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Prevention of valproic acid-induced neural tube defects by sildenafil citrate

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ABSTRACT

This study was undertaken to test the effects of sildenafil citrate (SC), a type 5 phosphodiesterase inhibitor, on valproic acid (VPA)-induced teratogenesis. On gestation day (GD) 8, ICR (CD-1) mice were treated by gastric intubation with SC at 0 (vehicle), 1.0, 2.5, 5.0 or 10 mg/kg. One hour later, animals received a teratogenic dose of VPA (600 mg/kg) or vehicle. Developmental endpoints were evaluated near the end of gestation. Twenty-eighth percent of fetuses exposed to VPA had neural tube defects (exencephaly). Pretreatment with SC at 2.5, 5.0 or 10 mg/kg significantly reduced the rate of VPA-induced exencephaly to 15.9%, 13.7%, and 10.0%, respectively. Axial skeletal defects were observed in 75.8% of VPA-exposed fetuses. Pre-treatment with SC at 10 mg/kg, but not at lower doses, significantly decreased the rate of skeletally affected fetuses to 61.6%. These results show that SC, which prolongs nitric oxide (NO) signaling action protects from VPA-induced teratogenesis.

1. Introduction

Valproic acid (2-propylpentanoic acid, VPA), with inhibitory activity of histone deacetylases (HDAC), is commonly prescribed to treat epilepsy and bipolar disorders. In addition, VPA is currently being tested as an anticancer agent [1]. Unfortunately, in utero exposure to VPA increases the risk of delivering children affected by major malformations, including neural tube defects, heart defects, craniosynostosis, and skeletal malformations [2–6]. Prenatal exposure to VPA has been linked not only to abnormal phenotypes, but also to increased risk of neurodevelopmental impairment [7,8]. The developmental toxicological profile of VPA has been well-characterized using experimental animal models. Teratogenic effects appear to be dose- and stage dependent with an elevated prevalence of exencephaly (the forerunner of anencephaly) [9–13]. VPA is known to bind to serum proteins and to freely cross placental barrier [14]. VPA itself and not its metabolites appear to be responsible for teratogenic effects [15]. Despite its long-standing usage, the mechanism of VPA teratogenicity is still poorly known. Several mechanisms have been proposed, including disturbances in folic acid metabolism, increased oxidative stress, and inhibition of HDAC [5]. We became recently interested in characterizing the effect of NO status on VPA-induced teratogenesis. A previous study from this laboratory evaluated the relationship between inhibition of NO synthesis and induction of congenital malformations by VPA, and found that pre-treatment of pregnant mice with a sub-teratogenic dose of the non-selective NO synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) enhances VPA-induced teratogenesis [17]. Phosphodiesterase inhibitors are a class of drug with the capacity of preventing the hydrolysis of cyclic guanosine monophosphate (cGMP) to guanosine monophosphate (GMP), and thereby enhancing NO/cGMP-mediated effects. In this study, we tested the hypothesis that SC, a type 5 inhibitor of phosphodiesterase, is able to prevent the teratogenic effects elicited by VPA. We undertook this study using an established mouse model of congenital malformations of the neural tube and of the axial skeleton induced by VPA.

2. Materials and methods

2.1. Animal husbandry and breeding procedure

Sexually mature and pathogen free ICR (CD-1) mice (24–26 g) were obtained from Harlan Italy (Udine, Italy). Upon arrival, animals were housed in polycarbonate cages and quarantined for at least one week before mating. Rodent laboratory chow (Altromin-MT®, Italy) and filtered tap water were provided ad libitum. Animal room was maintained at 22 ± 1 °C with a relative humidity of 55 ± 5%. The photoperiod was 12 h of artificial light and 12 h of darkness. Timed mating was produced by placing individual male

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Table 1
Developmental effects observed in ICR (CD-1) mice after combined treatment with valproic acid (VPA)* and sildenafil citrate (SC).**

	Sham treated	VPA	VPA + SC 1.0 mg/kg	VPA + SC 2.5 mg/kg	VPA + SC 5.0 mg/kg	VPA + SC 10 mg/kg
Number of pregnant animals treated	10	20	14	16	18	15
No. of implantation sites	126	320	242	257	286	217
No. of fetuses examined	110	244	186	176	205	180
Embryo/fetal lethality	15/126 (12.7%)	76/320 (23.8%)$^{\phi}$	56/242 (23.1%)	71/257 (27.6%)	81/286 (28.3%)	37/217 (17.0%)$^{¥}$
Mean fetal body weight per litter (g ± SD)	1.31 ± 0.11	1.13 ± 0.13$^{\phi}$	1.18 ± 0.11	1.16 ± 0.17	1.16 ± 0.08	1.20 ± 0.13
Fetuses with neural tube defects (exencephaly)	0	68 (27.8%)$^{\phi}$	47 (25.3%)	28 (15.9%)†	28 (13.7%)†	18 (10.0%)†
Fetuses with axial skeletal defects	0	185 (75.8%)$^{\phi}$	135 (72.6%)	128 (73.5%)	143 (76.8%)	111 (61.6%)$^{\bullet}$
Fetuses with facial/palate clefting	0	14 (5.7%)$^{\phi}$	15 (8.0%)	16 (9.0%)	4 (1.9%)‡‡	8 (4.4%)
Fetuses with limb defects	0	2 (0.8%)	0	0	0	0
Fetuses with anus imperforatus	0	2 (0.8%)	0	0	0	2 (1.1%)
Fetuses with short tail	0	3 (1.2%)	2 (1.1%)	0	0	0
Open eyelids	0	66/244 (27.0%)$^{\phi}$	53/186 (28.5%)	32/176 (18.2%)¶	43/205 (20.9%)	24/180 (13.3%)¶

* Injected intraperitoneally on gestation day 8 at 600 mg/kg.
** Administered by gavage on gestational day 8 at 1.0, 2.5, 5.0 or 10 mg/kg 1 h before the treatment with VPA.
§ Treated with vehicles.
$^{\phi}$ Statistically significant ($p < 0.05$) vs. sham treated group.
¥ Statistically significant ($p < 0.05$) vs. VPA and VPA + SC at 1.0, 5.0 and 2.5 mg/kg groups.
† Statistically significant ($p < 0.05$) vs. VPA, and VPA + SC 1.0 mg/kg groups.
‡‡ Statistically significant ($p < 0.05$) vs. VPA and VPA + SC at 1.0 and 2.5 mg/kg groups.
• Statistically significant ($p < 0.05$) vs. VPA, and VPA + SC 1.0 and 2.5 mg/kg groups.
¶ Statistically significant vs. VPA, and VPA + SC 1.0 mg/kg groups.

of the same stock into cages containing three/four females for the dark cycle. Detection of vaginal plug (taken as evidence of mating) at the end of the dark cycle (8:00 a.m.) was used to designate GD 0. The study was conducted in agreement with the Italian legislation on animal experiment and was approved by the ethical committee for animal experiment of the University of Chieti-Pescara.

2.2. Experimental procedure

VPA (sodium salt, sodium valproate; Sigma; Milan, Italy) was dissolved in sterile saline solution. SC (Viagra™), a product of Pfizer (New York, U.S.A.), was dissolved in distilled water. A dosage volume of 10 ml/kg was used for both agents. On GD 8 animals were administered by intra-gastric intubation (gavage) with a single dose of 0 (vehicle) 1.0, 2.5, 5.0 or 10 mg/kg of SC citrate. One hour after SC administration, mice received a single intraperitoneal injection of 0 (vehicle) or 600 mg/kg of VPA. The VPA dose was selected on the basis on a pilot study, as level of exposure with the capacity of inducing an elevate incidence of neural tube defects. Pregnancies were terminated near term, on gestation day 18, and the selected end-points, including embryo lethality, number of living fetuses, fetal weight and gross malformations were recorded. Fetuses were then prepared for double-staining skeletal examination using the methods of Inouye [18], Kimmel and Trammel [19] as modified by Kuczuk and Scott [20]. Briefly, skinned and eviscerated fetuses were placed in 95% ethanol for 3–5 days. The ethanol was then drained and replaced with Alcian blue stain (800 ml of 95% ethanol, 200 ml of glacial acetic acid, and 150 mg of Alcian blue) to stain cartilage. Twenty-four hours later, specimens were rinsed twice in 95% ethanol, and placed in 1% solution of potassium hydroxide (KOH) for 2–6 h to allow maceration of soft tissues. Thereafter, fetuses were exposed to alizarin red S (50 mg in 1 L of 2% KOH) for 1 h to stain the bone. Processing the specimens through a series of 2% KOH + glycerin solutions (60% KOH + 40% glycerin; 40% KOH + 80% glycerin; 20% KOH + 80% glycerin) served to clear the skeletal preparations. All morphologic evaluations were carried out under a stereomicroscope.

2.3. Statistical analysis

For statistical analysis, continuous data were compared using Student's *t*-test or ANOVA and post hoc Student–Newman–Keuls test for multiple comparisons. Binomial data were compared using the chi-square test. Differences were considered statistically significant when $p < 0.05$.

3. Results

Table 1 summarizes the gestational outcome observed in control and treated animals. Regarding embryo-lethality, a frequency of 23.8% was recorded in the VPA group. This level was significantly higher in comparison to the control (sham treated) group, that showed an embryonic loss of 12.7%. SC significantly decreased VPA-induced embryo lethality when administered at 10 mg/kg, but not at lower doses. The mean fetal weights of VPA-exposed fetuses were significantly lower in comparison to the sham treated group, and SC co-treatment did not prevented fetal weight restriction imposed by VPA. The teratogenic spectrum caused by VPA exposure consisted mainly in exencephaly (the forerunner of anencephaly) and axial skeletal defects (described in Table 2). Twenty-eighth percent (68/244) of fetuses from mother injected with VPA were exencephalic. While no significant reduction of exencephaly rate resulted by co-treatment with SC citrate at 1.0 mg/kg, SC at 2.5, 5.0 or 10 mg/kg significantly reduced the frequency of exencephalic fetuses to 15.9% (28/176), 13.7% (28/205) and 10.0% (18/180), respectively (Fig. 1). In several cases, exencephalic fetuses also displayed palate and/or craniofacial clefting. Cleft palate was also noted in fetuses without exencephaly. Twenty-seven percent of VPA exposed fetuses had open eyelids. SC significantly reduced the incidence of this abnormal phenotype when administered at 2.5 and 10 mg/kg. Observation of skeletal preparations revealed that 75.8% (185/244) of VPA-exposed fetuses had malformations of the axial skeleton. SC at 10 mg/kg resulted in a significant 14% reduction of the percentage of skeletally affected fetuses in comparison to the VPA group. No effect resulted from SC at 1.0, 2.5 or 5.0 mg/kg. The spectrum of vertebral anomalies included fused, asymmetric, and cleaved vertebrae, and vertebrae with asymmetric, cleaved, and dumbbell-shaped centrum. Abnormal vertebral phenotypes were found in thoracic, lumbar, sacral, and caudal vertebrae. Rib malformations consisted mainly of rib fusion.

4. Discussion

The gaseous molecule NO is synthesized from L-arginine by a family of three nitric oxide synthases (NOS), and mediates most of its action activating soluble guanyl cyclase to convert GTP to