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Morphological changes in the testis after long-term valproate treatment in male Wistar rats

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Uncertainty exists about the effect of antiepileptic drugs on gonadal function. In females, long-term valproate treatment has been shown to induce endocrine disturbances and an increased number of ovarian cysts. The aim of the present study was to investigate whether valproate can also induce morphological changes in the testis of male animals. In addition, possible morphological changes in the liver, heart, lungs, lymphatic nodes, pancreas, kidney or brain were studied. The carcinogenic implications were evaluated by the measurement of p53. Male Wistar rats were fed perorally with valproate mixture 200 mg kg^{-1} ($n = 15$) or 400 mg kg^{-1} ($n = 20$), or control solution ($n = 15$) twice daily for 90 days. Serum concentrations measured 4–6 hours after the last dose were 105 and 404 μmol l^{-1} in low- and high-dose valproate treated animals respectively. There was a highly significant, 51% decrease ($P < 0.001$) in testicular weight in the high-dose treated valproate rats with no changes in the other groups. There was widespread testicular atrophy with histologically verified spermatogenic arrest in 15/20 of the high-dose valproate treated animals. No changes in the testis were seen in the low-dose valproate treated rats, nor in the control rats. There were no morphological changes in the other investigated organs. None of the groups showed over-expression of p53. In conclusion, a dose-dependent effect of chronic valproate treatment was found on testicular morphology in rats. Caution must be taken before these results can be applied to humans.

Key words: valproate; testis; p53; rat.

INTRODUCTION

There is increasing concern about the effect of antiepileptic drugs (AEDs) on male reproduction. Sexual dysfunction, including reduced potency and hyposexuality is seen in as much as 38–71% of men with epilepsy[1–4]. This may, at least in part, be due to long-term use of AEDs which have been shown to induce disturbances in sex hormone levels[2,5–9] and also to reduce sperm quality[5,10–13]. These effects have so far not been related to specific drugs. In women, however, menstrual disorders, polycystic ovaries and hyperandrogenism were found in some recent studies after long-term valproate treatment[14–18]. This could indicate a drug-specific effect of valproate on ovarian morphology and female sex hormones.

In males, testicular atrophy has previously been shown in both rats and dogs after chronic valproate treatment[19]. This has, however, been attributed to doses far above those used when treating humans. In addition, chronic valproate treatment has also been shown to slow pubertal maturation by decreasing the rate of testicular growth and spermatogenesis in mice[20]. In the clinical setting there is sparse information over the possible hormonal effects of valproate in males. In a recent study, 12/20 male patients taking valproate showed increased androgen levels[21]. Further, in a case study Yerby and McCoy[13] found markedly reduced sperm quality in a man on valproate, which improved after change of medication to felbamate.

Recently, long-term treatment with valproate has

been shown to increase significantly the amount of polycystic ovaries in non-epileptic female Wistar rats at serum concentrations within the human therapeutic range[22,23]. By using a corresponding animal model, we wanted to study the effects of long-term valproate treatment also in male rats by investigating testicular morphology at serum concentrations within therapeutic range in humans. In addition, specific analyses were performed to investigate a possible relation between morphological changes and carcinogenicity. This was done by measuring the expression of the tumour suppressor gene p53, and by performing flow cytometric DNA measurements of the testis.

MATERIALS AND METHODS

Seventy male Wistar rats (Møllegaards Avlslaboratorium, Skensved, Denmark) were fed perorally through a gastric tube with valproate mixture or placebo solution twice daily for 90 days. All animals were at the age of 80 days at the beginning of the study. They were caged in triplets in Macrolon III cages at constant temperature and humidity on a 12 hour light and darkness schedule at an air exchange rate of 18 changes per hour. The rats were kept on B&K pelleted rat diet and given tap water *ad libitum*. The animals were tested negative for parasitical, bacterial and viral agents according to the recommendations of the Federation of Laboratory Animal Science Association[24]. Animal care was in accordance with institutional guidelines and national legislation. The animal ethical committee at the National Hospital, University of Oslo, Oslo, Norway approved the study.

A standard commercially available valproate mixture was used together with a placebo solution (gift from Desitin Pharma AS, Norway). The animals were separated into four different groups, receiving either valproate 200 mg kg^{-1} per dose ($n = 15$), valproate 400 mg kg^{-1} per dose ($n = 20$) or placebo solution ($n = 15$), twice daily. The animals were given half-daily doses the first week. They were weighed at the first day of the study, and the doses were later adjusted according to weight changes every week. After 90 days, all animals were weighed and thereafter killed. This was done by means of exsanguination under pentobarbital narcosis by abdominal artery puncture, 4–6 hours after their last medication intake. The blood was collected and serum frozen at $-20\,^{\circ}C$ until analysis. Each testicle was weighed separately and immediately cut in two pieces. One half was formalin fixed in 4% buffered formaldehyde for morphological and immunohistochemical examination. The other half of the testis was immediately stored at $-70\,^{\circ}C$ until further analysis.

Histology

In addition to testicular tissue, specimens of liver, pancreas, kidney, lung, heart, lymph nodes and brain including both hemispheres, brainstem and cerebellum, were fixed in 4% buffered formaldehyde. The specimens were routinely processed, embedded in paraffin, and 5 μm thick sections were cut and stained with haematoxylin and eosin for light microscopic evaluation. Two independent pathologists performed a blinded microscopic evaluation of the sections of the testes (AaB and KAB).

Immunohistochemistry

For immunohistochemistry 2–5 μm thick sections were mounted on silane-coated slides, deparaffinized and rehydrated. A mouse monoclonal p53 antibody (Oncogene Science) and a polyclonal rabbit anti-human testosterone antibody (BioGenex Laboratories, San Ramon, CA, USA) were used as primary antibodies.

Staining for p53

The slides were incubated with p53 antibody diluted 1:100. Staining was performed with labelled Avidin–Biotin (LAB)[25,26]. We microwaved the sections (2×5 min) in a citratebuffer prior to immunohistochemistry. Positive and negative controls included substitution of the primary antiserum with normal serum from the same species in which the antibody was made. Only nuclear staining was regarded as positive.

Staining for testosterone

A streptavidin alkaline phosphatase method was used. Sections were treated with a solution containing 30% fat-free dried milk (Nestlè SA, Vevey, Switzerland) for 20 minutes before incubation with a polyclonal antibody (BioGenex Laboratorium, San Ramon, CA, USA) diluted 1 : 50 in Tris buffered saline containing 2.5% bovine serum albumin (TBS/BSA). Biotinylated goat anti-rabbit Ig (DAKO A/S, Glostrup, Denmark) diluted 1 : 500 in TBS/BSA was used as a secondary antibody. Streptavidin alkaline phosphatase (Amersham plc, Bucks, UK) diluted 1 : 500 were applied to the sections. Incubations were in a dark moist chamber for 30 minutes, and washing between each of the steps was in TBS for 5 minutes. Alkaline phosphatase substrate containing Fast Red TR salt (Sigma-Aldrich Corporation, St Louis, MO, USA) was applied for 20 minutes. Subsequently, the sections were rinsed in tap water and counterstained with Mayer's modified haematoxylin. In the control sections, the primary antibody was replaced by normal rabbit serum diluted 1:100 in TBS/BSA.

Flow cytometric DNA measurements (FCM)

Nuclear suspensions were prepared from thawed, fresh frozen testicular specimens that were minced in slices in 10 mM phosphate buffer and filtered through a 50 μm filter. The preparation and staining of nuclei for FCM was performed according to the detergent–trypsin method developed by Vindeløv *et al.*[27] using solutions containing trypsin 0.003 mg ml^{-1} trypsin inhibitor 0.50 mg ml^{-1}, ribonuclease. A 0.01 mg ml^{-1}, spermin tetrachloride 0.01 mg/ml and propidum iodide 0.41 mg ml^{-1}. A single cell suspension from each specimen was mixed with a suspension of chicken and rainbow trout red blood cells for internal DNA standards.

The nuclear DNA content was measured in a FACS caliber flow cytometer. An argon ion laser from which the 488 nm line was used produced the excitation light. The output signals were sorted by a 256-channel analyser and were presented as histograms. The DNA amount was calculated in relation to the two internal standards, chicken and trout erythrocytes.

Valproate serum concentrations

Serum concentrations of valproate were analysed by a fluorescence polarization immunoassay system using an AxSym analyser (Abbott Diagnostic Division, Irving, TX). The sensitivity of the valproate assay was 5.0 μmol l^{-1}, and the intra-assay and inter-assay variations were 1.8% and 2.7% respectively.

Statistics

The mean and standard deviation for all analyses were calculated. The statistical analyses were performed with a one-way ANOVA technique using a commercially available software package (SPSS) for all data. Not normally distributed data, including serum VPA concentrations, were analysed with a non-parametric Kruskal–Wallis test (specified in tables). A chi-squared test was performed when appropriate.

RESULTS

All animals tolerated the treatment and the gastric tube feeding without any signs of discomfort, and none of the animals died during the study. There was no reduction in motor activity or any observed changes of the thriving in the animals.

Median serum concentrations of valproate at time of death 4–6 hours after the last dose were 105 and 404 μmol l^{-1} in low- and high-dose treated animals. Examinations of median valproate serum concentrations in 12 of the animals from the high-dose group at different time intervals were 1152, 599, 286 and 119 μmol l^{-1} measured 2, 4, 6 and 8 hours after the last dose respectively.

Morphological findings

Animal weight was unchanged in the low-dose group, while there was a statistically significant, 15% reduction in body weight in high-dose treated animals compared to control animals ($P < 0.001$, Table 1).

Table 1: Animal weight, testicular weight and ratio of testicular weight/animal weight after long-term treatment with valproate (VPA) and control solution in male Wistar rats.

	Control ($n = 15$)	Low-dose VPA ($n = 15$)	High-dose VPA ($n = 20$)
Animal weight			
Mean	440.60	422.20	374.50
St. dev	27.80	27.45	21.74
P	—	<0.001[c]	<0.001[a,b]
Testicular weight			
Mean	1.9051	1.8138	0.9288
St. dev	0.1664	0.1664	0.3531
P	—	<0.001[c]	<0.001[a,b]
Ratio			
Mean	0.0043	0.0043	0.0025
St. dev	0.0007	0.0004	0.001
P	—	<0.001[c]	<0.001[a,b]

[a] Compared to control; [b] compared to low-dose VPA; [c] compared to high-dose VPA.

Testicular weight in the high-dose treated animals showed a significant, 51% and 49% decrease ($P < 0.001$, Table 1) compared to control and low-dose treated animals respectively.

The ratio of testicular weight/animal weight was significantly lower in the animals receiving high-dose valproate ($P < 0.001$, Table 1) compared to all other groups. This ratio was unchanged in the low-dose valproate treated animals compared to control or lamotrigine treated animals (Table 1).

On the basis of the light microscopical findings in the testicles, the animals were now classified into four groups (Table 2). Groups A—normal findings, group B—slight atrophy, group C—moderate, diffuse atrophy and group D—extensive diffuse atrophy. Fifteen of the 20 high-dose valproate treated animals had moderate to severe testicular atrophy characteristics of groups C and D (Figs 1 and 2). These animals deviated significantly ($P < 0.001$) from both the controls and the low-dose treated animals. The tubuli seminiferi of the animals included in groups C and D were mostly atrophic. In the affected tubuli, the most advanced cell type was primary spermatocytes. There was no sign of

round spermatids in the tubuli categorized in groups C and D (Fig. 3). The Leydig cells did not appear to be affected. All the animals treated with low-dose valproate were found in groups 1 and 2, and this was not significantly different from the controls.

Table 2: Light microscopical findings in rat testicles after VPA treatment.

	Control ($n = 15$)	Low-dose VPA ($n = 15$)	High-dose VPA ($n = 20$)
A	14	14	1
B	1	1	4
C	0	0	8
D	0	0	7
C + D	0	0	15*

* $P < 0.001$, chi-squared test, compared to control. A: normal findings; B: slight atrophy; C: moderate atrophy; D: extensive atrophy.

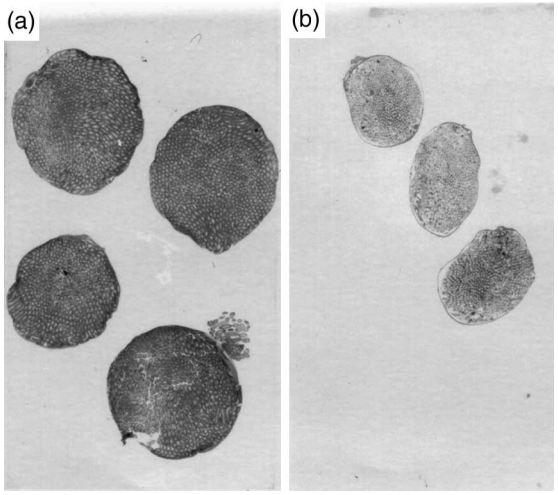

Fig. 1: Macroscopic cross-sections of the testicles of (*a*) a control rat and (*b*) a valproate high-dose treated rat.

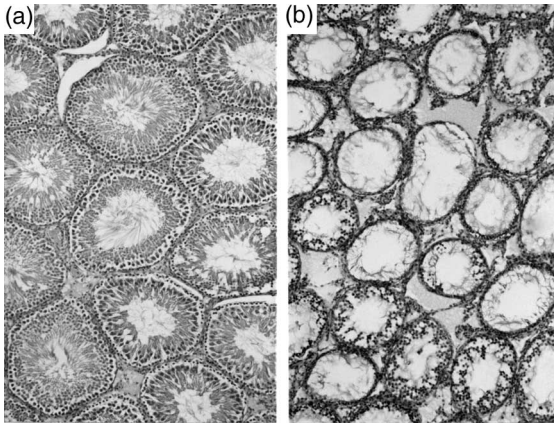

Fig. 2: Photomicrograph (×200) of cross-sections of seminiferous epithelium in (*a*) a control rat and (*b*) a valproate high-dose treated rat.

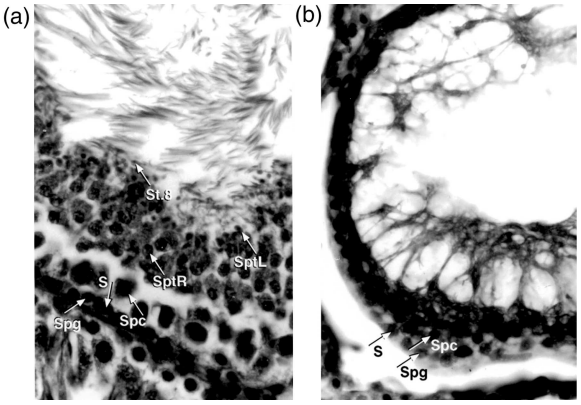

Fig. 3: (*a*) Photomicrograph (×400) of a partial cross-section of seminiferous epithelium showing intact epithelium in a control rat. (*b*) Photomicrograph (×400) of a partial cross-section of seminiferous epithelium demonstrating degenerated epithelium in a high-dose valproate treated rat. S = Sertoli cell, Spg = spermatogonium, Spc = spermatocyte St.8 = Stage 8[41].

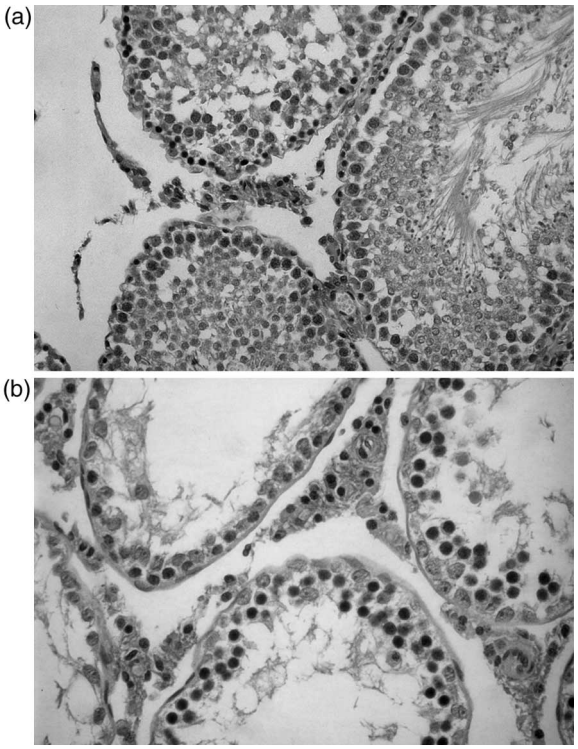

Fig. 4: (*a*) Testis of a control rat stained with an antibody against testosterone. Positive staining of interstitial cells is observed between normal seminiferous tubules. Streptavidin alkaline phosphatase method (× 260).
(*b*) Immunocytochemistry of a testis of a rat treated with high-dose valproate. Positive staining for testosterone is observed in interstitial cells. Note the severe atrophy of the adjacent seminiferous tubules. Streptavidine alkaline phosphatase method (× 260).

One of the low-dose treated animals had light microscopical findings of focal myocarditis, none of the high-dose treated animals showed similar changes.

This was not significantly different from the controls where two animals had focal myocarditis (Table 3). Four of the animals receiving low-dose valproate, and six that were given high-dose valproate, had an abundance of mast cells in the lymph nodes investigated. This did not differ from the controls where four animals had similar changes.

Table 3: Pathological findings in the heart, lung, lymphatic tissue, kidney, liver and CNS of male Wistar rats after long-term valproate treatment.

	Heart[a]	Lung	Lymphatic tissue[b]	Kidney	Liver	CNS
Control ($n = 15$) ($n = 15$)	2	0	4	0	0	0
Low-dose VPA ($n = 15$) ($n = 15$)	1	0	4	0	0	0
High-dose VPA ($n = 20$)	0	0	6	0	0	0

[a] Focal myocarditis; [b] abundance of mast cells.

There were no morphological changes in the lungs, the kidneys, liver or central nervous system (CNS) in any of the two valproate treated groups, similar to the findings in the controls.

Flow cytometric results

All specimens were diploid, mean CV 3.7 (2.7–5.2) (CV = coefficient of variations of signals within the main peak, providing a measurement of the peak width). There were no significant differences between the different treatment groups with mean CV in valproate low-dose treated animals 3.5; in valproate high-dose treated 3.2; and 3.9 in control treated animals. Aneuploid cell populations were not detected in either of the groups.

Immunohistochemistry

Neither the valproate high-dose treated, nor the valproate low-dose treated animals showed overexpression of p53 in the testis. This is in concurrence with the findings in the control rats.

Examination of sections of testes stained for testosterone showed positive reactivity in Leydig cells and some staining of interstitial connective tissue (Fig. 4). Differences in staining distribution and intensity of Leydig cells were not observed between the treated rats and the control animals. No staining was observed in the negative control slides.

DISCUSSION

Our main findings are the significant occurrence of testicular atrophy and the spermatogenic arrest after long-term treatment with high-dose valproate in rats. Walker *et al.*[19] demonstrated reduced or absent spermatogenesis and testicular atrophy in rats given valproate 1200 and 1600 mg kg^{-1} for 13 weeks. The doses were considerably higher than in our study, but serum concentrations were not measured. In the same study, dogs were given valproate 150–400 mg kg^{-1} for 13 weeks. These valproate doses were much lower, but again the principal pathologic finding was moderate to severe testicular atrophy, with degeneration of germ cells, suppression of spermatogenesis and interstitial fibrosis.

To evaluate the possible clinical implications of drug effects observed in animal studies, it is of crucial importance that the serum concentrations used can be applied to a clinical setting. The extensive testicular atrophy demonstrated in our study were found at serum valproate concentrations considered within, or even in the lower part of therapeutic range in humans, 4–6 hours after the last dose. Furthermore, the concentrations more than 6 hours after the last dose were regularly below therapeutic range. Although the maximum serum concentrations in our rats measured 2 hours after the last dose were about twice the maximum therapeutic range, it must be emphasized that the therapeutic range of 300–600 μmol l^{-1} in humans represents minimum concentrations. It is also well known that due to the kinetics of the drug, diurnal fluctuations in the range of several hundred percent occur[28,29]. It should also be noted that the doses given to achieve a certain concentration are considerably higher in rats than in humans, because of the much shorter half-life of the drug in rats. In addition, it is known that the serum concentration in mice and rats necessary for an anticonvulsant effect is actually nearly twice that of humans[30]. We therefore consider the concentrations achieved in our high-dose treated animals to be of clinical interest also when treating humans.

Previous studies have shown that epilepsy itself can induce hormonal disturbances in both males and females[32,33]. In women, a relation between left-sided temporal epileptic foci and the development of polycystic ovaries has even been found[34]. It has therefore been implied that the significant increase of polycystic ovaries and hormonal disturbances in women using valproate[14,15], is due to the epilepsy itself or by the selection of patients since laterality was not considered in these studies[35]. Randomly selected *non-epileptic* animals are used both in the present study, and in the previously published data in female rats[23]. Both studies demonstrate that valproate induces gonadal morphological changes. This supports the view that there is a drug-specific effect of valproate on gonadal morphology.

A drug-specific effect of valproate also gains some support from the study by Cohn *et al.*[11] in which male rats received different antiepileptic drugs for a period of 3 months. Both carbamazepine treated and valproate treated rats had diminished sperm content, but the valproate treated animals were the only ones with significantly decreased fertility. This does not, however, underscore the fact that changes in sex hormones and semen quality may also occur using a wide variety of other antiepileptic drugs[2, 8, 9, 11, 20, 36, 37].

Morphological changes could not be found in any other organ in any of the long-term valproate treated animals. This is identical to our findings in the newly published study of female rats[23]. Here we found a significant increase in polycystic ovaries in female Wistar rats given high-dose valproate, whereas other organs were spared. These studies together suggest a gonadal-specific effect of valproate. The results in our male animals are also in concurrence with the findings of Walker[19], who suggested that the testis could be a target organ for valproate toxicity.

The morphological findings in the gonads of male and female animals together with the well-known teratogenic effect of valproate may suggest a possible interference with gene expression. This possibility made it interesting also to study the expression of p53, and to perform flow cytometry. The p53 gene is regarded as one of the most frequently mutated genes associated with cancer development. More than 50% of human primary tumour cells over-express a variety of mutant p53 protein forms[38, 39]. Most seminomatous and non-seminomatous germ cell tumours exhibit increased p53 protein expression evaluated by immunohistochemistry[40]. The atrophic testis of the high-dose treated valproate animals in our study showed no over-expression of p53, weighing against a carcinogenic effect of the drug as evaluated by p53 expression. This is in accordance with the flow cytometric results; cells with abnormal DNA were not detected. Further differentiation of the cells on the basis of flow cytometry is inconclusive, most probably due to the frozen material. The light microscopical findings were, however, unambiguous.

The spermatogenesis was evaluated by way of histological verification of the different cells in the spermatogenetic cycle, which is an established method[31, 41]. In the atrophic tubuli none of the cells advanced beyond the stage of prophase of meiosis I which is known to be very sensitive to toxicological influence. Although no haploid cells were detected in any of the atrophied tubuli, different stages of the spermatogenesis could be visualized. Some tubuli had apparently an abundance of cells; they were clearly more irregularly situated than in the species with normal tubuli. In more advanced cases, empty spaces 'shadows' of epithelium were seen, corresponding to the space normally occupied by germ cells, due to lack of these cell lines in the spermatogenesis. The tubuli showing the most extensive atrophy resembled the pathological findings arising in other testicular degenerations due to toxic substances[42, 43].

Immunohistochemistry indicated an unchanged pattern of testosterone staining. This could be interpreted as a demonstration of unchanged number and activity of Leydig cells. The light microscopical findings, showing no morphological changes in the Leydig cells in either of the groups, supports this and is in accordance with the changes restricted to the germ cells in the study by Walker[19]. Atrophic seminiferous tubules were found in rats given toxic doses of valproate at 1600 mg kg^{-1} per day. There was a virtual total loss of germinal epithelium, while only minimal, if any, effect on the interstitial tissue was observed. Intact Leydig cell function might also be implicated in the case study of Yerby and McCoy[13], who showed that in a male patient changing medication from valproate to felbamate had an improvement in semen quality with no change in endocrine function as evaluated by FSH and LH.

In conclusion, the present study has shown a drug-specific effect of valproate on testicular morphology in non-epileptic male rats at serum concentrations within the therapeutic range in humans. However, caution must be taken before these results can be applied to humans. Our findings do however, encourage further, both animal and human, studies to reveal the clinical implications of these findings when treating epileptic patients.

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