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Short Contribution

Stage-dependent skeletal malformations induced by valproic acid in rat

ELENA MENEGOLA, MARIA LUISA BROCCIA, MARIANGELA PRATI, and ERMINIO GIAVINI*

Department of Biology, University of Milan, Milan, Italy

ABSTRACT In this work we study the skeletal teratogenic response in rats exposed to NaVP at different embryonic stages. Crl:CD female rats were treated subcutaneously with 400 mg/Kg b.w. NaVP at presomitic stage (group II) or nearly at 2, 6, 10, 14, 18 or 22 somites (groups III-VIII). The females on group I were treated with saline and served as controls. No treatment-related effects were observed at the level of resorptions, live fetuses and fetal or placental weight. The skeletal examination showed characteristic patterns of malformations strictly related to the period of treatment. In particular, groups II and III showed a significant increase of alterations of cervical vertebrae (mainly 1st to 3rd segment) and a decrease of the frequency of extra lumbar ribs in comparison to control. Group IV showed severe abnormalities localized at the 4th to 7th cervical segment and at the level of the 1st and 2nd thoracic segments, including duplications of thoracic segments 1, 2 or 3. The fetuses of group V were characterized by several alterations of the thoracic segments distributed without a clear specificity. In group VI, the thoracic region was also affected with some specificity at the level of the segments 4th to 9th; in group VII, last thoracic and lumbar segments were affected (mainly duplications) and in group VIII only lumbo-sacral abnormalities were recorded. These results confirm the specific effect of NaVP at the level of the axial skeleton and suggest a possible interaction with the expression of genes identifying the vertebral segments.

KEY WORDS: *valproic acid, skeletal malformations, teratogenicity, axial skeleton, fetus, embryo*

In laboratory animals the administration of VPA early in gestation is associated with an increased incidence of neural tube defects (exencephaly and spina bifida) in mice but not in rats and skeletal defects in all tested species (Kao *et al.*, 1981; Nau *et al.*, 1981; Ong *et al.*, 1983; Nau, 1985; Vorhees, 1987; Binkerd *et al.*, 1988; Hendrickx *et al.*, 1988; Ehlers *et al.*, 1992). Recently Menegola *et al.* (1996) reported that the administration of 150-300 mg/kg NaVP subcutaneously to pregnant mice or rats every 8 h during the first stages of somitogenesis is able to produce a very high incidence of malformations at the level of the axial skeleton: fusions of vertebrae and ribs, extra cervical vertebrae and extra thoracic vertebrae and ribs, agenesis of thoracic vertebrae, extra ribs inserted on the sternum. Some of these skeletal malformations are very similar to those obtained by Kessel and Gruss (1991) and by Kessel (1992) with retinoic acid in mice and by other authors in mice homozygous for Hox genes mutations (Krumlauf 1994; Horan *et al.*, 1995; Rancourt *et al.*, 1995; Saegusa *et al.*, 1996).

Due to the interest of this result, we carried out this experiment in order to better define the pattern of malformations induced at the level of the axial skeleton by NaVP when administered on selected stages of rat embryo development.

Crl:CD female rats were treated subcutaneously with 400 mg/kg NaVP at presomitic stage (group II) or nearly at 2,6,10,14,18 or 22 somite stage (groups III-VIII). The females on group I were treated with saline and served as controls.

Along the axial skeleton of control fetuses, characteristic morphological features have been chosen as landmarks to identify the reciprocal position of the axial segments. In particular, the atlas, the axis and the 6th vertebra, characterized by the presence of the *tubercula anteriora* (tubercula carotica) protruding ventrally, are easily recognizable at the cervical level (Fig. 1A). The thoracic segments are characterized by the presence of ribs, seven of which reach the sternum, inducing the formation of 6 sternebrae, whereas from the 8th rib on their cartilaginous part ends far from the sternum (Fig. 1B). The structure of the neural spine characterizes each thoracic segment: the 2nd vertebra has a very long and cranially-directed spine, from the 3rd to the 9th segment spines are caudally directed, the 10th has a short structure and from the 11th on the spines are cranially directed again (Fig. 1A). The lumbar region lacks ribs and is characterized by the typical transverse processes, cranially extended (Fig. 1C). The lumbar spines are similar to the thoracic ones and are cranially directed (Fig. 1D). The structure of transverse processes changes completely at the

Abbreviations used in this paper: VPA,valproic acid; NaVP, sodium valproate; ES, embryonic stage.

***Address for reprints:** Department of Biology, via Celoria 26, I-20133 Milan, Italy. FAX: 2-2362726. e-mail: tergia@imiucca.csi.unimi.it

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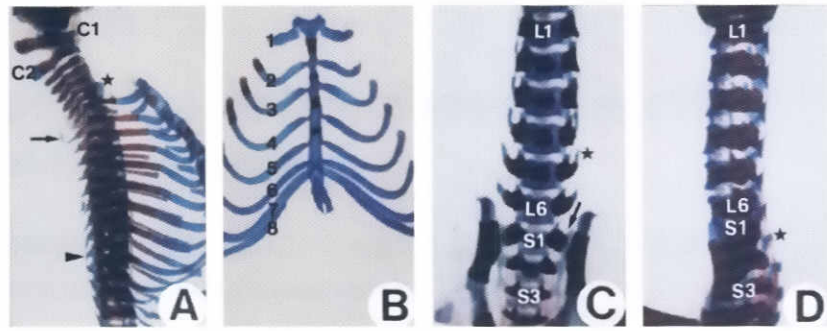


Fig. 1. Control fetuses (group I) stained with alcyan blue for cartilage and with alizarin red for bone. (A) *Lateral view of cervical (C1-C7) and thoracic (T1-T13) segments. Atlas (C1), Axis (C2) and C6 are easily recognizable. The asterisk shows the cartilaginous structure of the tuberculum anterior. At the thoracic level, the structure of the neural spines characterizes each segment. Note the 2nd one (arrow) and the 10th (arrowhead).* **(B)** *Isolated sternum of a control fetus. Seven ribs reach the sternum inducing the formation of six sternebrae (in this fetus the 5th and the 6th are not ossified). The 8th rib ends typically far from the sternum.* **(C)** *Ventral view of lumbar (L1-L6) and some sacral (S1-S3) segments. Transverse processes are cranially directed at the lumbar level (asterisk) and enlarged and directed toward the ileum at the sacral level (arrow).* **(D)** *Lateral view of lumbar (L1-L6) and some sacral (S1-S3) segments. Note the structure of neural spines, characteristically truncated and short at the sacral level (asterisks).*

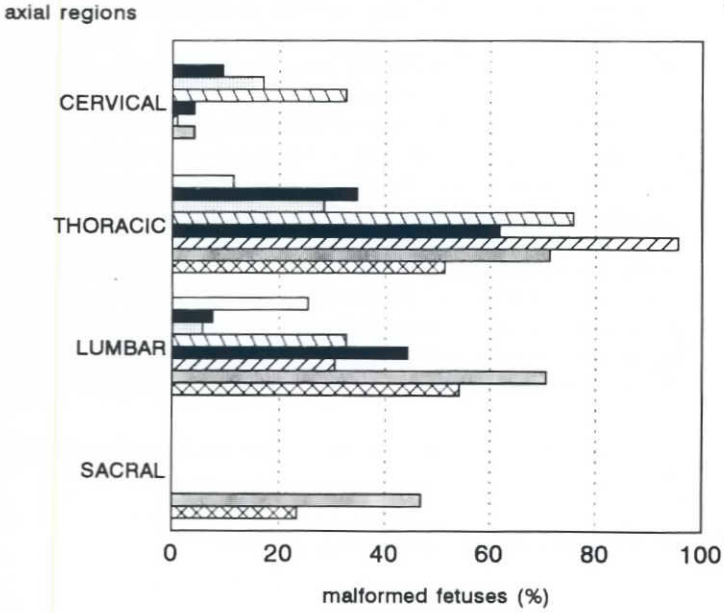
sacral level, where they are involved in the formation of the sacrum and appear enlarged and directed toward the ileum (Fig. 1C). In this region neural spines are typically short and truncated (Fig. 1D).

On the basis of those characteristics, all fetuses of the control group had 7 cervical, 13 thoracic, 6 lumbar and 5 sacral segments. These basic features served as parameter for the identification of morphological deviations in treated fetuses.

No differences were seen between the control and treated groups as far as the dead fetuses and the resorptions are concerned. The fetal weights were lower in all treated groups in comparison to the control one.

A significant increase in malformed fetuses has been observed in all treated groups. The identified malformations were localized only at the axial skeleton level. Their distribution in the different regions (cervical, thoracic, lumbar and sacral) appeared strictly stage-dependent. In particular, an antero-posterior distribution of malformations from group II to group VIII was seen (Fig. 2).

To better understand the complete morphological scenario of the dismorphogenetic effect of NaVP, we have studied the associations between the abnormalities for all embryos.



Group II (NaVP, day 9 h 8:00) and Group III (NaVP, day 9 h 16:00)

No characteristic dismorphogenetic scenario was detectable both for group II and for group III but very often a single malformation affected the fetuses.

The malformations induced by these two treatments were very similar: cervical fusions, removal of the 7th rib from the sternum, reduction in the frequency of fetuses with lumbar rib in comparison to control.

Fetuses from group IV-VIII were typically affected. A characteristic dismorphogenetic scenario was identified for each group.

Group IV (NaVP, day 9 h 24:00)

In this group, 28% of examined fetuses showed partial or complete fusion between the 1st and the 2nd rib, with the insertion of both on the manubrium (Fig. 3A). Ribs from 3rd to 7th resulted cranially shifted (the 3rd, 4th, 5th reached the sternum respectively under the 1st, 2nd, 3rd sternebra, the 8th rib approached or reached the sternal cartilage similarly to the 7th rib in normal fetuses) and the lack of one sternebra was evident. In addition, 17% of fetuses had a lumbar rib (Fig. 3A) and 3% had an extra thoracic vertebra and rib. An extremely similar scenario was also observed in 20% of analyzed fetuses of this group showing reduction or agenesis of the 1st rib (Fig. 3B). An anterior shift of the morphology of ribs 2nd-7th and the loss of one sternebra were related. The 8th rib reached or approached the sternum.

A clear duplication of the second thoracic segment was evident in 12% of examined fetuses (Fig. 3C). In some cases (7%) an extra sternebra was also induced.

Group V (NaVP, day 10 h 8:00)

Morphological alterations at the level of ribs (more often fusions at the 1st-4th rib) were observed in 35% of fetuses. The 8th rib was approaching the sternum (Fig. 3D) and an extra thoracic vertebra (25%) or a lumbar rib (10%) was seen.

Fig. 2. Distribution of the observed malformations in the different axial regions. *An antero-posterior distribution of malformations from group II to group VIII is shown.*

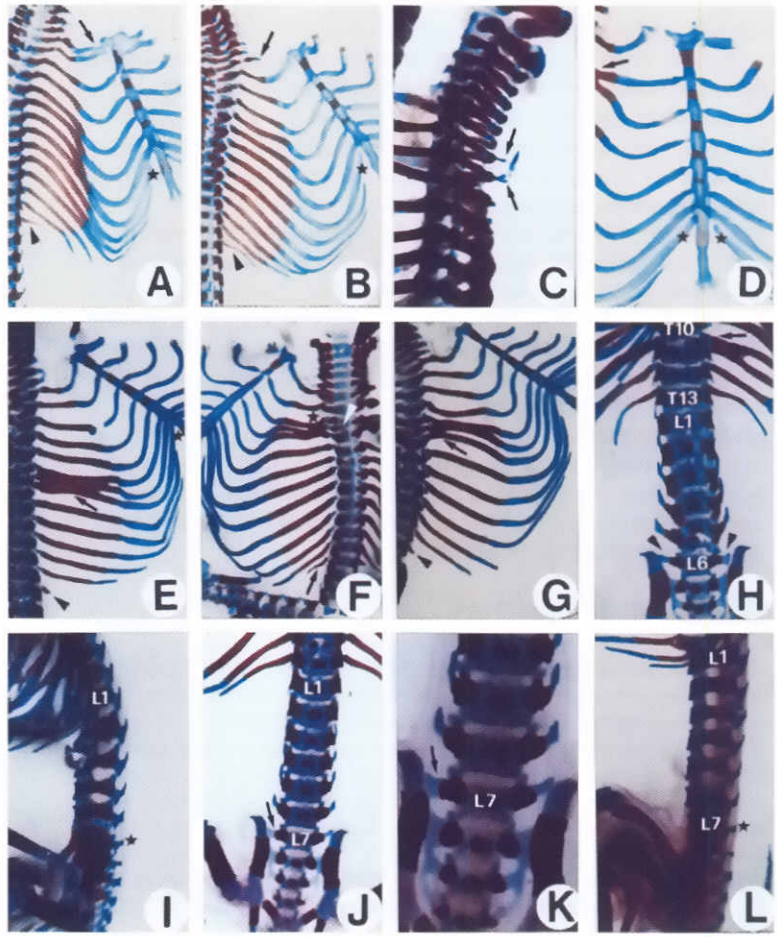


Fig. 3. Treated fetuses (groups II-VIII) stained with alcyan blue for cartilage and with alizarin red for bone. (A-C) *Group IV (NaVP, day 9 h 24:00).* **(A)** *Dorsal view of the thoracic region. Note the fusion between the 1st and the 2nd rib (arrow) and the shifted insertion on the sternum of ribs 3rd-7th. The 8th rib approaches the sternum (asterisk). The arrowhead shows a well developed lumbar rib.* **(B)** *Dorsal view of the thoracic region of a fetus. The reduction of the first rib (arrow), the shifted insertion of ribs 2nd-7th, the approach of the 8th rib to the sternum (asterisk) and the presence of a lumbar rib (arrowhead) are clearly visible.* **(C)** *Lateral view of a fetus showing duplication of the second thoracic segment. The duplication is well recognizable by the presence of two vertebrae with the typical long neural spine (arrows).* **(D)** *Sternum of a fetus of group V (NaVP, day 10 h 8:00). Note the fusions between ribs 2nd-3rd (arrow) and the typical approach of the 8th rib to the sternum (asterisk).* **(E-G)** *Group VI (NaVP, day 10 h 16:00).* **(E)** *Dorsal view of the thoracic region showing a fetus of group VI in which ribs 6-7-8 are fused (arrow). The 8th rib approaches the sternum (asterisk) and a lumbar rib is visible (arrowhead).* **(F)** *Ventral view of the thoracic region of a fetus showing monolateral duplication of the 3rd thoracic segment (arrowhead). Ribs 3-3bis-4 are fused (asterisk) and the 13th rib bilaterally reduced (arrowheads).* **(G)** *Dorsal view of a fetus with duplication of the 5th segment. Ribs 5-5bis-6 are fused (arrow). The 13th rib is reduced (arrowhead).* ***(H-I)*** *Group VII (NaVP, day 10 h 24:00).* **(H)** *Ventral view of last thoracic (T10-T13) and lumbar (L1-L6) segments of a fetus affected by monolateral duplication of one thoracic segment (arrow). The last lumbar segment shows transverse processes partially enlarged and directed toward the ileum (intermediate characteristics between lumbar and sacral, clearer on side where there is the thoracic duplication) (arrowheads).* **(I)** *Lateral view of the same fetus as I. The neural spine of L6 (asterisk) shows the typical shape characterizing the sacral segments.* **(J-L)** *Group VIII (NaVP, day 11 h 8:00).* **(J)** *Ventral view of the lumbo-sacral region. Note seven lumbar segments (L1-L7), the last of them with a monolateral transformation of the transverse process (enlarged and directed toward the ileum) (arrow).* **(K)** *Magnification of J.* **(L)** *Lateral view of the same fetus of J. The neural spine of L7 (asterisk) has the typical shape characterizing the sacral segments.*

Group VI (NaVP, day 10 h 16:00)

Rib abnormalities (in particular fusions) were observed at the level of thoracic segments 5th-8th (52%) (Fig. 3E). In association, 18% of fetuses showed lumbar rib (Fig. 3E) and 13% of fetuses reduced 13th rib.

Duplications at the thoracic level (often of the 5th thoracic segment) were also observed (38%). In addition, all these fetuses showed a clear reduction of the 13th rib (Fig. 3F,G). The removal of the 7th rib from the sternum was observed in 17% of cases.

Group VII (NaVP, day 10 h 24:00)

The thoracic abnormalities of group VII were at the level of the more posterior segments and characterized by duplications (often of the 10th segment) (63%). In association, 47% of fetuses had respecification of the morphology of the last lumbar vertebra (with an ambiguous shape of transverse processes and neural spina, very similar to those of sacral segments) (Fig. 3H,I), of which 42% were also missing the last sacral segment.

Group VIII (NaVP, day 11 h 8:00)

In group VIII, 54% of examined fetuses showed duplication of a lumbar segment with (23%) (Fig. 3J-L) or without association to a respecification of the last lumbar segment. Twenty-three per cent of these fetuses were lacking the last sacral segment. Interestingly, 20% of fetuses with lumbar duplication showed both the respecification of the last lumbar segment and the loss of the last sacral segment.

The results of this experiment show that the administration of NaVP during the formation of somites is able to transform the identity of axial segments of the skeleton. An anterior transformation is produced when the embryos are exposed at stages of about 2 to 10 somites, while a posterior transformation is produced when the embryos are exposed in more advanced stages of development.

Similar transformations of axial segments have been reported by Kessel and Gruss (1991) and by Kessel (1992) in mice after exposure in utero to retinoic acid. Because *Hox* genes encode homeobox containing transcription factors implicated in the specification of positional information along the anterior-posterior axis (Kessel *et al.*, 1990), the retinoic acid-induced transformations have been explained on the basis of activation of *Hox* genes during early stages of development or inhibition of *Hox* genes active in later stages of development. i.e., on the basis of interferences with the *Hox* code which controls the specification of the segmental skeleton (Kessel and Gruss, 1991, Kessel 1992). Knockout of some *Hox* genes has resulted in homeotic transformations of the axial skeleton in which skeletal structures assume a more anterior or posterior shape (Krumlauf, 1994, Rancourt *et al.*, 1995; Saegusa *et al., 1996*). But also knockouts of *Cdx1* (Subramanian *et al.*, 1995) and *Mll* (*Mixed-lineage leukemia* gene, Yu *et al.*, 1995)

resulted in Homeosis of axial structures, probably through the regulation of *Hox* expression in the somite mesoderm.

It is not clear at the moment how VPA induces these kinds of transformations of the axial segments. There are no data in the literature suggesting a direct effect of VPA on gene expression. However, two recent papers report a reduction of serum concentrations of endogenous retinoids in patients treated with anticonvulsants including VPA (Fex *et al.*, 1995; Nau *et al.*, 1995). It is well known that retinoic acid is a physiological morphogen acting during normal morphogenesis of vertebrate and invertebrate embryos (Hofmann and Eichele, 1994). Its main function is to activate particular genes through a link with specific nuclear receptors (Minucci and Ozato, 1996). The normal activation of these genes is related to physiological concentrations of retinoids. It has been demonstrated that higher concentrations of retinoids produce congenital malformations. Similarly, a reduction of retinoid concentration could be regarded as a possible mechanism of teratogenesis, as suggested by the experimental induction of congenital malformations in rats reared on Vitamin A deficient diets (Wilson and Warkany, 1948, 1949).

Experimental Procedures

CD: Crl rats (Charles River, Calco, Italy) were housed in a thermostatically maintained room (T=21±1°C, relative humidity=50±5%) with a regulated cycle of light (6:18) and free access to food (Italiana Mangimi) and tap water. After one week of acclimation, virgin females were caged overnight with males of proven fertility. The morning when the vaginal smears were positive was considered day 0 of gestation. Pregnant females were randomly distributed into experimental groups. At specific times of gestation, females were injected subcutaneously with saline (control group) or with a single injection of 400 mg/Kg NaVP. This dose level was chosen on the basis of literature data and on our previous results, as a level able to induce teratogenic effects with minimal embryonic death. The experimental design provides the following groups:

GROUP I - Control.
GROUP II - Treatment on day 9, h 8:00. ES = presomitic.
GROUP III - Treatment on day 9, h 16:00. ES = nearly 2 somites.
GROUP IV - Treatment on day 9, h 24:00. ES = nearly 6 somites.
GROUP V - Treatment on day 10, h 8:00. ES = nearly 10 somites.
GROUP VI - Treatment on day 10, h 16:00. ES = nearly 14 somites.
GROUP VII - Treatment on day 10, h 24:00. ES = nearly 18 somites.
GROUP VIII - Treatment on day 11, h 8:00. ES = nearly 22 somites.

At the end of gestation (20 day post coitum) the females were killed and the number of implantations, resorptions and live fetuses recorded. After external examination, fetal and placental weights were recorded. All fetuses were processed for skeletal examination according to the double staining method (alizarin red S for bone and alcyan blue for cartilage) of Kimmel and Trammel (1981).

Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons and chi-square test. The level of significance was $p<0.05$.

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