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Källén, Bengt

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Valproic Acid is Known to Cause Hypospadias in Man but does not Reduce Anogenital Distance or Causes Hypospadias in Rats

Bengt Källén

Tornblad Institute, University of Lund, Lund, Sweden

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Abstract: The use of valproic acid during human pregnancy increases the risk of hypospadias in the offspring. Rats exposed *in utero* to valproic acid did not develop hypospadias and even had a slightly increased anogenital distance in males 3–4 days after birth. A classical antiandrogenic drug, flutamide, caused hypospadias as well as a reduction of the anogenital distance in males. At the age of 3 months, rats exposed *in utero* with either valproic acid or flutamide showed a reduced testicular weight and hypoplasia of tubules, which seemed not to be related to the antiandrogenic activity of flutamide as it did not correlate with the presence of hypospadias. The mechanism through which valproic acid causes hypospadias in man and affects testicular development in rat is unknown. Hypospadias caused by valproic acid in man is apparently not due to anti-androgenic properties of the drug.

A standard technique in preclinical testing of chemical substances including drugs for antiandrogenic effects is the rodent anogenital distance test. In the newborn rat or mouse, anogenital distance is markedly larger in male than in female pups, and antiandrogenic substances reduce the anogenital distance in male pups. This has been shown for a number of antiandrogenic substances, e.g., cyproterone acetate (Clemens *et al*. 1978), finasteride (Clark *et al*. 1990 & 1993; Hib & Ponzio 1995), and flutamide (McIntyre *et al*. 2001).

Antiandrogenic effects on the human embryo or foetus could result in different outcomes. One which is relatively easy to detect at birth is hypospadias. At this condition the male urethra opens proximal to its normal position on the glans. This disturbance is regarded as being due to an impaired androgen influence from the foetal testicle which drives the normal closure of the urethral folds.

From epidemiological studies it is known that maternal use of valproic acid, an anticonvulsant with other teratogenic properties as well, increases the risk for infant hypospadias (Bradai & Robert 1998; Arpino *et al*. 2000). Animal developmental toxicity studies of valproic acid have concentrated on neural tube defects because of the increased risk of spina bifida observed in infants whose mothers used valproic acid during pregnancy. A search of the literature did not identify any study of the effect of valproic acid on anogenital distance in rodent pups.

A study was made on anogenital distance and testicular weight in rats after foetal exposure for valproic acid and (as a positive control) flutamide in order to see whether the capacity to cause hypospadias in man could be revealed in the rat model.

Author for correspondence: Bengt Källén, Tornblad Institute, Biskopsgatan 7, SE-223 62 Lund, Sweden (fax +46 46 222 4226, e-mail embryol@embryol.lu.se).

Materials and Methods

Rats of an inbred Fisher strain have been kept since 1974 in our laboratory with brother-sister mating. The strain originated from Møllegaard's Avlscentrum, Ejby, Denmark. In this experiment rats were fed standard laboratory food and water *ad libitum*. Breeding pairs (brother and sister) were selected at an age of about 3 months and were caged together.

In order to study anogenital distance in foetuses before parturition, vaginal smears were taken daily and analysed for cycle dating and presence of sperm. When a conception had taken place, the female was placed in a cage with a drinking bottle containing either water (controls), or a water solution of valproic acid (Ergenyl®) at a concentration of 3 mg/ml which will represent a daily dose of about 125 mg. This dose roughly corresponds to the teratogenic dose for sodium valproate described for instance by Ong *et al*. (1983). One series was also made with a doubled dose.

The dams were killed the day before the expected parturition. The number of resorption sites and the number of pups in each uterine horn was registered and also the position of each pup in the horn. Pups were weighted with a precision balance and the anogenital distances were measured under a preparation microscope with the use of an ocular micrometer. Measurements were made blindly and drug-treated and control rats were studied in parallel.

A second series was made using pups 3–4 days after delivery. Pregnant females were given water, valproic acid (as above), or flutamide (Sigma-Aldrich, 0.15 mg/ml) during the whole pregnancy until parturition. Pups were weighted and anogenital distances were measured as described above.

Some of the male pups from the second series were reared until the age of 3 months when they were killed and the testes were dissected and weighted. Penis was inspected under a dissecting microscope for signs of hypospadias. Testes were then fixed in Bouin's fluid and histological sections were made from each testicle and stained with haematoxylin-eosin. Using a standard microscope with a 10× objective and an 8× ocular, the number of tubuli per visual field was counted, one field from each testicle, and the mean number of tubuli was determined for each rat.

The experiments were approved by the animal ethics committee of Lund University.

Statistical considerations. Comparisons of frequencies were made using Fisher exact tests and expressed as odds ratios (OR) with 95% confidence intervals (95% CI). Means are presented with standard errors of the means (S.E.M.) and were compared with t-tests. A linear multiple regression analysis was used with anogenital distance as the dependent variable. As independent variables were used pup weight, drug treatment, and (in pups studied before parturition) presence or absence of an adjacent male pup. In some analysis of pups studied before parturition, mean anogenital distance and mean pup weight for each uterine horn was used instead and the number of pups per horn was added as an independent variable.

Testis weights were compared with a linear multiple regression model with testis weight as dependent and body weight (in 100 g) and drug treatment as independent variables.

SABER software (Center of Disease Control, Atlanta, USA) was used for calculations.

Results

Pups studied before parturition.

A total of 22 control dams and 12 valproic acid-treated dams were used. Implantation sites were found in 41 and 22 uterine horns, respectively. Thus, in three control dams and two valproic acid-treated dams, one horn was empty.

There were 173 implantation sites in the 22 control dams (7.9 implantations per dam) and 95 implantations in the 12 valproic acid-treated dams (also 7.9 implantations/dam). Among the implantation sites in the controls, nine (5%) were resorptions in five litters (range 0–4). In the valproic acid-treated dams, there were 21 resorptions (22%) in six litters (range 0–8). The odd ratios for having a resorption after valproic acid treatment was 5.2 (95% confidence interval 2.1–1.34), calculated on implantation sites.

The mean pup weights did not differ significantly between control (mean±S.E.M.=2.89±0.07 g) and valproic acid-treated (3.03±0.16 g), neither did anogenital distance: controls 2.51±0.05 mm, valproic acid 2.57±0.07 mm. P values were 0.16 and 0.27, respectively.

Table 1a summarises the linear multiple regression when untransformed pup weight was added as an independent variable. There was a highly significant effect of pup weight on anogenital distance. An adjacent male pup did not reduce anogenital distance significantly, and there was no effect by valproic acid on anogenital distance.

The analysis was repeated using mean anogenital distance and pup weight for each uterine horn with the number of pups per horn as a further explanatory variable. Similar results as above were obtained: the effect of valproic acid exposure was 0.04±0.19 (t=0.22, P=0.83).

A comparison between litters with and without resorptions showed no difference (t=−0.65, P=0.52).

Pups studied after parturition.

Anogenital distance was studied in 63 control male pups (16 litters) at the age of 3–4 days, 86 pups exposed to valproic acid (18 litters), and 54 pups exposed to flutamide (10 litters). The mean (with S.E.M.) anogenital distance among control pups was 4.24±0.07 mm, among pups exposed to valproic acid 4.47±0.06 mm, and among pups exposed to flutamide 4.06±0.12 mm. The mean body weights were 7.29±0.17 g, 7.57±0.13 g, and 7.75±0.20 g.

Table 1b shows the results of the multiple regression analysis. There was a marked effect of pup weight (partly as an expression of variation in age) on anogenital distance and also significant effects of both drug exposures. As ex-

Table 1.

Linear multiple regression analyses of outcomes after intra-uterine exposure for valproic acid or flutamide. Regression coefficients with standard errors are given and also t and P values.

Independent variable	Regression coefficient	t	P
a. Anogenital distance in pups before parturition			
Pup weight (g)	0.30±0.06	5.3	0.005
Adjacent male pup	−0.08±0.08	−1.0	0.31
Valproic acid versus control	0.01±0.09	0.09	0.92
b. Anogenital distance in pups, 3–4 days old			
valproic acid exposure			
Pup weight (g)	0.01±0.003	4.0	<0.001
Valproic acid versus control	1.90±0.87	2.2	0.03
Flutamide exposure			
Pup weight (g)	0.03±0.004	6.24	<0.001
Flutamide vs control	−2.99±1.18	−2.55	0.01
c. Testicular weight at 3 months of age			
valproic acid exposure			
Body weight (g/100)	0.17±0.03	6.0	<0.001
Valproic acid versus control	−0.03±0.01	−2.5	0.015
Flutamide exposure			
Body weight (g/100)	0.18±0.04	4.9	<0.001
Flutamide versus control	−0.04±0.01	−3.0	0.003
Among flutamide-exposed rats			
Body weight (g/100)	0.15±0.05	2.8	0.007
Hypospadias versus non-hypospadias	0.02±0.02	1.0	0.33

pected, exposure to flutamide reduced anogenital distance but exposure to valproic acid actually slightly increased it even though statistical significance is marginal (P=0.03).

A doubling of the valproic acid dose did not change the result. The multipe regression coefficient for valproate exposure was 0.14±1.25, t=0.11, P=0.91.

Rats studied at the age of 3 months.

The number of rats studied were 43 control rats, 48 rats exposed during development for valproic acid, and 47 rats exposed during development for flutamide.

The mean body weight was 236±2.7 g in control, 223±3.7 g in valproic acid-exposed, and 234±3.0 g in flutamide-exposed rats. The difference between control and valproic acid-exposed rats is statistically significant (t=2.97, P=0.006) but not the difference between control and flutamide-exposed rats (t=0.65, P=0.32). The mean testicular weight was 1.25±0.02 g among controls, 1.16±0.03 g among valproic acid-exposed, and 1.20±0.01g among flutamide-exposed rats. Both the latter groups have significantly low testis weights (t=3.07, P=0.004, and t=2.67, P=0.01, respectively).

Table 1c presents the linear multiple regression analysis which confirms a reduced testicular weight in both groups of drug-exposed rats.

None of the control or valproic acid-exposed rats had hypospadias. Among the 47 rats exposed to flutamide, 28 had hypospadias (60%, 95% confidence intervals 44–74%). Table 1c also shows a comparison between hypospadic and non-hypospadic rats exposed to flutamide. No difference in testicular weight is seen.

The mean number of tubuli per ocular field was determined for the three groups. For controls (n=44) it was 43.0±1.9, for valproic acid-exposed rats (n=27) it was 48.9±1.3, and for flutamide-exposed rats (n=44) it was 46.6±0.9. The difference between valproic acid and control was significant (t=2.23, P=0.03) but the difference between flutamide and control did not reach statistical significant (t=1.68, P=0.10).

Discussion

Valproic acid is rapidly metabolised in rats. In the present study, the drug was added to the drinking water. This will give an uncertainty in the amount of drugs given but at the same time increases the possibility for extended exposure. The amount of drug given can be estimated to correspond to a dose which has been shown as teratogenic in rats (Ong *et al*.,1983) and has also given marked effects: a significant increase in resorption rate (22%, of the same magnitude as that described by Hansen *et al*. (1995)), a reduction of 3 months testicular weight, and a weak *increase* in anogenital distance at the age of 3–4 days. It is therefore likely that if valproic acid reduced the anogenital distance it would be observed. A doubling of the dose did not change the result.

The treatment with flutamide (as a positive control) gave a significant reduction of anogenital distance but also a reduction of testicular weight (but not of body weight) at the age of 3 months. The reduced testicular weight seemed to be associated with tubular hypoplasia. Hypospadias was frequent after treatment with flutamide but was not seen after treatment with valproic acid.

As can be expected and as clearly shown by Gallavan *et al*. (1999), anogenital distance is a function of pup weight. As these authors point out, an effect on pup weight can mimic a reduction of anogenital distance (if the treatment decreases pup weight) or can hide such an effect (if the treatment increases pup weight). In the present experimental series, male foetuses were slightly heavier than controls (perhaps a function of the lower number of surviving pups per litter) and if valproic acid reduced anogenital distance, this could be hidden if no correction for pup weight had been made.

An effect of an adjacent male foetus on anogenital distance has been described repeatedly in mice (Van den Bergh & Huggett 1995) and rats (Richmond & Sachs 1984) and was indicated also in the present material, but statistical significance was not reached.

Valproic acid thus slightly increased (instead of decreased) anogenital distance and did not cause hypospadias in male rats while a clear-cut increased risk for hypospadias exists after valproic acid exposure in human pregnancy. A preclinical test, based on anogenital distance which is thought to reveal antiandrogenic properties, would thus not identify the potential risk for the human embryo. The drug may not cause hypospadias by an antiandrogenic effect. The mechanism may instead be a direct effect on the penis rudiment, on the embryonic testicle, or on the gonadotrophic stimulation of the early embryonic testicle to androgen production. In man, the latter occurs from placental gonadotrophins, in rats from hypophyseal gonadotrophins which could be one explanation for the species difference.

A long-time effect of valproic acid on the testicles could be seen at 3 months age. Testicular weight was reduced and the density of tubuli increased, indicating tubular hypoplasia. Testicular weight reduction has been described in the adult rat after long-term treatment with high doses of valproic acid (Roste *et al*. 2001). In our study, a testicular effect similar to that seen after valproic acid was also seen after foetal exposure to flutamide but this seemed unrelated to the antiandrogenic effect because there was no difference in testicular weight after flutamide exposure whether hypospadias was present or not. The mode of action is not known but could indicate that exposure to these drugs during pregnancy may have long-standing effects on testicular development and perhaps fertility also in man.

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