. These flow results were corroborated by TUNEL assay, which revealed increased apoptosis along the midline of the neural fold of the VA embryos with closed neural tubes. Dawson et al. (2006) observed changes in Bcl-2 family genes toward a proapoptotic pattern in VA-exposed embryos with both open and closed neural tubes with no significant difference between these two groups indicating VA disrupts apoptotic pattern in all exposed embryos. Apoptotic levels were not measured by these authors, however.

We observed, low TUNEL staining in control and IFN γ -only treated dams concomitant with low apoptotic levels detected by flow cytometry. This suggests that IFN γ treatment by itself did not affect apoptosis. IFN γ significantly reduced the rates of VA-induced NTDs from 45.3 to 21.2%. No significant difference in apoptotic levels was observed in embryos with

closed neural tube from IFN γ + VA treated dams compared to controls. Also, apoptotic TUNEL staining in these embryos was similar to controls. Levels of apoptosis similar to controls in these embryos suggest that prebreeding immune stimulation prevented VA-induced teratogenicity by increasing the resistance to VA-induced apoptosis in these embryos.

In embryos with open neural tubes from immune stimulated dams, apoptotic levels as determined by flow cytometry were found to be significantly higher compared to embryos from control (IFN γ only) dams. Apoptotic TUNEL staining also appeared darker along the neural fold edges in these embryos. This suggests that maternal immune stimulation failed to reduce apoptotic cells and also did not prevent NTDs in these embryos. One possible explanation for these results is that the embryos that did display NTDs had greater teratogenic damage (higher levels of apoptosis). The immune stimulation may prevent malformations in mildly affected embryos, but might not prevent malformations in severely affected embryos. This would cause embryos to exhibit relatively higher levels of apoptosis compared to a group containing both mildly and severely affected embryo. Alternatively, VA could have induced irreversible DNA damage to the neuroepithelial cells in these embryos that could not be repaired or prevented resulting in higher levels of apoptosis. Few studies have shown that HDAC inhibitors such as VA together with IFN γ initiate apoptosis in cancer cell lines. However in our study, we have seen the opposite effect where the IFN γ decreased the apoptosis in embryos that are exposed to VA (Häcker et al., 2009).

Overall we demonstrated increased apoptosis in embryos from VA-exposed dams by both flow cytometry and TUNEL assay. The increase in apoptosis was localized to midline of the neural tube closure in these embryos suggesting involvement of apoptosis in teratogenicity of VA in inducing NTDs. The observation of increased apoptosis in normal embryos from VA-exposed dams suggests that there may be other cellular processes, such as cell proliferation and differentiation, which might be affected in addition to apoptosis. Increased apoptosis together with inhibition of proliferation may have resulted in fewer cells available during neural tube closure resulting NTDs. Apoptotic levels in embryos with closed neural tubes from IFN γ + VA dams were similar to controls suggesting resistance to teratogen-induced apoptosis as a protective mechanism for maternal immune stimulation. This protection may be related to decreased apoptosis in these embryos. In the embryos that maternal immune stimulation failed to regulate apoptosis, exposure to VA resulted in NTDs.

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Fig. 1.

Incidence of neural tube defects ($\pm$ SE) seen in gestational day 9.5 mouse embryos caused by maternal valproic acid exposure (500 mg/kg IP), and reduction in these defects with maternal immune stimulation. Dams were immune stimulated with IFN γ (1000 units, IP injection). $N=9-12$ litters per group. Different letters indicate treatments are significantly different.

Fig. 2.

Percentage of apoptotic cells in embryo heads (GD 9.5) from saline control ($n = 10$ litters), VA ($n = 10$ litters), IFN γ ($n = 8$ litters), and VA + IFN γ ($n = 8$ litters) exposed dams. Embryos from VA and VA + IFN γ treatment groups were separated based on the open and closed neural tubes. Statistical significance was set at $p < 0.05$. Different letters indicate groups are significantly different, determined by one-way analysis of variance.

Fig. 3.

Apoptotic changes in the heads of GD 9.5 embryos exposed to VA with and without maternal immune stimulation. Histograms are from representatives of (A) embryos from control dams; (B) embryos with closed neural tubes from VA-exposed dams; (C) embryos with open neural tubes from VA-exposed dams; (D) embryos from IFN γ dams; (E) embryos with closed neural tubes from IFN γ + VA exposed dams; (F) embryos with open neural tubes from IFN γ + VA exposed dams. The x-axis represents Annexin-V FITC staining intensity and y-axis represents Propidium Iodide (PI) staining intensity. In each histogram, lower left quadrant shows viable cells (stained negative for Annexin-V or PI), lower right quadrant shows apoptotic cells (stained positive for Annexin-V only), and upper right quadrant shows dead cells (stained positive for both Annexin-V and PI). Increased apoptotic cells can be observed in (lower right quadrant) embryos from VA-exposed dams and embryos with open neural tubes from IFN γ + VA exposed dams compared to embryos from control, IFN γ -exposed dams, and also embryos with closed neural tubes from IFN γ + VA exposed dams.

Fig. 4. Identification of apoptotic regions in the heads of GD 9.5 mouse embryos from VA-exposed dams by Terminal Transferase dUTP Nick End Labeling (TUNEL) assay in different views. (AC) Embryos from control group; (D and F) embryos with closed neural tubes from VA group; (G–I) embryos with open neural tubes from VA group. Purple stained dots indicate apoptotic regions.

Fig. 5. Identification of apoptotic regions in the heads of GD 9.5 mouse embryos from IFN γ + VA group by Terminal Transferase dUTP Nick End Labeling (TUNEL) assay in different views. (J–L) Embryos from IFN γ group; (M–O) embryos with closed neural tubes from IFN γ + VA dams; (P–R) embryos with open neural tubes from IFN γ + VA dams. Purple stained dots indicate apoptotic regions.