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The effect of valproic acid on SLC5A8 expression in gonad-intact and gonadectomized rat thymocytes

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Milda Juknevičienė[1], Ingrida Balnytė[1], Angelija Valančiūtė[1], Jūratė Stanevičiūtė[1], Kęstutis Sužiedėlis[2] and Donatas Stakišaitis[1,2]

Abstract

Background: Valproic acid (VPA) pharmacological mechanisms are related to the anti-inflammatory and anti-viral effects. VPA is a histone deacetylases inhibitor and serves a role in its immunomodulatory impacts. VPA has complex effects on immune cell's mitochondrial metabolism. The SLC5A8 transporter of short fatty acids has an active role in regulating mitochondrial metabolism. The study aimed to investigate whether SLC5A8 expresses the sex-related difference and how SLC5A8 expression depends on gonadal hormones, VPA treatment, and NKCC1 expression in rat thymocytes.

Methods: Control groups and VPA-treated gonad-intact and gonadectomized Wistar male and female rats were investigated (n = 6 in a group). The VPA 300 mg/kg/day in drinking water was given for 4 weeks. The SLC5A8 (*Slc5a8* gene) and NKCC1 (*Slc12a2* gene) RNA expressions were determined by the RT-PCR method.

Results: The higher *Slc5a8* expression was found in the gonad-intact males than respective females (p = 0.004). VPA treatment decreased the Slc5a8 expression in gonad-intact and castrated males (p = 0.02 and p = 0.03, respectively), and increased in gonad-intact female rats compared to their control (p = 0.03). No significant difference in the *Slc5a8* expression between the ovariectomized female control and VPA-treated females was determined (p > 0.05). VPA treatment alters the correlation between *Slc5a8* and Slc12a2 gene expression in thymocytes of gonad-intact rats.

Conclusion: VPA effect on the *Slc5a8* expression in rat thymocytes is gender- and gonadal hormone-dependent.

Keywords

valproic acid, SLC5A8, thymocyte, sex-related difference

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Introduction

VPA is one of the most frequently prescribed anti-epileptic drugs.[1] VPA is an inhibitor of histone deacetylases (HDACs).[2] Studies raise the possibility of the therapeutic anti-viral potential of VPA.[3,4] The virus replication and survival depend on the produced mitochondrial energy; thus, the anti-viral therapy tactics may involve medicine preparations that change energetic mitochondrial functions and activate anti-

[1]Department of Histology and Embryology, Medical Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania
[2]Laboratory of Molecular Oncology, National Cancer Institute, Vilnius, Lithuania

Corresponding authors:
Donatas Stakišaitis, Laboratory of Molecular Oncology, National Cancer Institute, Santariskiu 1, LT 08660 Vilnius, Lithuania.
Email: donatas.stakisaitis@lsmuni.lt

Milda Juknevičienė, Department of Histology and Embryology, Medical Academy, Lithuanian University of Health Sciences, Sukileliu pr. 13, Kaunas, LT 44307, Lithuania.
Email: milda.girdauskaite-jukneviciene@lsmuni.lt

inflammatory mechanisms.[5] As a simple fatty acid, VPA is a substrate for the fatty acid β -oxidation pathway.[6] VPA bioactivation directs the metabolite 4-ene-VPA into the mitochondria, forming the 2,4-diene-VPA-CoA ester by β-oxidation.[7] VPA metabolites induce the significant decrease of pyruvate-driven oxidative phosphorylation in mitochondria by conflict with pyruvate transport.[6] Such VPA effect could interfere with the short fatty acid transporter SLC5A8 (Solute carrier family-5 member-8) activity. The SLC5A8, transporting short fatty acids as a substrate for the *β*-oxidation pathway in mitochondria, has a vital role in regulating cell metabolism.

SLC5A8 acts as electrogenic sodium (Na^+) and chloride (Cl^-)-dependent sodium-coupled transporter, transporting into the cell lactate, pyruvate, acetate, propionate, valerate, butyrate, and monocarboxylate drugs (dichloroacetate, nicotinate, salicylate, and 5-aminosalicylate) and ketone bodies.[8–12] Research of adult male wild-type *c/ebp*$\delta^{+/-}$ and *c/ebp*$\delta^{-/-}$ mice shows that the SLC5A8 physiologic function operates an essential role in the short-chain fatty acids transport into the cell.[13,14] If the transporter is not expressed, cells may not accumulate a substrate. Short fatty acids transported into immune cells through the SLC5A8 change the HDAC activity, exerting immunomodulatory effects: blockade of dendritic cell development, releasing cytokines and inducing T cell apoptosis, emphasizing the importance of SLC5A8 in the immune homeostasis, and suppression of inflammation.[15,16] SLC5A8 triggers cell apoptosis by pyruvate-dependent repression of HDACs.[17] The silencing of the *SLC5A8* gene is related to DNA methylation, and treating cancer cells with DNA-demethylating agents increases the SLC5A8 expression.[12] VPA could activate genes regulated by DNA methylation, and the VPA effect may be trigged to active DNA demethylation in cancer cells.[18,19] The SLC5A8 activity in the male rat duodenum enterocytes depends on Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) activity.[20] Changes in the NKCC1 activity might lead to mitochondrial function changes. In fibroblasts treated with NKCC1 inhibitor bumetanide, the increase in maximal respiration suggests enhanced substrate availability and mitochondrial oxidation.[21] VPA reduces NKCC1 RNA expression in males but not in female rat thymocytes.[22] Pro-inflammation-immune cells derive most of their energy from aerobic glycolysis rather than mitochondrial oxidation to generate more energy and support their intensified activity.[23] Sex differences in the immune response depending on the sexually dimorphic populations of mitochondria and gonadal hormones.[24]

The present study aimed at the following questions: (1) whether SLC5A8 transporter expresses the sex-related difference in rat thymocytes? (2) does VPA treatment change the SLC5A8 expression in thymocytes? (3) how SLC5A8 expression depends on gonadal hormones, VPA treatment, and NKCC1 expression in thymocytes? The answers to these questions are significant in dealing with personalized VPA therapy purposes.

Methods

Animals and treatment

Permission of the study was obtained from the State Food and Veterinary Service of Lithuania to use experimental animals for research (2017-01-02 No. G2-53). The preclinical and clinical research of pharmaceuticals regulatory guidelines indicates the importance of evaluating sex differences, stressing that medicine development should provide adequate information about the drug effects in both genders.[25] The VPA treatment effect on the thymus was started in the 4-to-5-weeks old Wistar rats: gonad-intact and gonadectomized controls and VPA-treated groups of both genders (n = 6 each). The animals were housed in standard colony cages with free entrance to food in the state of constant temperature (21°C ± 1°C), light/dark cycle (12-h/12-h), and humidity. Rats got a commercial pellet diet ad libitum. The orchidectomy and ovariectomy were performed at 28 ± 2 days of age (in the peripubertal period). Male gonadectomy was performed by orchiectomy using the scrotal approach, and female ovariectomy was performed by midline laparotomy. The following preparations for anesthesia were used for gonadectomy: Sedator 1 mg/kg intramuscular injection (i.m.) (Eurovet Animal Health B.V., Bladel, the Netherlands), Bioketan 75 mg/kg i.m. (Vetoquinol Biowet, Gorzów, Poland), and Atipam 2 mg/kg i.m. as an antidote of Sedator (Eurovet Animal Health B.V.). The accommodation period after the castration was 1 week. After the accommodation, animals were treated for 28 days with VPA 300 mg/kg/day in the drinking water as reported.[22] The only source of drinking for treated groups was the VPA solution, and the fresh tap water for the control groups was provided. One ovariectomized VPA-treated female was eliminated from the study due to a fistula formed after the operation.

Thymus preparation

After treatment, the animals were euthanized in a 70% CO_2 camera. The carotid arteries and the aorta were cut, and the animals exsanguinated to minimize the thymus contamination with red blood cells. After the euthanasia of the animals, their thymus was harvested, and the contaminating blood was removed by rinsing with Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Biological Industries, Israel). The thymus surrounding connective tissue was removed, and the left lobe samples were stored in the RNAlaterRNA stabilization reagent (Qiagen, Germany) at −80°C until further RNA extraction and analysis.

Extraction of RNA from the thymus

The frozen tissue was ground in liquid nitrogen. According to the manufacturer's instruction, the total RNA was extracted using the TRIzol Plus RNA Purification Kit (Life Technologies, USA). The RNA quality and concentration were assessed using a NanoDrop2000 spectrophotometer (Thermo Scientific, USA). The total RNA integrity was analyzed using the Agilent 2100 Bioanalyzer system (Agilent Technologies, CA) with an Agilent RNA 6000 Nano kit (Agilent Technologies, CA). RNA samples had the RNA integrity number (RIN) higher than 5. The samples of RNA were stored at −80°C until further analysis.

Determination of the Slc5a8 and Slc12a2 genes expression in thymocytes

RNA expression assays were performed for *Slc5a8* (Rn01503812_m1), *Slc12a2* (Rn00582505_m1), and *Gapdh* (Rn01775763_g1) genes. According to the manufacturer's instruction, the reverse transcription was performed with High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Applied Biosystems, USA) in 20 µL reaction volume containing 50 ng RNA using Biometra TAdvanced thermal cycler (Analytik Jena AG, Germany). The synthesized copy DNA (cDNA) was stored at 4°C until use or at −20°C for a longer time. Real-time polymerase chain reaction (PCR) was performed using an Applied Biosystems 7900 Fast Real-Time PCR System (Applied Biosystems, USA). The reactions were run in triplicate with 4 µL of cDNA template in a 20 µL reaction volume (10 µL of TaqMan Universal Master Mix II, no UNG (Applied Biosystems, USA), 1 µL of TaqMan Gene Expression Assay 20x (Applied Biosystems, USA), 5 µL of nuclease-free water (Invitrogen, USA) with the program running at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The amplification efficiency was about 100%. The amplicon length of TaqMan assays was 60 bp for the *Slc5a8* gene, *Slc12a2*—67 bp, and *Gapdh*—174 bp.

Statistical analysis

The statistical analysis was performed using the Statistical Package for the Social Sciences, version 23.0 for Windows (IBM SPSS Statistics 23.0). The figures were performed using GraphPad Prism 7 and IBM SPSS Statistics 23.0 software. The normality assumption was conducted by the Shapiro–Wilk test. Differences between the two independent groups were evaluated using the nonparametric Mann–Whitney *U* test. To investigate the SLC5A8 (*Slc5a8* gene) and NKCC1 (*Slc12a2* gene) RNA expression changes in the VPA-treated and control groups, the threshold cycle (CT) value was normalized with the control Gapdh gene, and ΔCT value was obtained. For the gene expression study, the ΔΔCT ($2^{-\Delta\Delta CT}$) Livak method was used to calculate the expression level between the VPA-treated (test) and the control conditions of the target gene when compared to the reference gene.[26] The Spearman's rank correlation coefficient (r) was used to assess relationships between the *Slc5a8* and *Slc12a2* genes (ΔCT values were used). R^2 linear was estimated by performing correlation plots. Differences at the value of $p < 0.05$ were considered significant.

Results

The VPA effect on Slc5a8 and Slc12a2 RNA expression in rat thymocytes

The *Slc5a8* gene expression differences in gonad-intact and gonadectomized VPA-treated and control groups of both genders are considered in the ΔCT value (Table 1 and Figure 1(a)). A significantly 1.5-fold higher RNA expression was found in the gonad-intact male control than in the female control. The VPA treatment caused a statistically significantly decreased RNA gene expression in gonad-intact males and increased in gonad-intact female rats compared with their controls (Table 1 and Figure 1(a)).

Comparing the thymocytes *Slc5a8* gene expression of the gonad-intact of both sexes of rats with the expression in the respective gonadectomized animal groups, there is a clear increase trend: castration is associated with the increased gene expression in ovariectomized female (1.5-

Table 1. RNA expression of SLC5A8 cotransporter in the thymus of study groups.

	CT mean				
Study group	*Gapdh*	*Slc5a8*	ΔCT	ΔΔCT	$2^{-\Delta\Delta CT}$
Gonad-intact female					
Control	23.530	33.406	9.876	−2.708	6.534
VPA-treated	23.083	30.251	7.168[a]		
Gonad-intact male					
Control	22.905	29.452	6.547[b]	4.034	0.061
VPA-treated	22.982	33.563	10.581[c]		
Gonadectomized female					
Control	22.675	29.403	6.728	0.046	0.969
VPA-treated	24.831	31.604	6.774		
Gonadectomized male					
Control	25.436	29.649	4.213	4.545	0.043
VPA-treated	22.319	31.077	8.758[d]		

[a] $p = 0.03$, significant compared with the gonad-intact female control.
[b] $p = 0.004$, significant compared with the gonad-intact female control.
[c] $p = 0.02$, significant compared with the gonad-intact male control.
[d] $p = 0.03$, significant compared with the castrated male control.

fold) and male (1.6-fold) groups; still, the difference is statistically insignificant. In castrated VPA-treated males, a statistically significant decrease of the *Slc5a8* gene expression in thymocytes was found compared with their control rats. Data showed no significant effects of VPA treatment on the gene expression level of the ovariectomized females (Table 1 and Figure 1(a)). The relative *Slc5a8* gene expression is shown in Table 1 and Supplemental material Figure 1(a).

The RNA expression of the *Slc12a2* gene was found significantly higher in the gonad-intact males than the gonad-intact females. The comparison showed significantly increased *Slc12a2* gene expression in gonad-intact VPA-treated females as compared with the control group. The *Slc12a2* expression level in the gonad-intact VPA-treated males was significantly decreased as compared with the control group. Compared with the controls, there were no significant *Slc12a2* gene expression changes in the gonadectomized female and male VPA-treated groups (Figure 1(b) and Table 2). The relative *Slc12a2* gene expression is shown in Supplemental material Figure 1(b).

The VPA effect on the correlation between Slc5a8 and Slc12a2 RNA expression in rat thymocytes of study groups

The *Slc12a2* gene expression, the correlation (*r*) between *Slc5a8 and Slc12a2* RNA expression in thymocytes of gonad-intact, and gonadectomized VPA-treated and their control groups of both genders are shown in Figure 2 and Table 2.

The gonad-intact females' thymocytes showed a significantly strong correlation between *Slc5a8* and *Slc12a2* genes. The gonad-intact VPA-treated female thymocytes did not express this relationship. R^2 linear value in the VPA-treated female decreases in the VPA-treated female group compared with the control (Table 2).

Ovariectomized VPA-treated females showed a significant correlation between *Slc5a8* and *Slc12a2* genes. Study data did not establish a substantial relationship between *Slc5a8* and *Slc12a2* in thymocytes of tested male groups (Table 2 and Figure 2).

Discussion

Pharmacological impacts of VPA show the association with the therapeutic anti-viral[7,27,28] and anti-inflammatory pathogenic mechanisms.[29] The immunomodulatory effect of VPA is related to the inhibition of class I and class II HDACs, which drives an increase in the acetylation of histones H2, H3, and H4, altering the gene expression related to immune cells activation.[30–33] VPA represses the generation of pro-inflammatory factors such as NF-κB, IL-6, and TNF-α and prevents macrophages migration.[34] Our previous work reported that the 4 weeks of VPA treatment did not significantly impact thymus weight in the study gonad-intact and gonadectomized rat groups of both genders.[22] Others noted that the 8 weeks VPA treatment significantly reduced the lymph node and spleen weight and cellularity compared to control in MRL/lpr(−/−) mice females.[35] VPA suppresses T cell proliferation *in vitro*[33] and induces apoptosis of the lymphocyte.[36] Rat gonadectomy induces thymus hyperplasia by increased thymocyte proliferation.[37] In male rats, age-related thymus involution depends on testosterone.[38] Supplementation of testosterone in aged rhesus macaque males increases the number of naïve T cells by raising thymic output.[39]

The present study of the VPA effect on rat thymocyte SLC5A8 transporter expression identified differences concerning gender, sex hormones, and NKCC1 expression

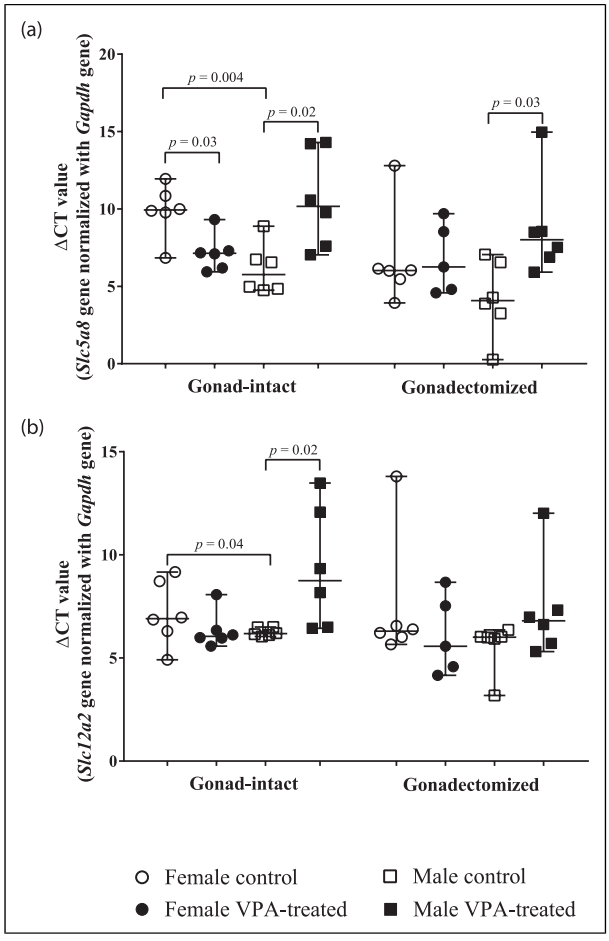

Figure 1. *Slc5a8* (a) and *Slc12a2* (b) genes expression in thymocytes of both gender gonad-intact and gonadectomized rats. Data after normalization with *Galph* gene. Delta threshold cycle (ΔCT) values were used for the graph (the horizontal bars represent the median; the short horizontal lines show the minimal and maximal values).

Table 2. Delta threshold cycle (ΔCT) values correlation (*r*) between *Slc5a8* and *Slc12a2* (NKCC1) genes, R^2 linear in the study groups.

	Gonad-intact female		Gonad-intact male		Gonadectomized female		Gonadectomized male	
Index	Control	VPA-treated	Control	VPA-treated	Control	VPA-treated	Control	VPA-treated
ΔCT *Slc12a2*	7.600[a]	6.344	6.229	9.332[b]	7.440	6.100	5.593	7.327
Spearman correlation (*r*)	0.941[c]	0	0.029	0.800	0.600	1[d]	0.700	0.800
R^2 linear	0.872	0.656	0.097	0.966	0.959	0.997	0.676	0.958

[a]p = 0.04, significant compared with gonad-intact male control.
[b]p = 0.02, significant compared with gonad-intact male control.
[c]p = 0.02, significant ΔCT values correlation between *Slc5a8* and *Slc12a2* genes in the study group.
[d]p = 0.02, significant ΔCT values correlation between *Slc5a8* and *Slc12a2* genes in the study group.

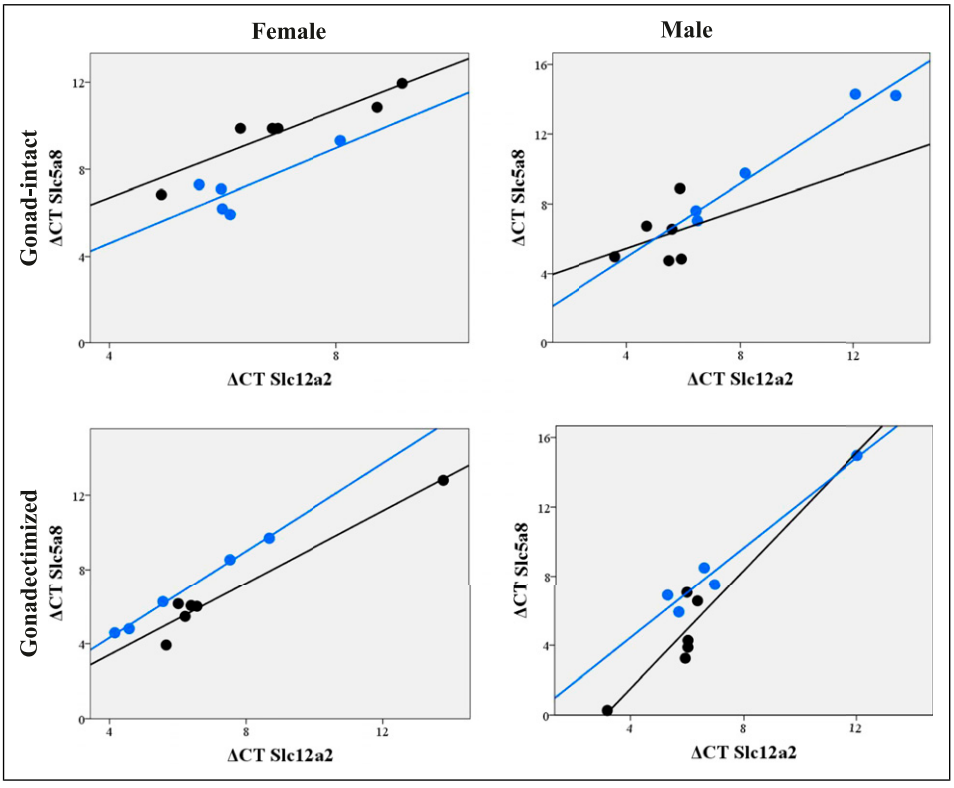

Figure 2. Correlation plots of the *Slc5a8* and *Slc12a2* (NKCC1) genes in the study rat groups. Black color represents control group, blue—VPA-treated group.

potential VPA gender-related effects on cell function. Our study revealed that gonad-intact male rat thymocytes express a significantly higher SLC5A8 RNA level than gonad-intact females; the research shows an insignificant increase of SLC5A8 RNA expression in gonadectomized males and female rat controls, with no sex-related difference. The VPA treatment caused an opposite effect on the SLC5A8 RNA gene expression in female and male rats' thymocytes. In gonad-intact females, the VPA treatment increased 2.7-fold of *Slc5a8* gene expression while did not effect on ovariectomized female thymocytes. In VPA-treated gonad-intact and castrated males, the thymocyte *Slc5a8* gene expression was decreased.

The SLC5A8 transporter function depends on NKCC1 cotransporter activity; its inhibition increases SLC5A8 activity and activates the mitochondrial function.[20,21] The NKCC1 RNA expression in the gonad-intact male rat thymocytes is higher than in females. VPA downregulates NKCC1 expression in gonad-intact male rat thymocytes, and no VPA effect on the NKCC1 was found in castrated male, gonad-intact, and ovariectomized female rat thymocytes.[22] Our study shows the natural gender-related

differences in the NKCC1 and SLC5A8 gene expression correlation in thymocytes. Such a biological gender-related relationship between SLC5A8 and NKCC1 gene expression differences disappeared in VPA-treated animals.

The SLC5A8 is a Na^+-coupled and electrogenic transporter, able to concentrate short fatty acids into the cell: with a Na^+ and substrate stoichiometry between 4:1 and 2:1.[40] The activity and expression of SLC5A8 and NKCC1 in the cell are related to the Na^+ and Cl^- ions transport into the cell; their intracellular and extracellular concentrations, which are sex-dependent,[41] and essential for immune-inflammatory, glycolytic processes.[42] A mice study shows that NKCC1 performs a novel role in acute lung inflammatory responses: a specific NKCC1 inhibition could benefit sepsis treatment.[43]

By mitochondrial mechanisms, VPA inhibits HDACs activity,[31] inducing DNA demethylation and increase the *SLC5A8* gene expression.[12,18,19] Pro-inflammatory-immune cells derive most of their energy from aerobic glycolysis rather than mitochondrial oxidation to generate higher energy and maintain their enhanced activity.[23] The virus-activated T cells starting accelerated growth and proliferation need glucose uptake and glycolysis.[44–46] VPA treatment decreases glucose blood concentration in animals and humans.[47,48]

VPA inhibits Th1 and Th17 cells and related pro-inflammatory cytokines.[49] VPA polarizes macrophages from M1 to M2 phenotype, which cannot induce naïve $CD4^+$ T cells differentiation into a Th1 profile, favoring a Th2 phenotype[50] by mitochondria affected immune cell metabolism changing from the down TCA cycle to β-oxidation.[51] VPA does not affect the viability and the activation of $CD8^+$ T lymphocytes exposed to viral peptides,[30] increased $CD4^+$ T cell level with no change in $CD8^+$ T cell level.[52] HDACs inhibition by VPA alters the gene expression related to cell differentiation and apoptosis.[33,36] In females, aging does not lead to T cell dysfunction, and older females generate strong T cell immunity; men aging leads to T cell dysfunction.[53]

VPA inhibits the aerobic glycolysis in tumor cells by reducing glucose uptake and decreasing lactate and ATP production via lowering the levels of E2F transcription factor 1; VPA affects E2F1 binding to the promoter of glycolytic genes GPI and PGK1.[54] Such VPA impact on glycolysis shows a new therapeutic strategy in researching the VPA effect on immune cells. The expression of SLC5A8 in T cells may likely affect the immune response and be related to the viral disease progression. It is known that the decreased SLC5A8 expression is the risk factor for *Helicobacter pylori* infection in children[55]; the low SLC5A8 expression is related to papillomavirus-related cancerogenesis.[56] The limitation of the study is that the influence of sex hormones on thymocytes SLC5A8 expression has not been studied. Further studies are needed to determine the role of SLC5A8 transporter expression with sex-specific risk mechanisms for disease progression.

Conclusions

Gonad-intact male rat thymocytes express a higher SLC5A8 RNA level than gonad-intact females. The effect of VPA treatment on the *SLC5A8* RNA gene expression in rat thymocytes depends on gender and gonadal hormones. The VPA treatment caused an oppositive effect on the *SLC5A8* gene expression in gonad-intact female and male rats thymocytes. In gonadectomized rats, VPA decreases the *Slc5a8* expression in castrated males, with no effect in ovariectomized female rat thymocytes.

Declaration of conflicting interests

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Ethics approval

Ethical approval for this study was obtained from the STATE FOOD and VETERINARY SERVICE of LITHUANIA (2017-01-02 No. G2-53).

Animal welfare

The present study followed international, national, and/or institutional guidelines for human animal treatment and complied with relevant legislation.

ORCID iD

Donatas Stakišaitis https://orcid.org/0000-0002-1547-7050

Supplemental Material

Supplemental material for this article is available online.

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