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The effect of feeding mice during gestation and nursing with *Rhodiola kirilowii* extracts or epigallocatechin on CD4 and CD8 cells number and distribution in the spleen of their progeny

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Abstract

Rhodiola kirilowii, a member of Crassulaceae family, grows wildly in Asiatic mountains and is also cultivated in some European countries. Its underground parts traditionally are used for enhance physical and mental performance of the body.

In our previous papers we reported immuno- and angio-modulatory effects of aqueous and hydro-alcoholic extracts of radix and rhizome of this plant in mice. In the present work we evaluated the effect of Rhodiola kirilowii water- (RKW) or hydro- alcoholic (RKW-A) extracts and epigallocatechin (one of the polyphenols present in these extracts) given to mice, during pregnancy and nursing period, on the number and localization of CD4+ and CD8+ cells in spleens of adult progeny mice. Previously, we observed several abnormalities in functionality of spleen cells collected from these mice. No differences in CD4+ T cells localisation or numbers were found between all tested mice groups. In contrast, CD8+ T cells localisation and staining were altered in progeny of water or alcohol extract-fed mice. CD8+ T cells were found not only in the PALS but also in the B cell follicle and in the red pulp. Furthermore, CD8+ T cells from T cell zones in the progeny of extract-fed mice showed much intensive staining for CD8 antigen and significantly higher numbers per area in comparison to control mice.

***Key words**: Rhodiola kirilowii, epigallocatechin, spleen, progeny, mice.*

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Introduction

Crassulaceae, a big family of plants, contains several of *Rhodiola* species which exhibit health benefits. They grow mainly in Asia and Eastern Europe, however they are also found in western Europe, especially in mountainous areas. These plants traditionally are used as dietary supplements for enhancing physical and mental performance of the body [1].

They contain many of the compounds which demonstrated a beneficial effect on the body. The anti-oxidative, immunomodulatory, anti-tumor and anti-microbial activity have been shown so far [2, 3]. Mechanism of action of various *Rhodiola* species depends on quality and quantity of the biological active compounds. It has been shown that the phytochemicals present in the plants (polyphenols, caretonoids, saponines, phytosterols, alkaloids) may also exhibit pro-oxidative activity and can directly induce apoptosis of cells. Gallic acid, the main component of the aqueous extract, found in *Rhodiola rosea,* induced apoptosis in monocytic cell line and inhibited lymphocyte proliferation [4]. Compounds from *Rhodiola* spp. may affect several immunocompetent cells and indirectly enhance health of the organism. Studies conducted with the hydro-alcoholic and aqueous extracts of the roots and rhizomes of the plants in mice or rats models, have repeatedly demonstrated stimulation of a variety of parameters of the cellular immune response [5-7]. Moreover, they may also influence chemokine production [8]. It has been also shown that the extract

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of *Rhodiola imbricata* stimulated Toll-like receptor 4, production of inflammatory mediators and Th1 cytokines [9].

Rhodiola kirilowii is one of the medical plants from the *Rhodiola* genus. The plant supplemented in adult organisms displays several beneficial activities. It was shown in mice model that *Rhodiola kirilowii* stimulated granulocyte activity and increased lymphocyte response to mitogens. It also increased respiratory burst activity (RBA), proliferative response to LPS (pigs) and number of blood granulocytes and lymphocytes (mice) which diminished the scale of *Pseudomonas aeruginosa* infection [10, 11]. The plant affects also other types of cells. It has been demonstrated that *Rhodiola kirilowii* supplementation in mice after grafting of L-1 sarcoma cells (TIA test) inhibited total number of newly formed blood vessels. Moreover, when used for *in vitro* test, *Rhodiola kirilowii* stimulated proliferation of endothelial cells, and suppressed proliferation of L-1 sarcoma cells [12].

Variety of biological activity of *Rhodiola kirilowii* prompted us to examine whether extracts from this plant may be useful for treatment of microbial infections in pregnancy and lactation period in mammalians. This is particularly important due to the fact that majority of antibiotics are dangerous to the developing fetus and due to increasing numbers of infection with antibiotic resistant bacteria. In present work we evaluated the effect of *Rhodiola kirilowii* water (RKW) or hydro-alcoholic (RKW-A) extracts and epigallocatechin (one of the polyphenols from these two extracts) given to mice during pregnancy and nursing period, on the number and localization of CD4+ and CD8+ cells in spleens of progeny mice. We made this analysis due to several abnormalities in functionality of spleen cells observed in our previous work [13].

Material and methods

The study was conducted in the Department of Regenerative Medicine, Military Institute of Hygiene and Epidemiology in Warsaw. The material for the histochemical study was obtained from the spleens of 38 mice, the progeny of mothers fed with the aqueous and aqueous-alcoholic extract of *Rhodiola kirilowii* or epigallocatechin.

Extract preparation

The roots and rhizomes of the ground plants of *Rhodiola kirilowii (Crassulaceae*) were collected and identified in Department of Botany, Breeding and Agriculture of Institute of Natural Fibres and Medicinal Plants, Poznań. The voucher specimen is kept in the herbarium of this department.

The plant material – finely powdered roots were extracted two times with water (first 2 hours and second 1 hour long) in the ratio raw material/solvents (1/5), at the temperature of 40-45°C. The supernatants were mixed together, spun and lyophilized (water extract – RKW). To prepare hydro-alcoholic extract (RKW-A), finely powered roots were extracted with ethanol/water solution (1/1, v/v) in the ratio raw material/solvent 1/10 by the percolation method. Then, the percolates were distilled of the ethanol at the temperature of 40-45°C and lyophilized. Dry extract ratio (DER) values were: 5.09/1 for RKW and 3.27/1 for RKW-A. To evaluate concentration of selected polyphenols and flavonoids both RK extracts were analyzed on HPLC, before experiments in animals, as previously described [14]. Extracts were lyophilized and stored at –70°C until used. Prior to use lyophilized extracts were dissolved in distilled water.

Epigallocatechin (EGC) was purchased from Sigma Aldrich (cat no: E3768-5MG), dissolved in distilled water and stored at – 70°C until used.

Animals

The study was performed on the 6-weeks old progeny of adult inbred females of Balb/c strain (Mossakowski Medical Research Centre Polish Academy of Science), 8-9 weeks old mated with adult males from the same strain. Females were fed during pregnancy and lactation with lyophilized RKW or RKW-A extract, dissolved in distilled water, (20 mg/kg b.m) or epigallocatechin (EGC, 0.2 mg/kg b.m.) daily. The dose of the extracts, given to 20 g of b.m. mouse corresponds to 100 mg (1.6 mg/kg) given to 60 kg b.m. person with accordance to the mouse/human converter. The daily dose of EGC matches the total content of epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate present in 400 micrograms (daily dose) of RKW-A extract. The control group received distilled water. For avoiding stress connected with gavage and handling the substances were placed on a corn crisp and served to the mouse in a Petri dish. Six weeks after birth, the progeny mice were weighed, anesthetized, (intraperitoneal injection of ketamine 120 mg/ kg of b.w. and xylazine 12 mg/kg of b.w. solution)bled from retro-orbital plexus and euthanized (pentobarbital 400 mg/kg). Sera were separated by 1-hour clotting (RT), centrifuged at 2000 × *g* for 20 min and stored at – 70°C until analysis.

Animals were handled according to the Polish law on the protection of animals and NIH standards. All experiments were accepted and conducted according to the ethical guidance of Local Bioethical Committee, (permission 73/2011).

Spleen analysis

Mice were anaesthetized, blood from retro-orbital plexus has been collected. Next spleens were isolated in aseptic conditions in laminar flow chamber. Spleens were fixed in 4% paraformaldehyde in PBS for 24h, then dehydrated and embedded in paraffin and cut into 6-µm sections on a microtome. The sections were further subjected to antigen retrieval in 0.1 M citrate buffer (pH 6.0) for 10 min. CD4 and CD8 antigens

were detected with biotinylated rat anti mouse CD4 antibody (4SM95) and biotinylated rat anti mouse CD8 antibody (4SM15) (e-Biosciences, San Diego, CA, USA) (1:100) for 1h in 1% bovine serum albumin/PBS and room temperature. Next, peroxidase labeled polymer conjugated was added for another 30 minutes. Sections were developed with 3,3'-diaminobenzidine (DAB) and counterstained with Harris's hematoxylin solution (Sigma Aldrich). The stained sections were dehydrated in graded series of ethanol followed by xylene and mounted with DPX (Sigma Aldrich). The images were captured with camera-equipped Zeiss Axio Imager.M1 microscope using ZEN 2 software. Numbers of CD4-positive and CD8-positive cells per each spleen section were counted on 10 slides. Results are showed as counts per 1000 cells.

Statistical analysis of data

One-way ANOVA and unpaired t test were used (GraphPadPrism v.5). The results are presented as mean +/- SEM. The significance level has been set to 5% ($\alpha = 0.05$).

Results

Animals

Progeny from mothers fed RKW-A extract and EGC exhibit mean decreased body mass in comparison to control or RKW group. No other differences were found (Table 1).

Histological analysis

In all tested groups, CD4+ and CD8+ T cells remained compacted mostly around the central arteriole in the peri-arteriolar lymphoid sheath (PALS) having limited overlap with the B cell follicular area. No differences in CD4+ T cells localisation or numbers were found between all tested mice groups (Figs. 1 and 2). In contrast, CD8+ T cells localisation and staining were altered in water or alcohol extract-fed mice. CD8+ T cells were found not only in the PALS but also in the B cell follicle and in the red pulp (Figs. 3 and 4). Furthermore, CD8+ T cells from T cell zones in extract-fed mice showed much intensive staining for CD8a antigen and significantly higher numbers per area in comparison to control mice.

Discussion

Whole parts and extracts obtained from plants have been used for centuries in medicine, cosmetics and phytotherapy [15]. That treatment did not confirm which individual components of the plant exhibit beneficial properties. Currently, it is known that the components of plant origin with the strongest biological activity include essential oils and polyphenols, classified as secondary metabolites of plants. Epigallocatechin gallate is one of such compounds (catechin derivative), known mainly due to its antioxidant and anti-inflammatory properties. The substance occurs in substantial quantities in chocolate, beans, green tea and *Rhodiola kirilowii* plant. It has been demonstrated that derivatives of catechins may influence the development of the zygote [16]. In previous studies we have proved that chocolate, theobromine and caffeic acid supplementation affect the immune response and the angiogenic activity in young mice [17-19]. Additionally, in a study performed by Skopiński *et al*. [20], chocolate administrated to pregnant mice caused decrease of the average length of the arm and thigh bones together with bone mineralization disturbances of their offspring. Moreover, in progeny whose mothers were fed during pregnancy and nursing with RK extracts, creatinine results were above the upper value of the confidence limit in comparison to the sera collected from the controls. Also alterations in the morphometric analysis of kidney were detected [21]. Therefore, it can be assumed that a diet rich in catechins, which were detected in *Rhodiola kirilowii* extracts, may affect fetal development and increase occurrence of post-natal anomalies including in the construction and functionality of spleen cells.

The spleen is an organ specialized in filtering blood antigens as well as phagocytosis and removing old or damaged red blood cells, platelets and white blood cells. The spleen combines the innate and adaptive immune system in a unique way. Releasing an immediate innate reaction to microbial penetration, but also an adaptive immune response which involves interaction of cells recognizing an particular antigen, implicating MHC molecules presented by antigen-presenting cells [22]. It consists of two morphologically and functionally different pulps: red and white [23]. Red pulp involves an extensive network of strings containing venous sinuses, which are responsible for mac-

Table 1. Mean ± SEM of body and spleen mass and relative spleen mass factor (mg of spleen/g of body mass)

	Control		RKW		RKW-A		EG	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
Body mass (g)	18.2	0.2	17.7	0.3	17.0**	0.2	16.9 **	0.4
Spleen mass (mg)	84.1	1.0	83.0	2.1	84.0	2.0	80.2	3.1
Relative spleen mass (mg/g)	4.7	0.1	4.8	0.2	5.0	0.1	4.8	0.2
Mice number	90		58		64		34	

*** p < 0.01; p – probability value in comparison to control group*
RKW – Rhodiola kirilowii water extract; RKW-A – Rhodiola kirilowii hydro-alcoholic extract; EGC – epigallocatehin

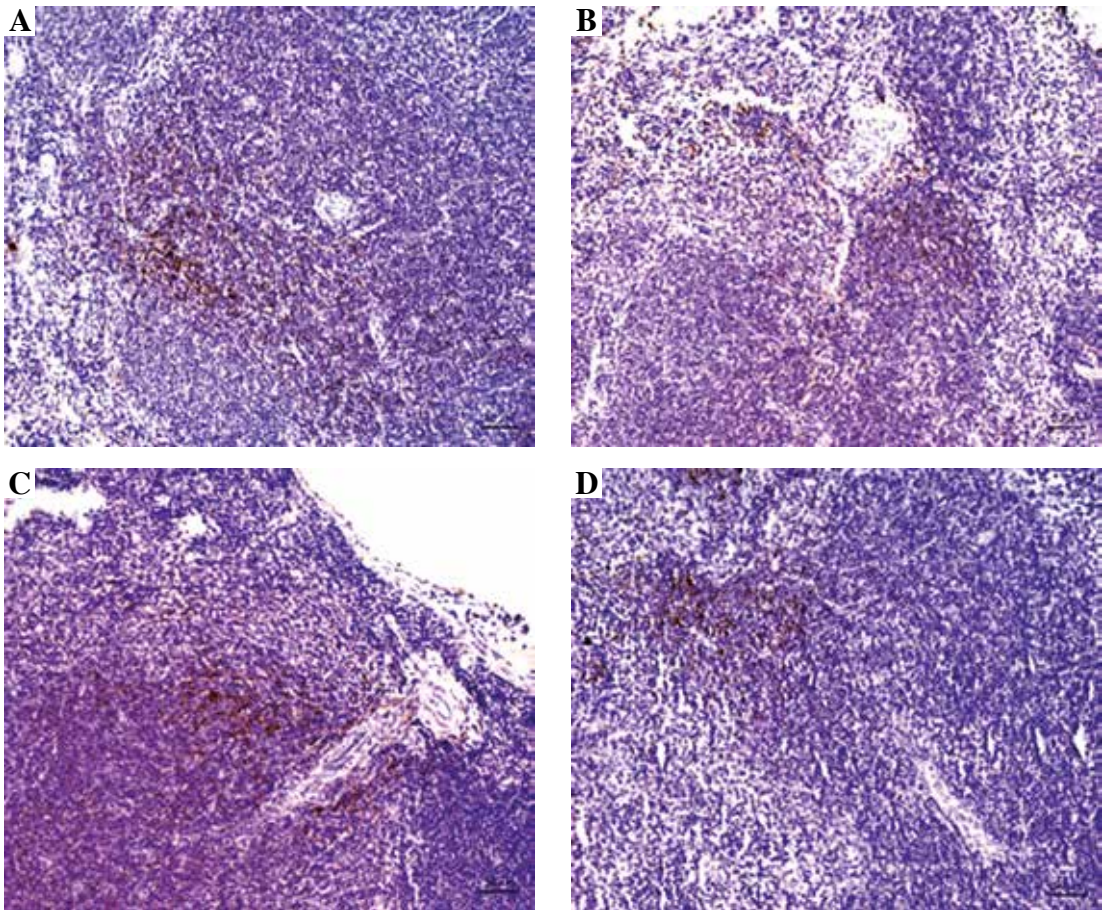
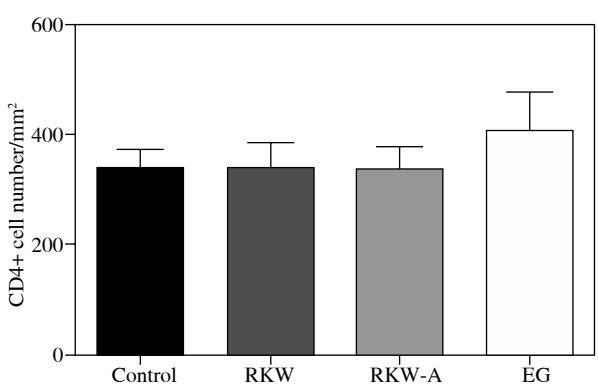


Fig. 1. Typical histological pictures from spleens of progeny. Spleens were counterstained with Harris's hematoxylin solution. CD4+ cells (brown color) were labeled with peroxidase conjugate mouse anit-CD4 antibody. A) control group; B) RKW group (*Rhodiola kirilowii* water extract); C) RKW-A group (*Rhodiola kirilowii* hydro-alcoholic extract); D) EGC group (epigallocatehin)

rophage phagocytosis of old red blood and other types of cells. White pulp is involved in the immune response against antigens which are in the blood. It comprises three regions: the T cell zone or periarteriolar sheath (PALS), B cell follicles, and the marginal zone. The PALS is further divided into inner PALS comprising mainly CD4+ T cells, some CD8+ T cells, interdigitating DC and migrating B cells. In lymphoid follicles after antigen activation, cooperation with T, B and dendritic cells occurs. Activated B cells differentiate into plasma cells and release a plurality of immunoglobulin. Lymphocyte activation is usually typically associated with the expression of IL-2, IL-4, IL-6, IL-10 and IL-17, and TNF-α. Interleukin 2 promotes proliferation and cytokine production by T cells and affects functioning of the B cells. Interleukin 4 takes part in the differentiation of naive T cells into Th2 cells. Interleukin 10 produced mainly by Th2 cells generates and promotes T cell tolerance by decreasing the activity of IFN-y, IL-2 and IL-5. In opposite to IL-4, IFN-γ activates macrophages and



Number of animals – 38
RKW – Rhodiola kirilowii water extract; RKW-A – Rhodiola kirilowii hydro-alcoholic extract; EGC – epigallocatehin

Fig. 2. Mean CD4+ cells number ± SEM in spleen of progeny

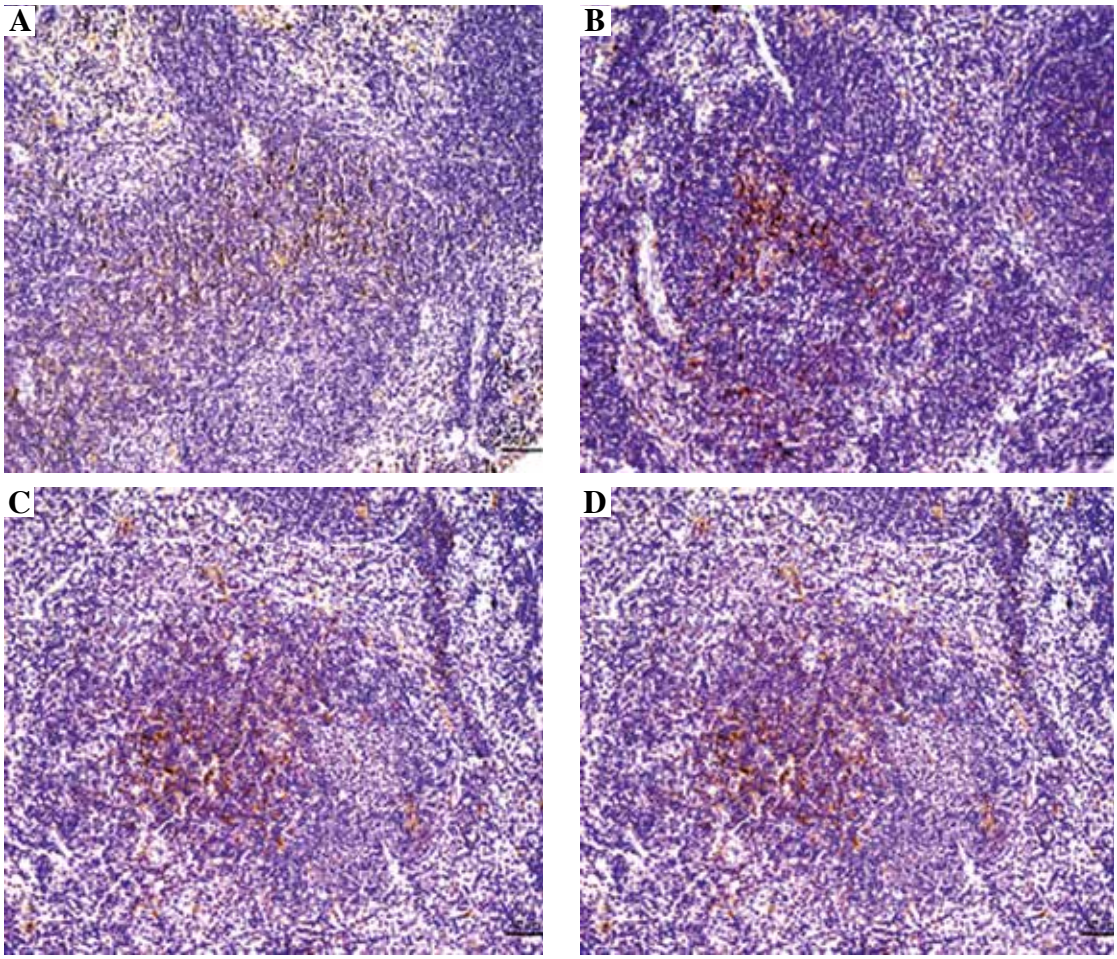


Fig. 3. Typical histological pictures from spleens of progeny. Spleens were counterstained with Harris's hematoxylin solution. CD8+ cells (brown color) were labeled with peroxidase conjugate mouse anit-CD8 antibody. A) control group; B) RKW group (*Rhodiola kirilowii* water extract); C) RKW-A group (*Rhodiola kirilowii* hydro-alcoholic extract); D) EGC group (epigallocatehin)

promotes differentiation of CD4+ T cells into Th1 cells. TNF-α is an important cytokine secreted by the cells of the spleens, which stimulates the differentiation of B cells, T cells, NK cells [24-27].

Most studies with *Rhodiolla kirilowii* extracts administration, performed in adult mice, showed positive effect on functionality of their immune system. The present work is a continuation of research project in which we would like to know whether RK extracts are safe for the progeny. We focused mainly on the various parts of the immune system. In the previous study we noted disturbances in the spleen cells functionality [13]. Both extracts of *Rhodiola kirilowii,* when administered to mothers, resulted in a diminished proliferation rate of progeny mice spleen cells after Concavalin A stimulation. A reduced proliferative response after lipopolysaccharide (RKW-A) or phytohemagglutinin (RKW) stimulation was also observed. Furthermore, significant differences in the percentage of spleen cell lymphocytes (CD4+ cells) between RKW and RKW-A were found. Therefore, we wanted to know if there are any abnormalities in macro- and microscopic analysis as well as in the level of CD4 and CD8 positive cells in spleens.

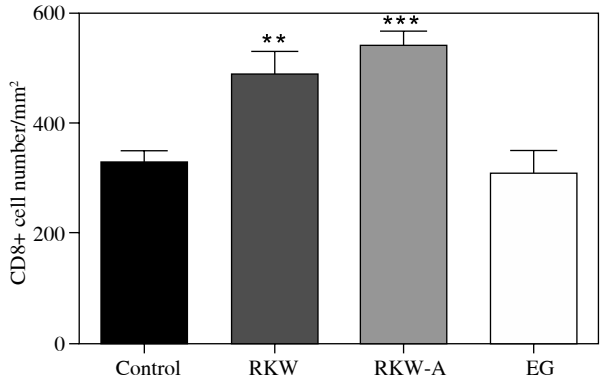
In the present study we did not observe any significant differences in the spleen mass. Offspring from mice fed during pregnancy and nursing with RKW-A extract or EGC exhibited a reduced weight compared to the control and RKW group. These results confirm earlier studies performed by Rogala *et al*. [28] that the polyphenols may impair fetal development. These observations are also in accordance with Zdanowski *et al.* [29] studies in which RKW-A extracts, given to pregnant and nursing mice caused mortality of some of their neonates.

We also observed this relationship in EGC group (unpublished data). What is interesting, *Rhodiolas* supple-

mentation likewise affect young organism development. Li *et al*. [30] on the model of growing broilers showed a decreased body weight in a group fed with the crushed roots of *R. crenulata*.

Differences in the body weight observed in the present study were not associated with changes in the mean spleen mass or relative spleen mass. Furthermore, there were no structural or morphometric changes in spleens between all studied groups. Number and localization of CD4 positive cells was also not affected in RK extracts or EGC groups in comparison to control. However, a significant increase the number of CD8 positive cells in both RKW and RKW-A groups was shown. It is in a minor contrast with our previous work where significant reduction of CD4+ in RKW-A group and no changes in CD8+ cells in spleen were found [13]. Probably, these opposite results are caused by various techniques used for examination. In the present work histological analysis was performed. Section of the spleen (in central part) caused that only a part of the cells were analyzed. In our previous work, we analyzed cell phenotype from the whole spleen cells suspension (cytometric analysis). Apart from the above considerations it is really promising, that we found CD8+ in various spleen regions. It means that both RK extracts positively affect mobility of these cells. It has been proven, with the use of mathematical modeling that spleen is a major source of effector CD8 T cells after influenza virus infection and antigen presentation might occur in the spleen [31]. Moreover, Sumida *et al*. [32] suggested that the reduced number of CD8+ T cells in the spleen may result in a lack of effective immune response to HCV.

Enhanced number of CD8+ cells in the central region of spleen, noted in the present work, may be an evidence of increased antiviral properties in pups whose mothers were fed during pregnancy and lactation with both *Rhodiola kirilowii* extracts. However, before a total recommendation of RK extracts supplementation in pregnancy, results from other components of the immune system should be considered.



*** p < 0.01, *** p < 0.001; p – probability value in comparison to control group; number of animals – 38*
RKW – Rhodiola kirilowii water extract; RKW-A – Rhodiola kirilowii hydro-alcoholic extract; EGC – epigallocatehin

Fig. 4. Mean CD8+ cells number ± SEM in spleen of progeny

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The authors declare no conflict of interest.

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