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Effects of valproic acid (VPA) and levetiracetam (LEV) on proliferation, apoptosis and hormone secretion of the human choriocarcinoma BeWo cell line

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Abstract:
Epilepsy has been associated with poor obstetric outcomes that may be the result of the epilepsy or a direct effect of anti-epileptic drugs on placentation. To investigate any direct effect of anti-epileptic drugs on cell proliferation, apoptosis and hormone secretion with focus on human chorionic gonadotropin (β-hCG), progesterone (P4) and 17β-estradiol (E2), BeWo cell line was cultured in the presence of different concentrations of sodium valproate (0.45, 0.6, 1.5 or 2 mM) or levetiracetam (0.07, 0.12, 0.3 or 0.5 mM) with appropriate solvent controls. Cell proliferation was measured using BrdU incorporation. Caspase-3 activity was used as a marker of cell apoptosis and was evaluated by a fluorometric assay. Additionally, hormone secretion was evaluated by ELISA kits. Dose-dependent action of VPA on cell proliferation occurred in parallel to stimulation of caspase-3 activity. LEV had no effect on cell proliferation, and after long term exposure to the drug, a decrease in caspase-3 activity was observed. A significant decrease in β-hCG, P4 and E2 production was observed when the cells were treated with VPA. LEV decreased β-hCG and E2 secretion but had no effect on P4 level. Direct inhibition of cell proliferation and hormone secretion along with apoptotic action suggest that exposure to VPA at therapeutic doses during early pregnancy should be approached with caution. Trophoblast cells appear to be less sensitive to LEV; however, further studies involving placental tissue are necessary to determine the safety of the drug.

Key words:
sodium valproate, levetiracetam, BeWo cell line, hormone secretion, proliferation, apoptosis

Introduction

Valproic acid (VPA) is the most widely prescribed anti-epileptic drug in the world [20]. In addition to the use of VPA as an anticonvulsant, it is being increasingly prescribed for a number of other conditions, including treatment of generalized and partial seizures, bipolar and schizoaffective disorders and even the prophylactic treatment of migraine [15]. The toxicity of this agent to the fetus and placenta remains a concern. Little human pregnancy exposure data are available; however, the current data indicate a high rate of spontaneous abortions and premature births following VPA exposure in pregnancy [16, 21]. Levetiracetam (LEV) is a relatively new anti-epileptic drug that has been extensively used during recent years [1], and preliminary animal experimental data and clinical reports suggest that levetiracetam use is safer than other

anti-epileptic drugs during pregnancy [11, 13, 14, 19], although it is known that anticonvulsants (including VPA and LEV) during pregnancy cross the placenta and attain pharmacologically active concentrations in the fetus [30]. Previous studies have focused on the known teratogenicity of anti-epileptic drugs [3, 32]; however, less attention has focused on the other obstetric complications associated with anti-epileptic drugs use in pregnancy.

The current study used a human choriocarcinoma cell line (BeWo) to assess the effects of a therapeutic doses of levetiracetam (from 0.07 to 0.47 mM) or sodium valproate (from 0.45 to 2.1 mM) [18] upon cell proliferation, apoptosis and hormone secretion (human chorionic gonadotropin, progesterone and 17β-estradiol). The human choriocarcinoma BeWo cell line was used because it has similar properties to a first trimester trophoblast in regards to morphology, physiology and hormone secretion [2].

Materials and Methods

Reagents

DMEM nutrient mixture F-12 Ham medium (DMEM/F12) without phenol red, insulin, trypsin, Ac-Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), HEPES, CHAPS, EDTA, glycerol, DTT and DMSO were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS, heat inactivated) and penicillin/streptomycin solution (penicillin 10,000 units/ml, streptomycin 10 mg/ml) were obtained from PAA Laboratories GmbH (Linz, Austria). Phosphate-buffered saline (PBS) was purchased from Biomed (Lublin, Poland). Sodium valproate (VPA) was obtained from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in sterile water. Levetiracetam (LEV) solution in sucrose (655 mg/ml) and purified water was prepared by the Pharmacy at Rikshospitalet University Hospital, Oslo, Norway and kindly gifted by prof. Erik Taubøll. The solutions were then further diluted in culture medium. These concentrations of VPA and LEV were chosen based on our previous findings [8, 27].

Cell culture

BeWo human choriocarcinoma cells (ATCC, Manassas, VA, USA) were routinely cultured in DMEM/F12 medium without phenol red supplemented with 0.01 mg/ml insulin and 10% heat-inactivated FBS, 100 IU/ml of penicillin and 100 μg/ml of streptomycin.

Experimental procedure

To investigate the effect of drugs on trophoblast cell proliferation and apoptosis, the cells were seeded in 96-well culture plates at a density of 1×10^3 cells/well, incubated in phenol red-free DMEM/F12 with 0.01 mg/ml insulin and 5% FBS and allowed to attach. After 48 h the medium was replaced with phenol red-free DMEM/F12 with 0.01 mg/ml insulin and 5% FBS containing different concentration of sodium valproate (0.45, 0.6, 1.5 or 2 mM) or LEV (0.07, 0.12, 0.3 or 0.5 mM) for 24, 48, 72 or 96 h. As a control for VPA we used medium alone and as a control for LEV we used medium supplemented with 0.5% sucrose solution (655 mg/ml).

To investigate the possible effects on hormone secretion the cells were seeded in 24-well culture plates at a density of 1×10^4 cells/well, incubated in phenol red-free DMEM/F12 with 0.01 mg/ml insulin and 5% FBS and allowed to attach overnight. After 48 h the medium was replaced with phenol red-free DMEM/F12 with 0.01 mg/ml insulin and 5% FBS and treated with different concentration of VPA (0.45, 0.6, 1.5 or 2 mM) or LEV (0.07, 0.12, 0.3 or 0.5 mM) for 24, 48, 72 or 96 h. Incubation media were collected and used for hormone analysis.

Cell proliferation

Cellular DNA synthesis rates were determined by measuring BrdU incorporation with the commercial Cell Proliferation ELISA System (Roche Molecular Biochemicals, Mannheim, Germany). After 24, 48, 72 or 96 h of incubation with reagents, cells were incubated for 3 h with a BrdU labeling solution (provided by the kit) containing 10 μM BrdU. The assay was performed according to the manufacturer's instructions. Absorbance values were measured at 450 nm using an ELISA reader (ELx808 Bio-Tek Instruments, USA). Culture medium alone was used as a control for nonspecific binding, and all samples were run six times in the same assay.

Caspase-3 activity assay

The activity of caspase-3, the primary executioner of apoptosis, was measured as described previously by Nicholson et al. [17] using caspase-3 fluorometric substrate Ac-DEVD-AMC. The specificity of the assay has been studied previously by using Ac-DEVD-CHO inhibitor [9]. After 24, 48, 72 or 96 h of incubation with reagents, culture media were replaced with caspase assay buffer containing 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM EDTA, 10% glycerol and 10 mM DTT (pH 7.4), and the cells were incubated on ice for 10 min. Next, the cell lysate was incubated at 37°C with Ac-DEVD-AMC at a final concentration of 10 μM. The amounts of fluorescent products was monitored at 460 nm using a fluorescence microplate reader (FLx800 Bio-Tek Instruments, USA) every 30 min until 5 h. Culture medium alone was used as a control for nonspecific binding. All of the samples were run six times in the same assay. Lysate protein levels were measured by the Bradford method as described in the Bio-Rad Protein Assay (Bio-Rad Laboratories Inc., CA, USA) with bovine serum albumin as standards.

Measurement of hormone level

The concentrations of progesterone (P4), 17β-estradiol (E2) and β-subunit of human chorionic gonadotrophin (β-hCG) in the media were determined by enzyme immunoassay using commercial ELISA kits (DRG Diagnostic, Germany). All samples were run in quadruplicate in the same assay.

The limit of progesterone assay sensitivity was 0.3 ng/ml; inter- and intra-run precisions had coefficients of variation of 4.34% and 6.99%, respectively. The sensitivity of 17β-estradiol assay was 25 pg/ml; inter- and intra-run precisions had coefficients of variation of 7.25% and 4.46%, respectively. The β-hCG assay sensitivity was 5 mIU/ml, and inter- and intra-run precisions had coefficients of variation of 6.72% and 2.71%, respectively.

Statistical analysis

Each experiment was repeated three times (n = 3). Data shown in graphs are the mean ± SEM of all 12 values derived from the three replicate experiments. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc., CA, USA). Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Differences (HSD) multiple range test.

Results

Action of VPA and LEV on cell proliferation

Cell proliferation of the control culture increased throughout incubation from 1.6 ± 0.16 relevant absorbance units at 24 h to 2.9 ± 0.16 relevant absorbance units in 96 h of culture. In VPA-treated cells, no effect of doses ranging from 0.45 mM to 1.5 mM during all times of exposure was observed. VPA at a dose 2.1 mM decreased cell proliferation beginning at 48 h

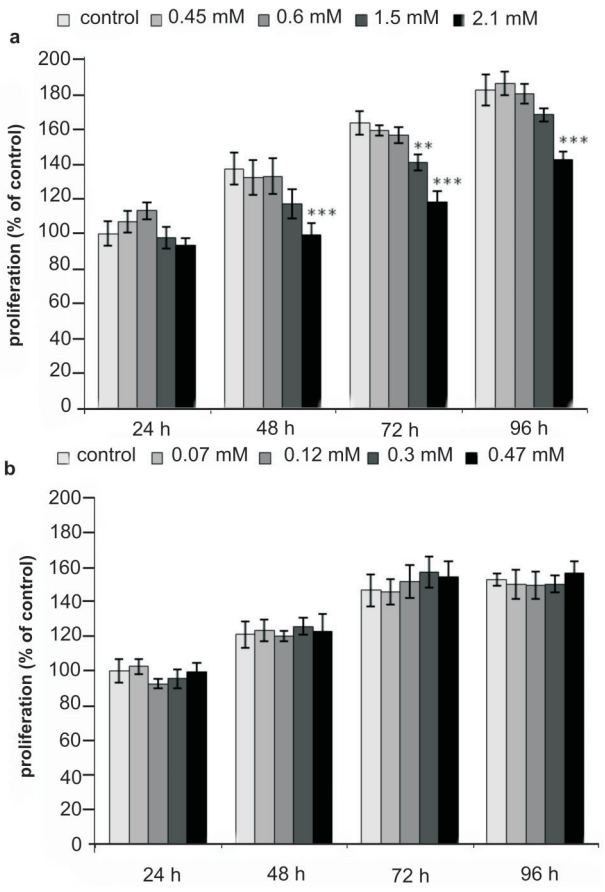

Fig. 1. The effect of sodium valproate (VPA) (**a**) and levetiracetam (LEV) (**b**) on cell proliferation. Each point represents the mean ± SEM of results from three independent experiments, each of which consisted of six replicates per treatment group. All means marked with ** ($p < 0.01$) and *** ($p < 0.001$) are significantly different from control

and lasted until 96 h (Fig. 1a). In LEV-exposed cells, no effect on cell proliferation was observed from 24 h to 96 h (Fig. 1b).

Action of VPA and LEV on caspase-3 activity

Caspase-3 activity in control culture increased throughout incubation from 39,000 ± 500 relevant fluorescence units at 24 h to 47,000 ± 900 relevant fluorescence units at 96 h of culture. In VPA-treated cells, no effect on caspase-3 activity was noted at 24 h of culturing. Beginning at 48 h of incubation, an increase of caspase-3 activity was observed under the influence of 1.5 and 2.1 mM VPA, and all of doses applied showed an increase in caspase-3 activity at 72 h and 96 h (Fig. 2a). In LEV-exposed cells, inhibitory action of low doses (0.07 and 0.12 mM) on caspase-3 activity was noted in 96 h of exposure (Fig. 2b).

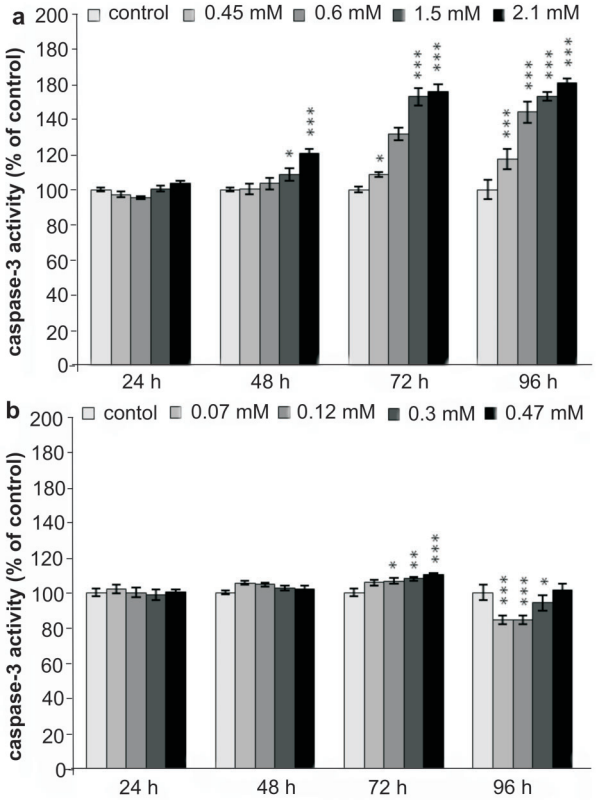

Fig. 2. The effect of sodium valproate (VPA) (**a**) and levetiracetam (LEV) (**b**) on caspase-3 activity. Each point represents the mean ± SEM of results from three independent experiments, each of which consisted of six replicates per treatment group. All means marked with * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) are significantly different from control

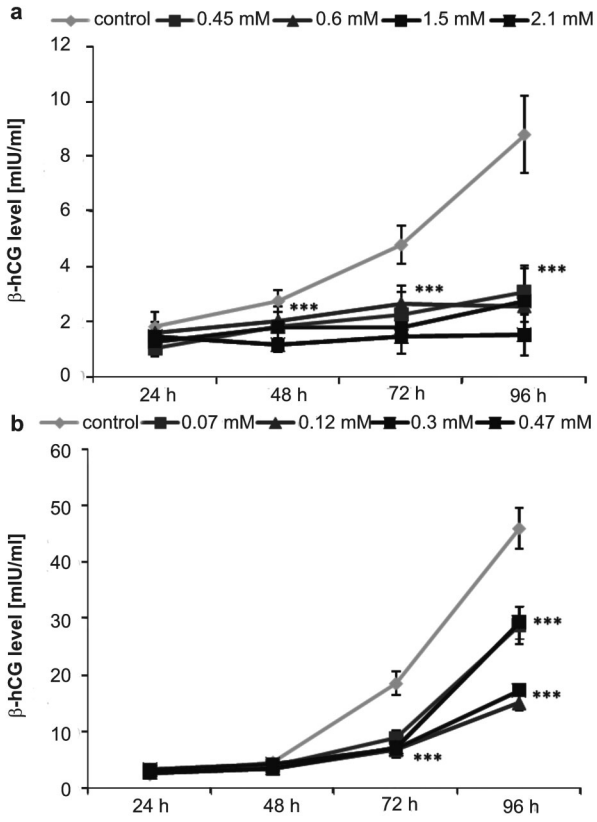

Fig. 3. The effect of sodium valproate (VPA) (**a**) and levetiracetam (LEV) (**b**) on human chorionic gonadotropin (β-hCG) secretion. Each point represents the mean ± SEM of results from three independent experiments, each of which consisted of four replicates per treatment group. All means marked with *** ($p < 0.001$) are significantly different from control

Action of VPA and LEV on β-hCG concentration

β-hCG concentrations increased throughout incubation from 1.8 mIU/ml at 24 h to 8.7 mIU/ml at 96 h of culture. A significant decrease in β-hCG production, with respect to control cultures, was observed with treatment of all VPA dosages at 48 h ($p < 0.05$), 72 h ($p < 0.01$) and 96 h ($p < 0.001$; Fig. 3a). Furthermore, a decrease in β-hCG production was witnessed at 72 h ($p < 0.05$) and 96 h ($p < 0.05$ and $p < 0.01$) in LEV-treated cells (Fig. 3b).

Action of VPA and LEV on steroid hormones secretion (progesterone and 17β-estradiol)

In control cultures, the concentrations of both P4 (from 1.5 ng/ml at 24 h to 11.3 ng/ml at 96 h of cul-

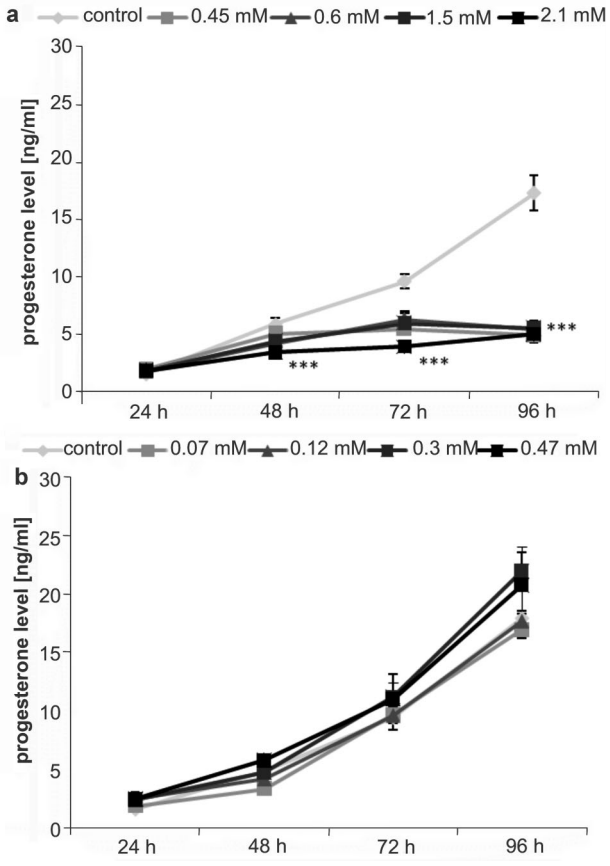

Fig. 4. The effect of sodium valproate (VPA) (**a**) and levetiracetam (LEV) (**b**) on progesterone secretion. Each point represents the mean ± SEM of results from three independent experiments, each of which consisted of four replicates per treatment group. All means marked with *** ($p < 0.001$) are significantly different from control

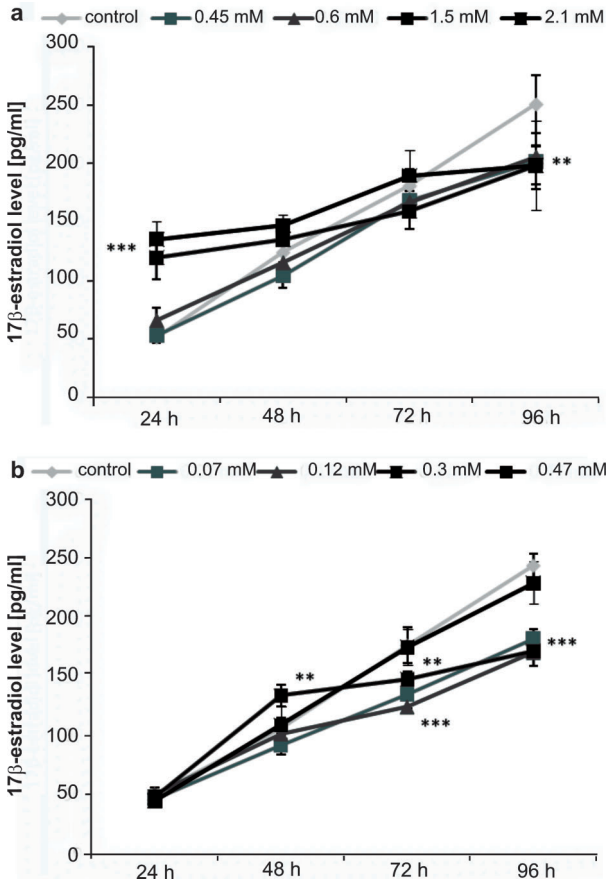

Fig. 5. The effect of sodium valproate (VPA) (**a**) and levetiracetam (LEV) (**b**) on 17β-estradiol secretion. Each point represents the mean ± SEM of results from three independent experiments, each of which consisted of four replicates per treatment group. All means marked with ** ($p < 0.01$) and *** ($p < 0.001$) are significantly different from control

ture) and E2 (from 51.5 pg/ml at 24 h to 250.5 pg/ml at 96 h of culture) increased throughout incubation.

In VPA exposed cells, a decrease of P4 secretion at 72 h ($p < 0.05$ and $p < 0.01$) and 96 h ($p < 0.01$) of exposure was noted under the influence of all applied doses (Fig. 4a). Transition stimulatory action on E2 secretion at 24 h of exposure in cells treated with 1.5 and 2.1 mM ($p < 0.05$) VPA was observed. The inhibitory action on E2 secretion at 48 h of exposure underwent a statistically significant decrease after 96 h of exposure ($p < 0.05$; Fig. 5a).

No differences in P4 secretion in cells exposed to all doses of LEV were observed (Fig. 4b). Additionally, no differences regarding the inhibitory action on E2 secretion of low doses (0.07, 0.12 and 0.3 mM) was noted at 96 h of exposure ($p < 0.05$; Fig. 5b).

Discussion

Our results showed differences in the action of VPA and LEV on cell proliferation and apoptosis. VPA at a dose of 2.1 mM decreased cell proliferation beginning at 48 h and lasted until 96 h of exposure. In all doses VPA caused an increase in caspase-3 activity beginning at 72 h of exposure. In LEV-exposed cells, no effect on cell proliferation was observed from 24 h to 96 h and, inhibitory action of low doses on caspase-3 activity was noted only at 96 h of exposure. Wu and Guo [31] showed that VPA is a histone deacetylase inhibitor and can suppress proliferation through induction of cell cycle arrest. To our knowl-

edge, our data are the first to show an effect of LEV on placental cell proliferation. Our results are in agreement with the data from Cansu et al. [5], who showed that VPA trigger apoptotic and degenerative effects on rat uterine cells. VPA also prevents implantation of the embryo to the uterus and causes abortion *via* endometrial eosinophil infiltration. Additionally, we previously showed that VPA and LEV treatment increase caspase-3 activity in human ovarian follicular cells [28]. Based on the data presented here, placental trophoblast cells are less sensitive to LEV.

Apoptosis is thought to be important for normal placental development; however, in complicated pregnancies such as preeclampsia or intrauterine growth restriction, a greater incidence of trophoblast apoptosis has been observed [7, 12], suggesting that alterations in the regulation of trophoblast apoptosis may contribute to the pathophysiology of these diseases.

The second part of our experiments evaluated the action of VPA and LEV on human trophoblast endocrine function. There are data showed an important role of hCG in the regulation of cell proliferation, differentiation and apoptosis during human placental trophoblast formation [33]. We demonstrated that VPA exposure beginning at 48 h and LEV exposure at 72 and 96 h decreased β-hCG secretion, a marker of trophoblast differentiation, and VPA at all applied doses increased caspase-3 activity, a marker of apoptosis [24]. Changes in β-hCG level in the first trimester are correlated with increased risk of pregnancy disorders e.g., hCG is present at reduced levels during spontaneous aborting and ectopic pregnancy. The low hCG levels most likely reflect a derangement of trophoblast function that will culminate in placental insufficiency and fetal growth restriction [6]. To our knowledge, there is no data showing β-hCG level during pregnancy in women treated with VPA or LEV.

The human placenta is the site of an important production of steroid hormones, primarily P4 and E2. Progesterone that is temporally secreted in large amounts and present at the fetomaternal interface may play a physiologic role in trophoblast differentiation [23]. Our data showed no effect of LEV on P4 secretion and inhibitory action of VPA, at all doses used, on P4 secretion from 72 h to 96 h of exposure.

Based on the data presented here, placental trophoblast cells may be sensitive to VPA, and inhibiting P4 secretion may help explain a high rate of spontaneous abortions and premature births following VPA exposure in pregnancy [16, 21]. Additionally, a study using female rats treated with LEV for 3 months reported side effects that impacted reproductive endocrine function (higher progesterone levels) [26]. In that study, the LEV effects observed did not increase with dose. Alternatively, for several endpoints, changes were actually more pronounced in animals given the lower LEV dose.

Estrogens increasingly produced by the fetoplacental unit may be implicated in the end-stage maturation and aging of the trophoblast. Previous studies showed that the placenta is an estrogen-target tissue and that estrogens have important physiological roles in regulating functional differentiation of the placental villous trophoblast [4, 22]. Our data showed transition stimulatory action on E2 secretion at 24 h of exposure for 1.5 and 2.1 mM VPA; however, we also observed inhibitory action at 48 h of exposure, leading to a significant decrease in E2 production after 96 h of exposure. Inhibitory action of low doses (0.07 and 0.3 mM) of LEV on E2 production was noted only at 96 h of exposure.

VPA has been shown to affect steroidogenesis and inhibit the conversion of testosterone to estradiol in a series of experiments with cultures of porcine ovarian follicular cells [8, 29]. VPA exposure resulted in a decrease in estradiol levels, while testosterone and progesterone levels were unaffected. Furthermore, VPA caused down regulation of expression of genes encoding enzymes involved early in steroidogenesis [10]. Bukovsky et al. [4] showed that estrogens may play a role in the stimulation of cytotrophoblast differentiation into syncytiotrophoblast; therefore, we hypothesise that the inhibition of E2 secretion by VPA may be responsible for disruption of syncytiotrophoblast formation, but further studies are necessary to confirm this hypothesis.

Until recently, levetiracetam endocrine side effects had not been described. A study using ovarian follicular cells from prepubertal pigs demonstrated that LEV may affect basal steroid hormone secretion, indicating a possible effect of the drug on steroidogenesis [27]. However, Gustavsen et al. [10] reported no effect on sex hormone production in human *in vitro* cells exposed to LEV. Furthermore, this result was confirmed by Svalheim et al. [25] that showed no apparent sexual or endocrine side effects in the women being treated with LEV.

Summarizing this part of the experiments, falling β-hCG and E2 secretion by cells exposed to LEV without effects on P4 secretion indicate possible ef-

fects on spontaneous abortion. P4, E2 and β-hCG is decreased under the influence of VPA, suggesting that it plays a role in multiple processes such as differentiation, maturation and aging.

In conclusion, the inhibited proliferation of trophoblast cells after longer exposure to VPA and the direct effect on hormonal homeostasis of both anti-epileptic drugs suggest that therapeutic administration of all doses of VPA during early pregnancy should be approached with caution. Trophoblast cells appear to be less sensitive to LEV; however, further studies involving placental tissue are necessary to decide the safety of the drug.

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