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The decrease in number of splenic lymphocytes in mice fed *Rhodiola kirilowii* during pregnancy and lactation concerns mainly CD19+ and CD4+ cells

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Abstract

In previous work we described the decline in the number of splenocytes of mice which during pregnancy and lactation were fed Rhodiola kirilowii. In this work we present the size of individual subpopulations of splenic lymphocytes in these mice. Experiments were performed on adult inbred female Balb/c mice, 8-9 weeks old, 20-22 g b.m., mated with adult males from the same strain. Females, from when the copulatory plug was noted up to the 28th day after delivery, were supplemented daily with lyophilized aqueous (RKW) or 50% hydro-ethanolic (RKW-A) extract (20 mg/kg b.m.) dissolved in distilled water. Then, mice were euthanized, spleens dissected, cells counted and the total numbers of CD3+, CD19+, CD4+, CD8+ and CD335+ splenic lymphocytes were evaluated by cytometry.

The number of CD3+ lymphocytes per 1 g of splenic tissue was higher in RKW-A than in RKW spleens and did not differ from the control. The number of CD3+ lymphocytes in RKW spleens was lower than in the controls. The number of CD19+ and CD4+ cells was lower in both experimental groups than in the controls. The number of CD335+(NK) cells was lower in RKW spleens than in the control.

Key words: *mice, pregnancy, spleen, Rhodiola kirilowii, lymphocyte populations.*

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Introduction

Rhodiola kirilowii, *Crassulaceae* (RK), a medicinal plant native to high mountain regions of Tibet and China, a perennial herb with its distribution from the southeastern Qinghai-Tibetan Plateau and the Hengduan Mountains to adjacent northern China and central Asia, was traditionally used as a tonic, adaptogen, antidiarrheal, cardioprotective and anti-inflammatory drug [1-3]. Recently studies in rats and mice demonstrated, that RK extracts enhance cellular immunity [4-7]. *In vitro* aqueous and hydro-alcoholic extracts stimulated granulocyte activity and increased the lymphocyte response to mitogens in mice. *In vivo* both extracts stimulated immunological angiogenesis in mice and promoted angiogenesis in the myocardium of rats with acute myocardial infarction. Feeding mice with both extracts for 7 days lowered the intensity of *Pseudomonas aeruginosa* infection, increased the number of blood leukocytes and modulated their metabolic activity. Also antimicrobial properties of this plant were described against HCV and *Mycobacterium tuberculosis* [8, 9].

In order to obtain a herbal remedy for pregnant women, a safe alternative to antibiotics, we started research on immunotropic effects of RK in pregnant mice. The current work builds on research described previously, regarding the spleens of mice that were fed extracts of *Rhodiola kirilowii* rhizomes during pregnancy and lactation [10]. We demonstrated in that research that long-term administration of aqueous or aqueous-alcoholic *Rhodiola kirilowii* extract to pregnant mice caused a drastic decrease in the number of leukocytes in their spleens. The aim of the present study was to present what lymphocyte subpopulations were involved in this phenomenon.

Material and methods

Plant extracts

The roots and rhizomes of *Rhodiola kirilowii* were collected from field cultivations of the Institute of Natural Fibers and Medicinal Plants (Poznań, Poland). The raw material was washed, cut into thick slices, dried in natural

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conditions and next powdered. Water and hydro-alcoholic extracts of *Rhodiola kirilowii* were prepared as previously described [10].

Animals

Experiments were performed on 8-9-week-old inbred Balb/c females which were mated with adult males from the same strain.

Females from when the copulatory plug was noted up to the 28th day after delivery were fed daily with lyophilized RKW or RKW-A extracts (20 mg/kg b.m.) dissolved in distilled water. The control group received distilled water as placebo. Mice were housed separately and to avoid stress connected with gavage the substances were applied on a corn crisp and served to the female in a Petri dish.

For all performed experiments animals were handled according to the Polish regulations concerning the welfare of laboratory animals. All experiments were accepted and conducted according to ethical guidance of the Local Bioethical Committee (permission 73/2011). Mice were maintained under conventional conditions (room temperature 22.5-23.0°C, relative humidity 50-70%, 12 h day/night cycle) with free access to breeding rodent feed (Labofeed H, Wytwórnia Pasz "Morawski") and water.

Spleen cell analysis

Isolation of spleen and preparation of the cell suspension were performed as previously described [10]. Cell number was counted in a hematological analyzer (Exigo, Boule Medical AB). Spleen cell suspensions (100 µl, 1 x 10^6 cell/ml) were labeled by surface staining with fluorochrome-conjugated anti-mouse antibodies (Mouse T Lymphocyte Subset Antibody Cocktail (BD Biosciences): CD3e PE-Cy7, CD4 PE, CD8a APC, Mouse B Lymphocyte Activation Antibody Cocktail: CD 19 APC and panel 3 – CD3 FITC, CD 335 PE), according to the manufacturer's protocol (BD Biosciences). Percentage distribution of spleen cell phenotypes was determined by flow cytometry (FACSCalibur, BD). The total number of adequate spleen populations was then calculated. Results are presented as mean number of cells x 10^6 per g of splenic tissue ± SEM (standard error of the mean).

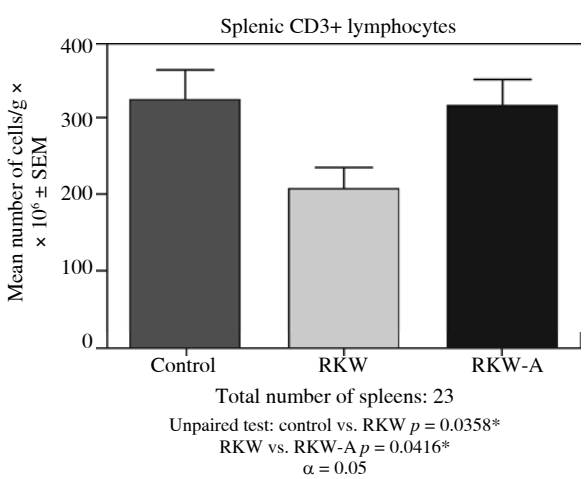


Fig. 1. Mean number of CD3+ cells × 10^6/g of splenic tissue. Spleen donors: mice (mothers), fed for 48-50 days after mating with *Rhodiola kirilowii* extracts. RKW – mice supplemented with water extract, RKW-A – mice supplemented with 50% hydro-alcoholic extract, Control – mice supplemented with placebo. Statistical analysis: unpaired *t* test, SEM – standard error of the mean

Chemical analysis of flavonoids in RK extracts and spleen tissue

Total extracts' polyphenol/flavonoids concentration was assayed by applying the HPLC system (Dionex) equipped with the CoulArray electrochemical detector (ESA Inc) as previously described [11].

Statistical analysis

Statistical evaluation of the results was performed using the Shapiro-Wilk normality test, unpaired t test, one-way ANOVA with Tukey's or two-way ANOVA with Bonferroni multiple-comparison post-tests (Graph Pad Prism).

Results

Spleen cells analysis

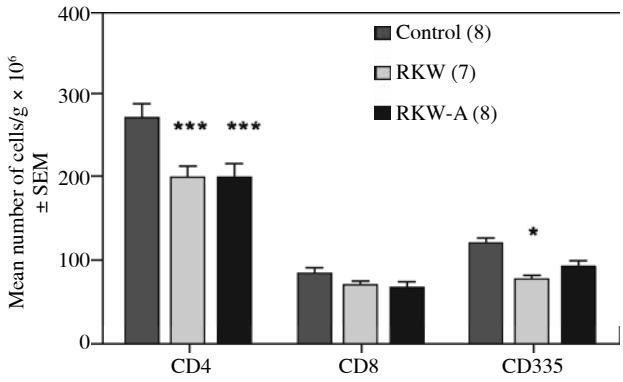
Both extracts significantly reduced the number of spleen cells in mice mothers. There were observed some differences between RK groups.

Mice from the RKW group exhibited a significant reduction of the number of T-cells (CD3+) in comparison to the control and RKW-A groups (Fig. 1). This was associated with significant reduction of CD4-positive cells. Spleens from mice fed RKW-A extract also had a lower number of CD4+ cells, but the number of CD3+ cells did not differ from the control. The number of CD8+ cells was not changed in all groups (Fig. 2).

Reduction of CD3+ positive spleen cells in the RKW group was also connected with a reduction of natural killer (NK) cells (CD335+, Fig. 2). The number of NK cells in spleen was not affected by RKW-A supplementation. Opposite to differences observed for T and NK cells, both extracts significantly reduced the number of spleen CD19+ cells (B lymphocytes, Fig. 3).

Chemical analysis of flavonoids in spleen tissue

In the control group a low concentration of epicatechin (0.068 ng/g of spleen) and a high concentration of quercetin (1.170 ng/g of spleen) were observed (Fig. 4). Kaempferol was not detected. In spleens from the RKW



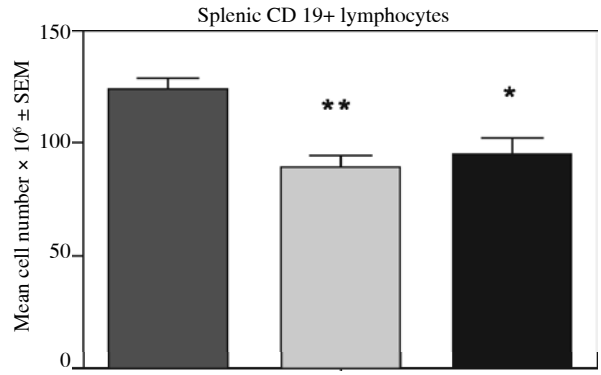
Two-way ANOVA		
Source of variation	% of total variation	*p* value
Interaction	2.26	0.0539
Extract	6.53	< 0.0001
CD phenotype	77.51	< 0.0001

Bonferroni posttests

Control (8) vs. RKW (7)	
CD4	$p < 0.001$
CD8	$p > 0.05$
CD335	$p < 0.05$

Control (8) vs. RKW-A (8)	
CD4	$p < 0.001$
CD8	$p > 0.05$
CD335	$p > 0.05$

Fig. 2. Mean number of CD4+, CD8 + and CD335 + cells × 10^6/g of splenic tissue. Spleen donors: mice (mothers), fed for 48-50 days after mating with *Rhodiola kirilowii* extracts. RKW – mice supplemented with water extract, RKW-A – mice supplemented with 50% hydro-alcoholic extract, Control – mice supplemented with placebo. Statistical analysis: two-way ANOVA, Bonferroni post-test; $\alpha = 0.05$. SEM – standard error of the mean. Number of mice in parentheses



One-way analysis of variance	
p value	0.0043

Tukey's multiple comparison test	Significant? $p < 0.05$?	Summary
Control (8) vs. RKW (7)	Yes	**
Control (8) vs. RKW –A (8)	Yes	*
RKW (7) vs. RKW-A(8)	No	NS

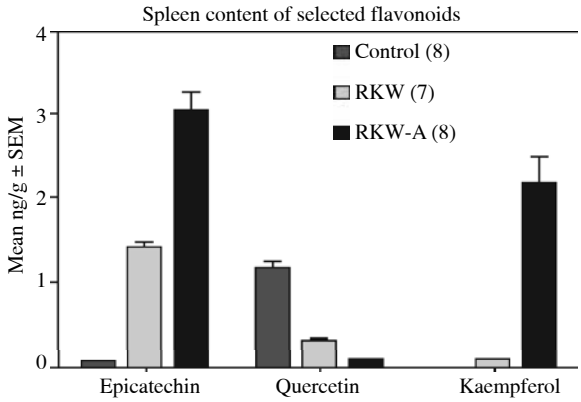
Fig. 3. Mean number of CD19+ cells × 10^6/g of splenic tissue. Spleen donors: mice (mothers), fed for 48-50 days after mating with *Rhodiola kirilowii* extracts. RKW – mice supplemented with water extract, RKW-A – mice supplemented with 50% hydro-alcoholic extract, Control – mice supplemented with placebo. One-way ANOVA, $\alpha = 0.05$. SEM – standard error of the mean. Number of mice in parentheses

group epicatechin concentration was about 21 times higher than that in the control group and two times lower than in the RKW-A group (3.040 ng/g of spleen). In spleen from the RKW-A group kaempferol concentration was over 25 times higher (2.200 ng/g of spleen) than that in the RKW group (0.080 ng/g of spleen). In contrast, the highest quercetin concentration was found in the control group, then RKW (0.320 ng/g of spleen) and RKW-A (0.07 ng/g of spleen).

Discussion

Many plants and their derivatives (extracts, brew etc.) are used in natural medicine. Chemical compounds found in those plants exhibits different effects on cells and tissues. Only the proper proportions of compounds in the plant or its derivatives allow to use in treatment. We had hoped that one such plant may be *Rhodiola kirilowii*, in the form of aqueous and alcoholic extracts. Unfortunately, as we previously described, daily supplementation with aqueous or hydro-alcoholic extracts of *Rhodiola kirilowii* (20 mg/kg b.m.) significantly decreased the number of cells in the spleen [10]. In the present work we wanted to determine which populations of spleen cells were reduced and which compounds may be associated with this phenomenon. We chose three flavonoids whose concentrations significantly differed in the studied groups: epicatechin, kaempferol and quercetin. Catechins are well-known inhibitors of cell proliferation [12]. Epicatechin inhibits two major enzymes: mammalian DNA polymerase and topoisomerase, which are necessary for cell replication [13]. Epigallocatechin-3-gallate also directly suppresses T cell proliferation [14]. This effect was associated with impaired IL-2 utilization [15]. In the present work high concentrations of epicatechin were found in the spleen of both groups of mice fed *Rhodiola kirilowii*, which might explain the decreased number of B-cells in spleens of these mice. However, the number of CD3+ and CD335+ cells was reduced only in spleens from the RKW group, which contains a 2-fold lower concentration of epicatechin.

Therefore one might suppose that other substance would be responsible for this phenomenon. Kaempferol in a very high concentration (200 μM) exerts strong anti-proliferative effect [16] and also affects cell metabolism [17]. In contrast, kaempferol in lower concentrations had no



RKW (12) vs. RKW-A (8)

Epicatechin	$p < 0.001$
Quercetin	$p > 0.05$
Kaempferol	$p < 0.001$

Two-way ANOVA		
Source of variation	% of total variation	*p* value
Interaction	46.28	< 0.0001
Treatment	30.00	< 0.0001
Compound	14.96	< 0.0001
Source of variation	*p* value summary	Significant?
Interaction	***	Yes
Treatment	***	Yes
Compound	***	Yes

Fig. 4. HPLC analysis of the spleen in control and experimental pregnant and afterwards lactating mice, recipients of *Rhodiola kirilowii* extracts. RKW – mice supplemented with water extract, RKW-A – mice supplemented with 50% hydro-alcoholic extract. Statistical analysis: two-way ANOVA, Bonferroni post-test; $\alpha = 0.05$. SEM – standard error of the mean. Number of mice in parentheses

influence on murine embryo stem cell proliferation [18]. The opposite effect of epicatechin and kaempferol corresponds to a negative correlation between concentration of those compounds in splenic tissue, as described in a previous publication [10]. In the spleens of mice belonging to the RKW-A group, abundant content of kaempferol may play a protective role against the adverse effect of catechins, and, probably, of other unidentified substances.

An important protective role against the anti-proliferative action of epicatechin may also be played by quercetin. This flavonol has dual, pro- and anti-proliferative action. Quercetin in high concentration (above 50 μM) inhibited proliferation of cells and may induce their death [19], while in lower concentration may stimulate proliferation [20]. Previously, we observed a positive correlation between spleen cellularity and quercetin content [10].

Finally, it cannot be ruled out that other compounds present in *Rhodiola kirilowii* extracts might also be responsible for the observed reduction in the number of splenocytes. An example of such a compound is lotaustralin, a cyanogenic glycoside. Hydrogen cyanide is an effective inhibitor of cytochrome oxidase activity and is toxic to animals. Lotaustralin is synthesized from isoleucine, and was found in various edible plants as well as others used as herbal medicines, for example *Rhodiola* species [21-23]. According to Gryszczyńska *et al*., *Rhodiola kirilowii* aqueous extract contained more lotaustralin (74.791 mg/100 g of dry powdered material) than a hydroalcoholic extract. (53.773 mg/100 g of dry mass). What is interesting, both spleens obtained from mothers administered RKW and RKW-A exhibited similar numbers of CD4+ and CD8+ cells. This indicates that the number of CD3-positive cells in RKW-A spleen, comparable to the control, may be related to an increased CD3+ CD4-, CD8- (CD3+DN) population in the spleen. The role of this population is not fully understood, but it probably exhibits some regulatory actions [24].

Moreover, the CD3+ CD4-, CD8- population was found in the states of pathological activation of the immune system. Increased size of this population was noted in granzyme B deficient (GrB –/–) mice after acetaminophen liver injury [25]. It also could be induced *in vitro* after 4-5 times stimulation of CD4+ T cells (which mimics chronic immune hyper-activation) [26]. Some protective role against negative immune system activation, associated with elevation of CD3+ CD4-, CD8 cells, may be played by quercetin, which possesses strong anti-inflammatory capacities [27]. In conclusion, both tested extracts had an adverse effect on various subpopulations of splenic lymphocytes, in particular CD19+ and CD4+ cells, which is a dangerous situation. Therefore we do not recommend administration of *Rhodiola kirilowii* extracts to pregnant and lactating mothers, especially for such a long period of time.

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The authors declare no conflict of interest.

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