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MORPHOLOGICAL AND MORPHOMETRIC ALTERATIONS INDUCED BY VALPROIC ACID ON RAT FETUSES' MECKEL'S CARTILAGE, LINGUAL MUSCULATURE, AND SUBMANDIBULAR GLAND

ALTERACIONES MORFOLÓGICAS Y MORFOMÉTRICAS INDUCIDAS POR ÁCIDO VALPROICO EN CARTÍLAGO DE MECKEL, MUSCULATURA LINGUAL Y GLÁNDULA SUBMANDIBULAR DE FETOS DE RATAS

***,**Selma Siéssere; *Marisa Semprini; *Ruberval A. Lopes; *Miguel A. Sala; & *Maria G. C. Mattos**

* College of Dentistry of Ribeirão Preto / São Paulo University, Ribeirão Preto, SP, Brazil.
** College of Dentistry of Uberaba / Uberaba University, Uberaba, MG, Brazil.
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Correspondence to:

SUMMARY:Valproic acid, an antiepileptic drug, is a well-known teratogenic agent; its main target organ is the neural tube, though organ malformations have also been described. The aim of the present work was to analyze the effects of valproic acid on embryo/fetal oral structures: Meckel's cartilage, muscles of the tongue, and submandibular gland. Rats received a intraperitoneal injected of Valproic acid (300mg/Kg) on the 10th day of their gestation. Females were sacrificed on day 20 post coitus, and fetuses were examined.

No gross malformations were observed. The following morphological alterations were observed: lower body and placental weight; shorter umbilical cord length; submandibular gland parenchyma less differentiated with larger cellular cord and terminal buds, and increased nuclear volume; lingual muscles fibers disorganized and of smaller size, and smaller nuclear volume; rudimentary Meckel's cartilage with small chondrocytes and more abundant matrix. These data suggest that alterations in oral tissue, induced by valproic acid, result from a direct effect of the drug on tissues causing a delayed differentiation.

KEY WORDS: 1. Valproic acid; 2. Rat; 3. Meckel's cartilage; 4. Lingual musculature; 5. Submandibular gland.

RESUMEN:El ácido valproico es una droga antiepiléptica, bien conocida como agente teratogénico; su principal órgano blanco es el tubo neural, aunque malformaciones de otros órganos también han sido descritas. El propósito del trabajo fue analizar los efectos del ácido valproico en las estructuras orales embriofetales: Cartílago de Meckel, músculos de la lengua y glándula submandibular. Las ratas recibieron una inyección diaria intraperitoneal de ácido valproico (300 mg/Kg), durante 10 días de su gestación. Las ratas hembras fueron sacrificadas al 20 día post coito y fueron examinados los fetos. No fueron observadas malformaciones macroscópicas. Fueron observadas las siguientes malformaciones: bajo peso del cuerpo y de la placenta; cordón umbilical corto; parénquima de la glándula submandibular menos diferenciada con gran cordón celular y ramificaciones terminales e incremento del volumen nuclear; las fibras de los músculos de la lengua desorganizados y de menor tamaño y de volumen nuclear pequeño; cartílago de Meckel rudimentario, con pequeños condrocitos y muy abundante matriz. Estos datos sugieren que alteraciones en el tejido oral, inducidos por ácido valproico, como resultado de un efecto directo de la droga en los tejidos, provoca una diferenciación retardada de ellos.

PALABRAS CLAVE: 1. Acido valproico; 2. Rata; 3. Cartílago de Meckel; 4. Musculatura lingual; 5. Glándula submandibular.

INTRODUCTION

Valproic acid (VPA) has been shown to be a teratogenic in animals and man. In man, VPA exposure during pregnancy may be related to an increased incidence of ocular abnormalities (Glover *et al*., 2002), lumbosacral neural tube defects (bifid spine); as well as a spectrum of major malformations of the skeleton and at the level of the cardiovascular and urogenital system (Koch *et al*., 1983; Winter *et al*., 1987; Rodriguez-Pinilla *et al*., 2000; Kozma, 2001).

Multiple congenital malformations, including generalized hypertrichosis with gum hypertrophy, were observed in a child that had been exposed to valproic acid while in the womb (Stoll *et al*., 2003).

There are records of neural tube defects (bifid spine and exencephaly) in mice (Ehlers *et al*., 1992), and occult bifid spine in rats (Ceylan *et al*., 2001).

Skeletal defects, both facial and in the axial skeleton, have been observed in all tested species: mice, rats, and rabbits (Ong *et al*., 1983; Nau, 1985; Petrere *et al*., 1986; Ehlers *et al*.; Menegola *et al*., 1996).

The goal of the present study was to investigate the histopathological alteration caused by VPA in rats, on their submandibular gland, Meckel's cartilage, and tongue musculature, using appropriate karyometric and stereologic techniques.

MATERIAL AND METHOD

Virgin female Wistar rats were housed in plastic cages and maintained in climate-controlled room under an alternating 12-hr light/dark cycle. Timed pregnancies were obtained by placing 2 females with singly caged males over night. Detection of vaginal spermatozoids was used to designate gestational day 1.

Pregnant females were treated intraperitoneally with saline (control) and with 300mg/Kg of VPA (3 times a day) on the 10th day of gestation. All females were killed on day 20 of gestation by ether inhalation, the abdomen was opened, and uterine contents were removed. The fetuses were collected, which were immediately immersed in a fixative solution consisting of alcohol (85ml), formalin (10ml), and acetic acid (5ml), and fixed for as long as 24 hours. After fixation, the heads were separated from the bodies, embedded in paraffin, cut into serial 6µm sections and stained with hematoxylin and eosin. The following karyometric parameters were measured in the drawing of each nucleus of submandibular acini and ducts, and of lingual muscular fibers: longest, shortest, and mean diameters, the relation between longest and shortest diameters, perimeter, area, volume, the relation between volume and area, form coefficient, contour index and eccentricity (Sala *et al*., 1994).

The outlines of 50 chondroplasts projected with a camara lucida (Zeiss Jena) at a final magnification of 1000X, were drawn with soft pencil. The following morphometric parameters were used: axis, perimeter, area, and volume. Data were analyzed statistically by Mann-Whitney non-parametric test.

RESULTS

The fetuses that received valproic acid during pregnancy showed significantly lower body weight (1.32g), placental weight (320.6mg), and umbilical cord length (1.9cm) than control fetuses (4.78g, 541.9mg, and 2.4cm, respectively), but no gross malformations were observed in this work.

Histopathological analysis revealed submandibular gland parenchyma less differentiated in the valproic acid-treated fetuses. The submandibular glands of treated-fetuses consisted of cellular cord and larger terminal buds and increased nuclear volume (Fig. 1). Table I gives mean values for the nuclear parameters: it is possible to observe that all parameters studied showed significant differences between control and treated group. All these findings showed a delayed differentiation of the analyzed gland in treated animals.

The lingual muscles fibers of treated fetuses were disorganized and of smaller size; the round nuclei were of smaller volume (Fig. 2). The mean values of karyometric parameters are seen in Table I. It is possible to observe that all parameters studied showed significant differences between control and treated group.

The Meckel's cartilage of VPA treated fetuses was rudimentary, with small chondrocytes with smaller nuclei. The cartilaginous matrix is more abundant (Fig. 3). Table II gives mean values for chondroplast morphometric parameters: it is possible to observe smaller values for axis (longest, shortest, and geometric), perimeter, area, and volume. These findings showed a delayed differentiation of the analyzed cartilaginous tissue in the treated fetuses.

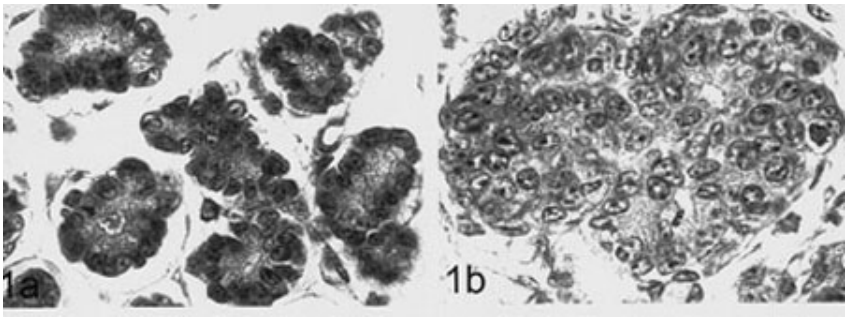

Fig. 1. Histological aspect of the submandibular gland in rat fetuses from the control group (a) and from the treated group (b). Note the formation of acini and ducts evident with visible light, and the beginning of acinic secretion in a; acini and ducts of immature morphology, higher volume nuclei and abundant conjunctive tissues in b. Hematoxylin and eosin (340X).

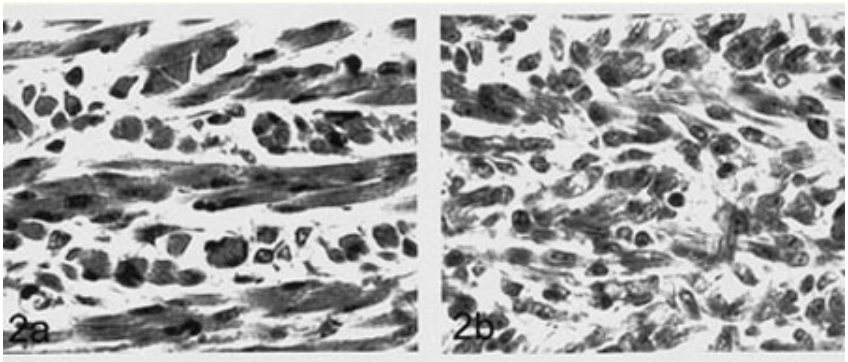

Fig. 2. Histological aspect of tongue muscles of rat fetuses from the control group (a) and from the treated group (b). Note the disposure of bundles of muscular fibers in different directions and the elongated form of its nuclei in a; fibers disorganized and of a smaller size, nuclei with a more rounded form and with smaller volume in b. Hematoxylin and eosin (340X).

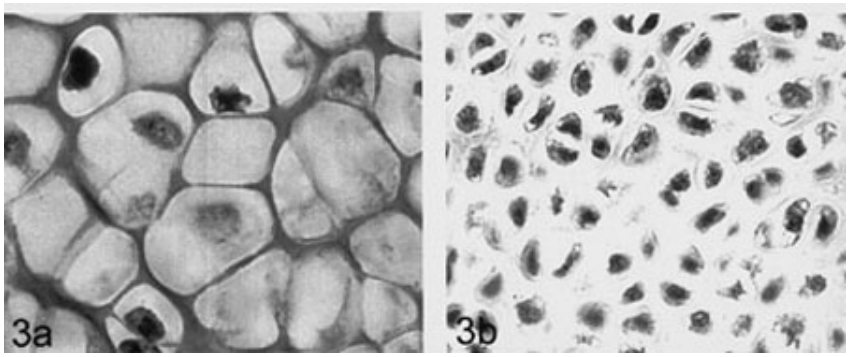

Fig. 3. Histological aspect of the Meckel's cartilage in rat fetuses from the control group (a) and from the treated group (b). Note the matrix's thin trabeculate, with the presence of wide chondroplasts in a; the presence of small chondroplasts and a more abundant matrix in b. Hematoxylin and eosin (340X).

Table I. Mean values of karyometric parameters of submandibular gland acinus, duct, and muscle fiber cell nuclei for control (C) and valproic acid treated (T) Wistar rat fetuses. Mann-Whitney test.

Parameter	Acinus		Duct		Muscle fibers	
	C	T	C	T	C	T
Longest diameter (µm)	5,50	7,44*	5,25	6,80*	11,18	9,67*
Shortest diameter (µm)	4,25	5,18*	4,06	4,66*	3,77	3,93**
Mean diameter (µm)	4,82	6,18*	4,60	5,60*	6,44	6,13**
Ratio D/d	1,32	1,45*	1,31	1,48*	3,08	2,54*
Perimeter (µm)	15,42	20,03*	14,71	18,19*	29,31	16,87*
Area (µm2)	18,43	30,49*	16,87	24,97*	33,28	29,86**
Volume (µm3)	60,44	129,29*	53,34	95,43*	87,61	71,40**
Ratio V/A	3,21	4,12*	3,06	3,74*	2,52	6,45*
Form Coefficient	0,96	0,94*	0,96	0,93*	0,49	1,30*
Contour index	3,61	3,65*	3,61	3,66*	5,14	3,10*
Eccentricity	0,54	0,69*	0,53	0,69*	0,93	0,90*

* Statistically different from the respective control ($\propto$ =0,01)
** Statistically different from the respective control ($\propto$ =0,05)

Table II. Mean values of morphometric parameters of Meckel□s cartilage chondroplast for control (C) and valproic acid treated (T) Wistar rat fetuses. Mann-Whitney test.

Parameter	C	T
Longest diameter (µm)	29,64	13,71*
Shortest diameter (µm)	22,35	9,21*
Mean diameter (µm)	25,65	8,93*
Perimeter (µm)	82,20	36,65*
Area (µm2)	536,85	105,95*
Volume (µm3)	9880,41	934,97*

*Statistically different from the respective control ($\propto$ =0,01)

DISCUSSION

The VPA treated fetuses were developmentally delayed as compared to the controls, with all fetuses having lower body weight, placental weight, and umbilical cord length. Decreased fetal body weight was also observed in mice (Hansen *et al*., 1995; Menegola *et al*.) as well as in rats (Hansen *et al.*).

The concentration of VPA in placental and embryonic tissues were higher than those observed in maternal blood, as observed in monkeys (Dickinsson *et al*., 1979), suggesting that this drug affects the placental tissues, resulting in a smaller placenta, as observed in this work. Smaller placenta has a decreased blood flow, resulting in significant fetal hypoxia and delayed development (Emmanouilides *et al*., 1972).

VPA probably has a direct effect on the organization of the cartilaginous tissue of Meckel's cartilage, resulting in delayed differentiation, as observed in this work, with a reduction in the collagenous concentration and a decrease in the synthesis of proteoglycans (Aulthouse & Daron, 1994).

The mechanism for the drug's muscular and glandular effects is unknown. Probably the VPA exert its effect directly on lingual and submandibular structures. Our results confirm the VPA induced oral alterations previously described in rat fetuses' floor of the mouth (Freitas *et al.*, 1999), tongue (Iyomasa *et al*., 1999) and palate mucous (Sousa *et al.*, 1999), i.e., a picture of delayed differentiation tissues.

Several theories on the mechanism of VPA teratogenesis have been presented; the theory receiving the most experimental attention is that VPA acts through a deficiency of the folic acid vitamin (Lammer *et al*., 1987; Finnel & Dansky, 1991). Padmanabhan & Shafiullah (2003) suggest that plasma levels of folic acid and B 12 have to be kept substantially elevated and maintained high throughout the organogenesis period to protect embryos against VPA induced neural tube defects. A number of studies have noted that VPA doesn't affect glycolysis, DNA, or protein synthesis (Turner *et al*., 1990). In 1996, Barnes *et al.* hypothesize that a candidate set of molecular targets of teratogens are the Pax family of pattern-forming genes, specifically Pax-a, which has been previously demonstrated to be an important regulator of axial skeletal patterning at the somite level.

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Correspondence to:

Prof. Dra. Marisa Semprini
Faculdade de Odontologia de Ribeirão Preto USP
Departamento de Morfologia, Estomatologia e Fisiologia
Avenida do Café, s/n Bairro: Monte Alegre
CEP: 14040-904
Ribeirão Preto SP - BRASIL

e-mail: msemprin@forp.usp.br

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Casilla 54-D

Temuco - Chile

Tel.: (56-45) 232 5571

Fax: (56-45) 232 5600

e-Mail
ijmorpho@ufrontera.cl