Long-term levetiracetam treatment affects reproductive endocrine function in female Wistar rats

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KEYWORDS
Levetiracetam; Hormones; Rats; Ovary morphology

Summary
Purpose: Several antiepileptic drugs (AEDs) induce changes in endocrine function in women with epilepsy. Levetiracetam (LEV) is one of the newer AEDs, and to date no endocrine side-effects have been reported in humans. However, a recent study on ovarian follicular cells from prepubertal pigs showed that LEV affected basal steroid hormone secretion. The aim of the present study was to investigate possible effects of the drug on endocrine function and ovarian morphology in non-epileptic rats.

Methods: Thirty female Wistar rats were fed per-orally with either 50 mg/kg LEV ($n = 15$) or 150 mg/kg LEV ($n = 15$) twice daily for 90–95 days. Twenty rats received a control solution. The rats were killed in the dioestrus phase of the oestrous cycle. Serum concentrations of testosterone, $17\beta$-oestradiol, progesterone, follicle stimulating hormone (FSH), luteinizing hormone (LH) and LEV were measured, and the ovaries examined histologically.

Results: Mean ovarian weight showed a significant, dose-dependent increase after LEV treatment. Mean numbers of ovarian follicular cysts were not changed, but the numbers of corpora lutea and secondary follicles were significantly higher in the treated animals. Serum testosterone was significantly increased in treated animals (0.50 nmol/l versus 0.16 nmol/l in controls, $p < 0.05$), while oestradiol was reduced (67.4 compared to 257.5 pmol/l in controls, $p < 0.05$). The low-dose group had
Introduction

Women with epilepsy are at risk of reproductive health disorders, including menstrual disturbances, fertility problems, polycystic ovaries and hormonal changes. The explanation for this is multifactorial. Epilepsy itself, antiepileptic drugs (AEDs) and psychosocial factors are all known to affect endocrine function. Enzyme-inducing AEDs, such as phenytoin, carbamazepine and phenobarbital, reduce the levels of biologically active sex steroid hormones, while valproate (VPA) has been associated with increased occurrence of hyperandrogenism, polycystic ovaries and menstrual disorders. In animal studies, VPA treatment has been demonstrated to affect ovarian morphology by increasing the number of cysts and reducing the number of corpora lutea in non-epileptic rats. VPA has also been shown to affect steroidogenesis and inhibit the conversion of testosterone to oestradiol in a series of experiments with cultures of porcine ovarian follicular cells.

Levetiracetam (LEV) is a relatively new, broad-spectrum AED with positive reports regarding both effects and side-effects. Recent studies have shown a promising effect of LEV on generalized epilepsies like tonic–clonic, absence and myoclonic epilepsy, for which VPA has previously often been the drug of choice. Safety data indicate that LEV is very well tolerated by the majority of patients, and endocrine side effects have not been reported in humans to date. If these data are confirmed in further studies, LEV may become an appropriate alternative to VPA for many patients. This is of particular interest to women within the fertile age range, for whom VPA has well-known disadvantages such as reproductive endocrine disorders and teratogenicity.

However, a recent study, utilizing ovarian follicular cells from prepubertal pigs, demonstrated that LEV affects basal steroid hormone secretion, indicating an effect of the drug on steroidogenesis. These data indicate that LEV may influence endocrine function, and this possibility should be explored in both animal models and humans before LEV can be recommended as a safe alternative to VPA.

The aim of this study was to investigate possible effects of long-term LEV treatment on ovarian morphology and sex hormones in female rats. We used an in vivo model, which has been used to demonstrate side-effects in other studies of AEDs and in which it has been possible to extrapolate from the results of these studies to humans.

Methods

Experimental animals and AED administration

The experiments described were carried out in agreement with the provisions enforced by the National Animal Research Authority (NARA). Sixty-two female Wistar rats (Taconic, Denmark) were used. The animals were approximately 80 days of age at the beginning of the study. They were housed in polycarbonate type 4 B&K cages in groups of three, in a closed system. Beekay bedding (Scanbur BK) was used. Temperature and humidity were regulated (21°C ± 1°C; 55% ± 10%), and a 12 h light and darkness schedule used at an air exchange rate of 20 changes/h. Rats were fed a standard pellet rat diet, and tap water was provided ad libitum.

The animals were fed per-orally through a gastric tube with LEV solution 50 mg/kg (n = 15), 150 mg/kg (n = 21) or control solution (n = 26) twice daily for 90–95 days. Variations in serum concentrations of LEV after a single dose were measured by repeated blood sampling in a subset of the animals (Fig. 1). The lower dose was chosen to achieve LEV concentrations well within the therapeutic range, while the higher dose was chosen to achieve concentrations slightly above the therapeutic range without being toxic.

At the end of the study, the animals were killed by exsanguination under pentobarbital narcosis by abdominal artery puncture, 3–4 h after the last dose. Blood was collected, and the serum frozen at −70°C until analysed. Internal organs (heart,
kidneys, lungs, liver and ovaries) were collected from 50 animals. Twelve animals from which internal organs were to be used for other purposes were perfusion-fixated according to the method described by Lehre et al. These 12 perfusion-fixated animals were only used in the morphologic ovary analyses.

For standardisation of cycle stage at sacrifice, vaginal smears were taken daily from days 90 to 95, and the animals were killed as soon as they reached the dioestrus phase of the oestrus cycle. Vaginal smears were taken between 06:30 and 08:30 a.m., and all animals were killed between 9:00 and 11:00 a.m. Sacrifice was done at random, independent of treatment groups.

Analyses

Body weight and ovary weight were measured. Tissue samples from the ovaries, liver, kidneys, lungs and myocardium were fixed in formalin, and subjected to routine procedures for paraffin—wax embedding. Five micrometers sections were cut and stained with haematoxylin and eosin for evaluation by light microscopy. A blinded examination of the ovarian sections was performed and the numbers of cysts, follicles and corpora lutea were counted. The other organs were examined for histopathological lesions by routine light microscopic evaluation.

Testosterone serum levels were determined by solid-phase RIA (Coat-A-Count, Diagnostic Corporation, LA, USA). Detection limit was 0.14 nmol/l.

17β-Oestradiol concentrations were analysed using the kit "Coat-A-Count", Oestradiol (Diagnostic Corporation, LA, USA). Detection limit was 50 pmol/l.

Serum levels of progesterone were determined by solid-phase RIA (Spectria, Progesterone 125J Coated Tube RIA, Orion Diagnostica, Espoo, Finland). Detection limit was 0.3 nmol/l. Concentrations of plasma and pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH) were measured by RIA using reagents supplied by the NIDDK. The reference preparations used were rat LHRP-1 and rat FSHRP-3, and the minimum detectable concentrations were 0.2 and 1.2 ng/ml for LH and FSH, respectively.

Serum concentrations of LEV were measured by an isocratic liquid chromatographic method.

Statistics

Morphological studies: Means and standard deviations for the analyses were calculated. The statistical analyses were performed by one-way ANOVA and means were compared by Student's t-test.

Hormonal studies: Non-parametric statistics were used due to the lack of normal distribution. Median and quartile deviations (QD: the difference between the 25 and 75 percentile divided by 2) are given in Table 3. Statistical analyses were performed using the Kruskall—Wallis test for multiple comparisons, followed by Mann—Whitney U-test. When multiple comparisons were done, Bonferroni corrections for multiple comparisons were used. A parametric ANOVA analysis was also per-
formed after log transformation of the endocrine data.

Large between-animal variations in hormone concentrations were found and indicated that some animals were not killed in dioestrus as intended. As there was a possibility that not all animals were killed in dioestrus phase as intended, the statistical analyses of the endocrine data were recalculated after excluding animals in probable heat (oestrogen values more than 2S.D. above median, and progesterone values less than 2S.D. lower than median). The number of animals excluded from the analyses was as follows: from the low-dose group, 1/15 animals; from the high-dose group, 4/21 animals; and from the controls, 5/26.

Results

All animals accepted the gastric tube feeding without signs of discomfort or reduced motor activity, except four (one low dose, three controls) that died during the experiment period.

Mean LEV concentrations at exsanguination, 4 h after the last dose, was 122 μmol/l in the low-dose and 277 μmol/l in the high-dose group (Table 1). A graph showing variations in serum LEV concentrations after a single dose is provided (Fig. 1). Mean body weight was the same in all three groups (Table 1).

Mean ovary weight showed a dose-dependent and significant increase after LEV treatment (Table 2; controls versus high dose: \( p = 0.02 \). The number of corpora lutea was significantly higher in all LEV-treated animals, and in the low-dose group, than in controls (Table 2; \( p = 0.045 \) and 0.034, respectively). In the high-dose group the number of corpora lutea was higher than in the controls, but not significantly so (Table 2; \( p = 0.298 \)). The number of secondary follicles was significantly higher in all LEV-treated animals compared to controls (Table 2; \( p = 0.034 \)). The mean number of cysts per section was lower in the group of LEV-treated animals than in control animals (Table 2).

Tissue sections from the liver, kidney, lung and myocardium were examined by light microscopy for presence of histopathological changes, such as degeneration, necrosis and inflammation. No such lesions were observed.

LEV-treated animals differed significantly from the control group with respect to the serum concentrations of several hormones (Table 3). Serum testosterone concentrations were significantly higher in LEV-treated animals than in the control group (Table 3; \( p = 0.002 \)). Serum oestradiol concentrations were significantly lower in LEV-treated animals than in controls (Table 3; \( p < 0.001 \)). This difference was more pronounced in the low-dose group, which differed significantly from the control animals (\( p < 0.0001 \)), than in the high-dose group, in which the difference from the control animals was not significant. Serum progesterone concentrations were higher after LEV treatment. Again, the difference was only statistically significant in the low-dose group (\( p = 0.045 \)). Serum FSH concentrations were significantly lower in treated animals than in controls (\( p = 0.004 \) (all animals) and 0.012 (high-dose group only)). No significant difference was found in serum LH concentrations between the different treatment groups. Using a parametric ANOVA analysis after log transformation of endocrine data did not reveal any further significant values.

A reanalysis of the data, excluding animals which might not have been exsanguinated during dioestrus, did not change the pattern of results, except for progesterone concentrations, which demonstrated no significant difference between low-dose animals and controls following recalculation (Table 3).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>All treated</th>
<th>Low dose</th>
<th>High dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal weight (mean S.D.) (mg)</td>
<td>253.8 (15.17)</td>
<td>254.8 (19.87)</td>
<td>253.6 (19.87)</td>
<td>255.6 (22.31)</td>
</tr>
<tr>
<td>Serum LEV concentrations (mean S.D.) (μmol/l)</td>
<td>0</td>
<td>202 (95.6)</td>
<td>122 (40.9)</td>
<td>277 (65.2)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>All treated</th>
<th>Low dose</th>
<th>High dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovary weight (mean S.D.) (mg)</td>
<td>94.0 (18.0)</td>
<td>104.1 (21.7)</td>
<td>97.3 (21.5)</td>
<td>110.4* (20.6)</td>
</tr>
<tr>
<td>Corpora lutea (mean S.D.) (number/section)</td>
<td>9.8 (3.0)</td>
<td>11.8 (4.5)</td>
<td>13.3 (5.5)</td>
<td>10.9 (3.5)</td>
</tr>
<tr>
<td>Secondary follicles (mean S.D.) (number/section)</td>
<td>6.8 (2.8)</td>
<td>8.6 (3.5)</td>
<td>8.3 (3.6)</td>
<td>8.8 (3.5)</td>
</tr>
<tr>
<td>Cysts (mean S.D.) (number/section)</td>
<td>0.8 (1.7)</td>
<td>0.4 (1.1)</td>
<td>0.0 (0.0)</td>
<td>0.7 (1.4)</td>
</tr>
</tbody>
</table>

*Significantly different from controls (\( p \leq 0.05 \); one-way ANOVA, followed by Student’s t-test).
Discussion

These data suggest that long-term LEV-treatment significantly affects reproductive endocrine function, as well as ovarian morphology, in non-epileptic rats. The shown endocrine effects of LEV seem to differ from those previously reported with other AEDs. The only other AED that has been associated with morphological changes in the ovary is VPA. Røste et al.\textsuperscript{12} demonstrated that Wistar rats treated with VPA for 90 days had an increased number of ovarian cysts, a reduced number of corpora lutea, and reduced ovary weight compared to controls, which correspond with the findings of polycystic ovaries in women taking VPA.\textsuperscript{1,10,26} In the present study, LEV treatment caused different effects to these, as ovarian weight and numbers of corpora lutea increased, whereas the number of cysts decreased. Also the hormonal changes contrasted with the findings from the VPA study,\textsuperscript{12} since there was no effect of VPA on progesterone or testosterone, whilst oestradiol concentrations were reduced. Gonadotrophins are not affected by VPA in either humans or animals,\textsuperscript{1,12} whereas in the present study FSH was decreased. With respect to the VPA findings with the same experimental model, there were close similarities between animal and human findings.\textsuperscript{1,10–12}

A major problem with all animal studies is how to extrapolate to the human clinical situation. Extrapolating to possible clinical implications from animal studies has obvious limitations, and before this can be considered a number of questions must be addressed. Firstly, is the animal model used relevant to the human situation? Secondly, is the treatment period sufficient for studying long-term effects? Thirdly, are the drug concentrations used in the animal study relevant to the clinical situation?

The laboratory rat has been a major contributor to our understanding of reproductive biology, not only in the rat itself, but also in other animals and in humans.\textsuperscript{27} Although predictions about drug-related effects across animal species should be made with great care, the results presented here clearly demonstrate the need for further studies on possible side effects caused by LEV treatment.

It is important that the treatment period is sufficient to mimic long-term drug treatment. In female rats, 90 days represents about 20 oestrous cycles. In humans, this number of menstrual cycles would correspond to approximately 2 years, which is well above that which has been used as an inclusion criterion for women participating in studies on endocrine effects of AEDs.

The therapeutic concentration range for LEV in humans is between 40 and 130\,\mu{mol}/l, although many patients tolerate much higher concentrations. However, these are trough morning values and represent the minimum concentration of the therapeutic range during the day. Diurnal fluctuations are marked for LEV and mean concentrations during the day are therefore higher than trough morning levels. In humans using 1000\,mg LEV twice daily, the mean serum concentration after an overnight fast is 70\,\mu{mol}/l (range: 29–218) and mean $C_{\text{max}}$ is 188\,\mu{mol}/l (135–235). In humans using 1500\,mg

### Table 3. Serum hormone levels after levetiracetam treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>All treated</th>
<th>Low dose</th>
<th>High dose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Testosterone (median QD)</strong> (nmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.16 (0.27)</td>
<td>0.50$^\ast$ (0.19)</td>
<td>0.48 (0.13)</td>
<td>0.52$^\ast$ (0.87)</td>
</tr>
<tr>
<td>B</td>
<td>0.08 (0.10)</td>
<td>0.46$^\ast$ (0.10)</td>
<td>0.45$^\ast$ (0.10)</td>
<td>0.47$^\ast$ (0.10)</td>
</tr>
<tr>
<td><strong>Oestradiol (median QD)</strong> (pmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>257.5 (126.8)</td>
<td>67.4$^\ast$ (54.5)</td>
<td>55.8$^\ast$ (14.9)</td>
<td>145.3 (98.7)</td>
</tr>
<tr>
<td>B</td>
<td>160.1 (89.6)</td>
<td>62.1 (22.8)</td>
<td>52.9 (12.4)</td>
<td>102.4 (52.0)</td>
</tr>
<tr>
<td><strong>Progesterone (median QD)</strong> (nmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>34.7 (18.1)</td>
<td>47.4 (15.4)</td>
<td>56.8$^\ast$ (26.5)</td>
<td>41.8 (18.4)</td>
</tr>
<tr>
<td>B</td>
<td>46.5 (13.8)</td>
<td>53.4 (18.9)</td>
<td>57.6 (25.4)</td>
<td>43.0 (11.8)</td>
</tr>
<tr>
<td><strong>FSH (median QD)</strong> (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>5.5 (1.2)</td>
<td>3.3$^\ast$ (0.8)</td>
<td>3.4 (1.3)</td>
<td>3.3$^\ast$ (0.6)</td>
</tr>
<tr>
<td>B</td>
<td>5.4 (2.3)</td>
<td>3.2 (0.8)</td>
<td>3.6 (1.2)</td>
<td>3.2 (0.4)</td>
</tr>
<tr>
<td><strong>LH (median QD)</strong> (ng/ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1.24 (0.34)</td>
<td>1.31 (0.49)</td>
<td>1.29 (0.31)</td>
<td>1.37 (0.49)</td>
</tr>
<tr>
<td>B</td>
<td>1.23 (0.36)</td>
<td>1.27 (0.32)</td>
<td>1.35 (0.33)</td>
<td>1.24 (0.20)</td>
</tr>
</tbody>
</table>

A: all treated animals; B: all treated animals, except those in heat.

$^\ast$ \( p < 0.05 \) compared to control. Kruskall–Wallis test for multiple comparisons and Mann–Whitney U-test. Bonferroni corrections used for comparing low- and high-dose animals with controls.
twice a day, the mean serum concentration is 94 µmol/l (41–200) and mean C_{max} is 265 µmol/l (212–370).^{28} In our study, human therapeutic concentrations of LEV were reached in the rats treated with the low dose. The animals treated with the high dose of LEV were exposed to drug concentrations above that commonly used in humans. However, two daily doses of LEV, as used in this study, indicated that serum LEV concentrations fluctuate considerably, and the serum LEV concentrations measured in the high-dose group were only slightly above, or within, the therapeutic range for most of the day. We therefore believe that the results observed in our study were achieved at clinically relevant drug concentrations, particularly for those animals in the low-dose treatment group.

The observed effects could be mediated at several levels of the hypothalamic—pituitary—gonadal (HPG) axis. An effect at the hypothalamic—pituitary level could be argued, because of the lowered FSH levels. A suppressive effect of LEV on FSH could affect follicular development and might also disturb ovarian steroid secretion. The mean plasma testosterone concentration was increased approximately threefold in the present study, and this could be because testosterone is the principal ovarian secretory product in follicles deprived of FSH. However, this explanation is not supported by the increased numbers of corpora lutea and higher progesterone levels found in LEV-treated animals. It is possible that the maintained corpus luteum function could be due to the induction of a pseudo pregnancy-like state in which corpora lutea are activated by prolactin and persist for up to 13 days before regressing. During this time follicle growth and oestriadiol levels are reduced.^{29}

Consequently, a direct effect of LEV on the ovary should be considered. This possibility is supported by the increased basal testosterone and reduced oestriadiol secretion noted with LEV-treatment of isolated porcine ovarian follicular cells in vitro.^{23} To the best of our knowledge there are no other reports published describing similar effects in vivo.

The data suggest that the LEV effects observed did not increase with dose. On the contrary, for several endpoints, changes were actually more pronounced in animals given the lower LEV dose. So far there is no obvious explanation for this finding. Methodological errors in the timing of sample and tissue collection could be suspected, however, there was consistency between hormonal and morphological findings. Further, it is important to be aware that the dose level used in the low-dose group (where the greatest effects were seen) resulted in serum concentrations within the human therapeutic range.

In conclusion, the present study indicates a drug-specific effect of LEV on reproductive endocrine function and ovarian morphology in female Wistar rats at clinically relevant doses following long-term drug treatment. Clinical studies on LEV, however, have not shown any clinical symptoms indicating endocrine side effects,^{20,30} and further studies using both in vivo and in vitro models are needed to elucidate if, how, and to what degree, LEV may affect reproductive endocrine functions in humans.

Acknowledgement

We want to thank Svein Johannessen, The National Center for Epilepsy, Sandvika, Norway for analysing levetiracetam concentrations.

References

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