УДК 612.12.085.2:546.34-38
https://doi.org/10.20538/1682-0363-2021-3-21-28

Estimation of the effect of lithium salts on cytokine production by blood cells in *in vitro* experiments

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ABSTRACT

Aim. To study the effects of lithium salts on production of cytokines by immunocompetent cells in the whole-blood culture of patients with alcohol dependence and affective disorders.

Materials and methods. The study materials were blood samples from 25 patients with alcohol dependence (AD) and 12 patients with bipolar disorder (BD). Blood diluted 1:1 with complete RPMI-1640 medium (Gibco, UK) was added to the wells of the culture plate, then new lithium salts (succinate, fumarate, pyruvate, ascorbate) and a reference salt – lithium carbonate at a final concentration of 1.2 mmol / l per lithium ion – were added. In parallel, control samples without lithium salts were tested; the samples were incubated for a day. The concentration of cytokines (interferon (IFN) γ, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17A, tumor necrosis factor (TNF) α) was determined in the culture supernatants on the MAGPIX multiplex analyzer (Luminex, USA) (Center for Collective Use "Medical Genomics", Tomsk NRMC) using the Human Cytokine / Chemokine Magnetic Bead Panel (Merck, Germany).

Results. All lithium salts had a unidirectional effect on the production of cytokines by immunocompetent cells (ICC), except for lithium ascorbate and IL-8. The concentrations of cytokines in the supernatants of loaded and control samples (spontaneous production) were comparable, which indicates an absence of stimulating or suppressing effects of salts on the functional activity of ICC under the experimental conditions. The effect of lithium ascorbate as an IL-8 inducer was detected: the production of IL-8 induced by lithium ascorbate was 2.3–2.5 times higher than its spontaneous production.

Conclusion. The obtained results, as well as the previously revealed antioxidant and cytoprotective properties of new lithium salts, confirmed that they are promising for development of pharmacological agents with combined action.

Key words: lithium salts, cytokines, blood cells, alcoholism, bipolar disorder.

Conflict of interest. The authors declare the absence of obvious or potential conflict of interest related to the publication of this article.

Source of financing. The study was carried out with the financial support of the Russian Science Foundation (project No. 17-75-20045), partly (recruitment of patients) – within the financing of the research project No. 0550-2019-0007.

Conformity with the principles of ethics. All patients signed an informed consent to participate in the study. The study was approved by the local Ethics Committee at Mental Health Research Institute of Tomsk NRMC (Protocol No. 361 of 23.10.2017).

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For citation: Vetlugina T.P., Epimakhova E.V., Savochkina D.N., Plotnikov E.V., Boiko A.S., Ivanova S.A., Bokhan N.A. Estimation of the effect of lithium salts on cytokine production by blood cells in *in vitro* experiments. *Bulletin of Siberian Medicine.* 2021; 20 (3): 21–28. https://doi.org/10.20538/1682-0363-2021-3-21-28.

Оценка влияния солей лития на продукцию цитокинов клетками крови в опытах *in vitro*

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РЕЗЮМЕ

Цель – изучить влияние солей лития на продукцию цитокинов иммунокомпетентными клетками в культуре цельной крови больных с алкогольной зависимостью и аффективными расстройствами.

Материалы и методы. Материалом для исследования послужили образцы крови 25 больных с алкогольной зависимостью и 12 пациентов с биполярным аффективным расстройством. В лунки культурального планшета вносили кровь, разведенную 1 : 1 полной средой RPMI-1640 (Gibco, Великобритания), добавляли новые соли лития (сукцинат, фумарат, пируват, аскорбат) и соль сравнения – карбонат лития в конечной концентрации 1,2 ммоль/л в расчете на ион лития, параллельно ставили контрольные пробы без солей лития; пробы инкубировали в течение суток. В супернатантах суточной культуры на мультиплексном анализаторе MAGPIX (Luminex, США) (ЦКП «Медицинская геномика», Томский НИМЦ) определяли концентрацию цитокинов (интерферона γ, интерлейкина (ИЛ) 1β, ИЛ-2, ИЛ-4, ИЛ-6, ИЛ-8, ИЛ-10, ИЛ-17А, фактора некроза опухоли α) с использованием наборов реагентов Human Cytokine/Chemokine Magnetic Bead Panel (Merck, Германия).

Результаты. Все соли лития оказывали однонаправленное действие на продукцию спектра цитокинов иммунокомпетентными клетками (ИКК) за исключением аскорбата лития и ИЛ-8. Концентрации цитокинов в супернатантах нагрузочных и контрольных проб (спонтанная продукция) были сопоставимы, что свидетельствует об отсутствии стимулирующего или супрессирующего действия солей на функциональную активность ИКК в условиях эксперимента. Обнаружен эффект аскорбата лития как индуктора ИЛ-8: индуцированная аскорбатом лития продукции ИЛ-8 в 2,3–2,5 раза превышала его спонтанную продукцию.

Заключение. Полученные результаты, а также выявленные ранее антиоксидантные, цитопротекторные свойства новых солей лития подтверждают их перспективность для разработки фармакологических средств комбинированного действия.

Ключевые слова: соли лития, цитокины, клетки крови, алкоголизм, биполярное аффективное расстройство.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Исследование выполнено при финансовой поддержке Российского научного фонда (проект № 17-75-20045), частично (набор пациентов) – в рамках финансирования темы НИР № 0550-2019-0007.

Соответствие принципам этики. Все пациенты подписали информированное согласие на участие в исследовании. Исследование одобрено локальным этическим комитетом НИИ психического здоровья Томского НИМЦ (протокол № 361 от 23.10.2017).

Для цитирования: Ветлугина Т.П., Епимахова Е.В., Савочкина Д.Н., Плотников Е.В., Бойко А.С., Иванова С.А., Бохан Н.А. Оценка влияния солей лития на продукцию цитокинов клетками крови в опытах *in vitro*. *Бюллетень сибирской медицины.* 2021; 20 (3): 21–28. https://doi.org/10.20538/1682-0363-2021-3-21-28.

INTRODUCTION

Lithium-containing drugs are often used as mood stabilizers in the complex therapy of patients with mood disorders and alcohol dependence complicated by affective disorders; lithium carbonate is commonly used for such treatment [1–3]. At the same time, the data of biological studies indicate a significant role of inflammation and oxidative stress factors in these diseases [4–8]. This determines the relevance of developing combined action drugs targeted to the main links in the pathogenesis of affective spectrum disorders.

Mental Health Research Institute of Tomsk NRMC is engaged in search for lithium salts with combined normothymic, neuroprotective, cytoprotective, and antioxidant effect. To select the most promising compounds as the basis for combined action drugs, studies are being carried out on the biological effects of new lithium salts synthesized on the basis of ascorbic acid and citric acid cycle substrates (succinate, fumarate, pyruvate).

The aim of this work was to study the effect of lithium salts on the production of cytokines by immunocompetent cells in the whole-blood culture of patients with alcohol dependence and affective disorders.

MATERIALS AND METHODS

The material for the study was blood samples from 25 men with alcohol dependence (AD) according to the International Classification of Diseases, Tenth Revision (ICD-10) ("Mental and behavioral disorders due to alcohol use: dependence syndrome – F10.21 and withdrawal syndrome – F10.30"), aged 29 to 60 (46.82 ± 9.48) years and 12 patients (2 men, 10 women) diagnosed with ICD-10 "Bipolar disorder – F31" (BD), aged 20 to 59 (35.61 ± 13.63) years; the level of statistical significance of age differences between the groups $p = 0.0152$.

The patients were admitted for treatment to the Addictive States and the Affective States Departments of the Mental Health Research Institute clinic, Tomsk NRMC. Blood sampling from the patients was carried out from the cubital vein in the morning on an empty stomach before the start of standard therapy; Vacutainer systems with EDTA anticoagulant were used. All patients signed an informed consent to participate in the study. The study was carried out in compliance with ethical standards developed in accordance with the Declaration of Helsinki of the World Medical Association and approved by the local Ethics Committee at Mental Health Research Institute of Tomsk NRMC (protocol No. 361 of 23.10.2017).

Lithium salts (lithium succinate – $Li_2\ C_4H_4O_4$; lithium fumarate – $Li_2\ C_4H_2O_4$; lithium pyruvate – $LiC_3H_3O_3$; lithium ascorbate – $Li\ C_6H_7O_6$) were synthesized at Research School of Chemical and Biomedical Technologies of National Research Tomsk Polytechnic University. Lithium carbonate – Li_2CO_3 (Sigma-Aldrich, USA) was used as a reference salt, since this salt is the basis of most lithium preparations used in medical practice. Lithium salts were dissolved in physiological saline, and stock solutions were obtained with a convenient concentration for further addition to experimental samples. The final standard concentration was 1.2 mmol / l per lithium ion (Li^+), which correlated with the therapeutic dose of lithium in treatment of affective spectrum disorders.

Peripheral venous blood was sampled in BD Vacutainer tubes with EDTA anticoagulant. Blood was diluted 1: 1 with the RPMI-1640 medium (Gibco, UK) with addition of inactivated fetal bovine serum (Gibco, UK), HEPES (Sigma-Aldrich, USA), and gentamicin (Dalkhimpharm, Russian Federation). A certain volume of diluted blood was added to the wells of the Cell Culture Plate (24-Well, Eppendorf). The samples were added with stock solutions of lithium salts to a final Li^+ concentration of 1.2 mmol / l (loaded samples). To evaluate the spontaneous production of cytokines by immunocompetent cells (ICC), in parallel with loaded samples, control samples without addition of lithium salts were used.

The samples were incubated for 24 hours at 37° C in 5% carbon dioxide in a CO_2-incubator. After incubation, the supernatants were carefully taken, poured into aliquots, and stored in a low-temperature chamber at –80° C until cytokines were determined.

The level of cytokine production was assessed by their concentration in the supernatants of loaded and control samples using the LuminexxMAP technology on a MAGPIX multiplex analyzer (Luminex, USA) (Center for Collective Use "Medical Genomics", Tomsk NRMC). The concentration of cytokines (interferon γ (IFNγ), interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17A, and tumor necrosis factor (TNF) α) was determined using the MILLIPLEX MAP Human Cytokine / Chemokine Magnetic Bead Panel (Merck, Germany) The analysis was performed in a standard 96-well plate according to the instructions for the kits. The results were presented in pg / ml.

Statistical analysis was performed using the STATISTICA software package for Windows, version 12.0. Descriptive statistics were presented by the me-

dian and the interquartile range *Me* (*LQ–UQ*). For intergroup comparison, the Mann – Whitney test was used. The differences were considered statistically significant at $p < 0.05$.

RESULTS

During the cultivation of blood samples from patients with AD, most lithium salts did not have either a stimulating or inhibitory effect on the production of cytokines, except for lithium ascorbate and IL-8 (Table 1). There were no statistically significant differences in the level of cytokines in the loaded and control samples, which indicates the presence of cytokines spontaneously produced by ICC in the samples with lithium salts, including lithium carbonate. The highest concentration in blood cell culture supernatants was found for the anti-inflammatory cytokine IL-4, the median values in all the samples ranged from 19.52 to 19.85 pg / ml. The lowest concentrations were found for proinflammatory cytokines IL-2 (1.43–1.50 pg / ml) and IL-6 (1.54–1.69 pg / ml).

At the same time, the addition of lithium ascorbate to the whole-blood culture resulted in a pronounced increase in the production of IL-8 (Table 1), and its content in the supernatants of the loaded samples exceeded the corresponding value in the samples with other lithium salts (the level of statistical significance of the differences between the samples ranged from 0.0060 to 0.0003). In addition, the concentration of IL-8 in the samples with lithium ascorbate was 2.5 times higher than the corresponding value of its spontaneous production in the control samples (25.37 (17.19–36.04) and 10.07 (8.41–15.99) pg / ml, respectively, $p_c = 0.0010$).

Table 1

Cytokine concentration in the supernatants of diurnal blood cell culture of patients with alcohol dependence, pg / ml, *Me (LQ–UQ)*

Cytokines, pg / ml	Loaded samples, Li^+ dose – 1.2 mmol / l					Control 6 (n = 25)	*p*
	1 (*n* = 25)	2 (*n* = 23)	3 (*n* = 25)	4 (*n* = 24)	5 (*n* = 24)		
IFNγ	6.62 (5.72–7.59)	6.93 (6.00–7.41)	6.69 (6.02–6.96)	6.92 (6.24–7.41)	6.58 (6.02–7.46)	6.46 (5.94–7.18)	$p > 0.05$
IL-1β	4.41 (2.83–6.56)	4.06 (3.24–5.54)	3.78 (2.95–5.28)	3.44 (2.74–4.81)	4.00 (3.10–6.21)	4.10 (3.03–5.08)	$p > 0.05$
IL-2	1.47 (1.30–1.69)	1.43 (1.27–1.68)	1.45 (1.26–1.60)	1.50 (1.38–1.67)	1.49 (1.29–1.57)	1.46 (1.27–1.60)	$p > 0.05$
IL-4	19.85 (18.25–21.30)	19.65 (18.25–21.20)	19.65 (18.21–21.50)	19.68 (18.24–21.15)	19.62 (18.23–20.77)	19.52 (18.25–20.28)	$p > 0.05$
IL-6	1.69 (1.31–2.25)	1.62 (1.36–2.07)	1.54 (1.39–2.13)	1.65 (1.36–2.07)	1.66 (1.34–2.55)	1.64 (1.36–2.33)	$p > 0.05$
IL-8	12.66 (8.57–20.26)	11.05 (8.66–18.04)	11.01 (8.52–12.64)	11.57 (9.02–17.88)	25.37 (17.19–36.04) $p_c = 0.001$	10.07 (8.41–15.99)	0.006 (between samples 1 and 5); 0.002 (between samples 2 and 5); 0.0003 (between samples 3 and 5); 0.004 (between samples 4 and 5)
IL- 10	7.29 (6.88–8.9)	7.29 (6.59–7.98)	7.27 (6.59–8.35)	7.30 (6.92–8.66)	7.23 (6.80–8.33)	7.29 (6.49–7.94)	$p > 0.05$
IL-17	5.56 (5.01–6.07)	5.37 (5.16–5.91)	5.56 (5.20–5.88)	5.59 (5.19–6.06)	5.74 (5.21–6.18)	5.64 (5.00–5.95)	$p > 0.05$
TNFα	5.81 (4.7–7.31)	5.52 (4.88–7.33)	5.43 (4.96–7.00)	5.71 (4.91–6.40)	5.81 (4.42–6.54)	5.62 (4.64–7.30)	$p > 0.05$

Note: 1 – lithium succinate; 2 – lithium fumarate; 3 – lithium pyruvate; 4 – lithium carbonate; 5 – lithium ascorbate; 6 – control samples.
p_c – the level of statistical significance of differences in relation to sample 6 (control);
p – the level of statistical significance of differences between the samples 1 and 5; 2 and 5; 3 and 5; 4 and 5.

Similar results were obtained in a series of experiments with blood samples from patients with bipolar disorder (Table 2), although this group of patients differed from the group of patients with alcoholism in terms of age and sex characteristics.

In the diurnal cell culture supernatants of patients with BD, as well as in the first series of experiments with blood samples from patients with AD, no statistically significant differences were found between the samples loaded with lithium salts (except for ascorbate and IL-8) and the control samples. In all the samples, IL-4 (18.38–20.09 pg / ml) and IL-17A (14.15–15.12 pg / ml) had the highest average values of the cytokine content (*Me*), whereas IL-2 (1.70–1.85 pg / ml) and IL-6 (1.47–1.69 pg / ml) were characterized by the lowest values.

Table 2

Cytokine concentration in the supernatants of diurnal blood cell culture of patients with bipolar disorder, pg / ml, *Me (LQ–UQ)*

Cytokines, pg / ml	Loaded samples, Li^+ dose – 1.2 mmol / l					Control 6 (n = 12)	p
	1 (n = 12)	2 (n = 12)	3 (n = 11)	4 (n = 12)	5 (n = 11)		
IFNγ	5.88 (4.50–6.99)	5.16 (4.71–6.41)	5.29 (4.41–6.58)	5.35 (4.75–7.37)	5.77 (4.56–6.93)	5.35 (4.45–8.51)	$p > 0.05$
IL-1β	2.8 (2.37–4.44)	3.12 (2.56–3.49)	2.68 (2.09–2.98)	2.88 (2.32–3.22)	3.22 (2.43–3.44)	2.83 (1.98–3.27)	$p > 0.05$
IL-2	1.76 (1.68–1.90)	1.74 (1.64–1.92)	1.74 (1.56–1.81)	1.70 (1.60–1.87)	1.81 (1.60–1.96)	1.85 (1.60–2.02)	$p > 0.05$
IL-4	19.89 (18.25–20.10)	18.38 (18.29–20.06)	19.08 (16.51–20.44)	19.14 (18.21–20.03)	20.09 (18.20–21.84)	19.85 (18.25–20.13)	$p > 0.05$
IL-6	1.67 (1.47–2.04)	1.61 (1.54–1.96)	1.47 (1.35–1.74)	1.58 (1.47–1.84)	1.67 (1.39–2.24)	1.69 (1.54–1.99)	$p > 0.05$
IL-8	10.00 (6.63–13.00)	10.52 (8.33–16.45)	8.83 (6.72–13.30)	10.66 (7.39–20.33)	19.80 (18.84–31.74) ***$p_{c=}$ 0.002***	8.48 (6.66–10.30)	0.002 (between samples 1 and 5); 0.01 (between samples 2 and 5); 0.005 (between samples 3 and 5)
IL-10	5.63 (5.32–6.05)	5.42 (5.11–5.85)	5.28 (4.87–5.72)	5.75 (5.21–7.17)	5.58 (5.15–6.43)	5.31 (5.07–5.75)	$p > 0.05$
IL-17	14.62 (13.28–15.34)	14.25 (13.35–15.02)	14.15 (12.84–15.24)	14.69 (13.17–15.32)	15.12 (14.04–15.77)	14.63 (13.60–15.19)	$p > 0.05$
TNFα	2.96 (2.74–3.17)	3.09 (2.81–3.23)	2.86 (2.61–3.01)	3.16 (2.76–3.19)	2.96 (2.76–3.26)	3.06 (2.50–3.36)	$p > 0.05$

Note: p_c – the level of statistical significance of differences in relation to sample 6 (control);
p – the level of statistical significance of differences between the samples 1 and 5; 2 and 5; and 3 and 5.

In this series of experiments, the ability of lithium ascorbate to enhance the production of IL-8 by ICC was confirmed. The concentration of IL-8 in the supernatants of the samples loaded with lithium ascorbate significantly exceeded the corresponding value both in the control samples (p_c = 0.002) and in the samples with other lithium salts (the levels of statistical significance of differences were from 0.010 to 0.002). The mean (*Me*) lithium ascorbate-induced IL-8 production / the mean spontaneous IL-8 production ratio was 2.33.

DISCUSSION

Despite the fact that lithium has been used as a mood stabilizer for more than half a century, it remains the gold standard for treatment of affective spectrum disorders, as evidenced by a number of reviews over the past decade [9–11]. Along with a high therapeutic effect of lithium therapy, it poses a risk of toxicity, to decrease which various ways are proposed, in particular, to reduce the dose of the drug and monitor constantly the level of lithium in patients' blood.

To reduce toxicity and increase the therapeutic effect, one of the approaches is to expand the spectrum of lithium salts synthesized on the basis of various anionic components. We previously identified antioxidant and cytoprotective effects in certain lithium compounds synthesized from substrates of the citric acid cycle (succinate, fumarate, pyruvate) and ascorbic acid in plasma and blood cell models [12, 13].

Considering the role of inflammation in the pathogenesis of affective spectrum disorders, this study continued to evaluate the biological effects of new lithium salts, as well as the reference salt of lithium carbonate, on the production of cytokines by ICC in the diurnal whole-blood culture of patients with AD and BD in the complete RPMI-1640 medium. Analysis of the data showed that almost all the salts had a unidirectional effect on the cytokine production in blood samples from patients with different pathologies, except for the lithium ascorbate effect on the production of IL-8.

At the same time, there were no statistically significant differences in the level of cytokines in the supernatants of the loaded and control samples. Thus, the analyzed salts did not have negative effects on blood cells, which, under the experimental conditions,

retained the ability to spontaneously produce cytokines. A new property of lithium ascorbate was discovered – to enhance the production of IL-8 by ICC. It was found that the level of IL-8 in the samples loaded with lithium ascorbate was 2.3–2.5 times higher than the level of its spontaneous production, regardless of the pathology and gender and age characteristics of patients.

IL-8 (CXCL8) belongs to the group of CXC chemokines involved in the regulation of the biological activity of almost all ICC; the main role of IL-8 is enhancing the chemotaxis of leukocytes to the focus of infection, its localization and removal. The role of chemokines in the development of an immune response to the pathogen is widely covered in the literature [14–16]. The effect of lithium ascorbate on IL-8 synthesis, revealed in our experiments, may be due to the unique properties of ascorbate in regulating redox processes [17, 18].

In the blood culture medium with lithium ascorbate in the presence of iron ions, a prooxidant system can be created. This leads to stimulation of leukocytes and expression of CXCR1 and CXCR2 receptors, which initiate the early phase of a nonspecific immune response and are able to bind to CXCL1, CXCL2, CXCL5, CXCL6, CXCL7, and CXCL8 [16, 19]. To some extent, the obtained results may depend on the experimental conditions (drug dose, incubation time, etc.). It should be noted that the revealed effect of lithium ascorbate was manifested at a Li^+ dose of 1.2 mmol / l, corresponding to the therapeutic dose in treatment of affective disorders.

High levels of IL-8 are typical of a wide range of chronic inflammatory diseases [20–22]. At the same time, production of IL-8 associated with innate immunity activation does not weaken subsequent adaptive mechanisms of antigen-specific immune defense [23]. On the contrary, suppression of IL-8 secretion in the initial phases of inflammation can result in a decrease in the influx of neutrophils into the foci of inflammation, impaired elimination of the pathogen, and transition of the infectious process to a chronic course due to immunodeficiency [24]. The revealed property of lithium ascorbate makes it possible to expand the arsenal of IL-8 / CXCL8 inducers – a regulator of natural immunity, which is especially necessary in the initial phases of inflammation.

CONCLUSION

Lithium succinate, lithium fumarate, lithium pyruvate, lithium ascorbate, and lithium carbonate (the reference salt) have a unidirectional effect on the production of cytokines by immunocompetent cells during 24-hour incubation in the RPMI medium of blood samples from patients with alcohol dependence and bipolar disorder. Cytokine concentrations in the supernatants of all loaded and control samples (spontaneous production) were comparable. This indicates the absence of both stimulating and suppressive effects of these compounds on the functional activity of ICC and can be considered as their positive characteristic. This characteristic also applies to lithium ascorbate. In addition, the property of lithium ascorbate as an IL-8 inducer was discovered.

The data obtained in this work and the previously revealed antioxidant and cytoprotective properties of some new lithium salts confirm the relevance of further study of their biological effects in order to select the most promising compounds for the development of import-substituting combined action drugs both for treatment of affective spectrum disorders and for possible wider use in other diseases, the pathogenesis of which involves inflammation and oxidative stress.

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Vetlugina T.P. – development of experimental technology and research design, analysis and interpretation of data, critical revision of the manuscript for important intellectual content, drafting of the manuscript. Epimakhova E.V. – carrying out of the experimental part of the study, statistical analysis of data. Savochkina D.N. – carrying out of the experimental part of the study. E.V. Plotnikov – synthesis of lithium preparations, editing of the manuscript. Boiko A.S. – determination of the cytokine concentration using the Luminex xMAP technology. Bokhan N.A. – critical revision of the manuscript for important intellectual content, editing of the manuscript, final approval of the manuscript for publication.

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Received 01.08.2020
Accepted 28.12.2020