

Effect of quercetin on immunocompetent cells in mice

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Background. The aim of our study, as part of a complex investigation of the influence of flavonoids on *in vivo* and *in vitro* systems, included the effect of quercetin supplementation on the hematological parameters and immune surface markers of lymphocytes in murine blood and spleen.

Methods. The effect of prolonged (4 weeks) dietary quercetin excess was studied in BALB/c mice. The control group was supplied with pure drinking water and the experimental group with 100 mg/l of quercetin solution. The weight of body and organs was measured and hematological analysis was performed at the end of the experiment. Blood and spleen samples were analysed flow-cytometrically for lymphocyte markers (CD3⁺, CD19⁺, NK-1.1⁺, CD4⁺CD8⁻, CD8⁺CD4⁻, CD4⁺CD8⁺, NK-1.1⁺CD3⁺).

Results. The tendency of lung weight increment (0.1 > P > 0.05) was revealed in the experimental group of mice. Hematological analysis showed that the excess of quercetin increased the hemoglobin level by 11.8% (P < 0.05). Multiparameter flow cytometry analysis demonstrated that quercetin increased NK-1.1⁺ (1.3-fold, P < 0.05), NK-1.1⁺CD3⁺ (1.9-fold, P < 0.05) in the spleen and CD4⁺CD8⁺ (1.6-fold, P < 0.05) in the blood but suppressed NK-1.1⁺CD3⁺ cells (2.0-fold, P < 0.05) in the blood.

Conclusions. Our results have shown that a prolonged supplementation of quercetin to mice increases blood hemoglobin. The percentage of lymphocyte CD4⁺CD8⁺ and NK-1.1⁺CD3⁺ subpopulations in the blood and spleen are affected and redistributed differently in the compartments of mice after prolonged quercetin supplementation.

Key words: quercetin, lymphocyte, flow cytometry, mice

BACKGROUND

Flavonoids are polyphenolic compounds that occur in a variety of food and beverages of vegetable origin. Fruit, vegetables and beverages are the sources of flavonoids. It has been reported that the human intake of flavonoids from the diet is about 20 mg to 1 g every day (1).

Flavonoids have been recognized for their interesting clinical properties, such as anti-inflammatory, antiallergic, antiviral, antibacterial, and antitumoral activities (2–5), but the molecular mechanisms of their biological responses remain to be elucidated. In fact, the pharmacological effects of many traditional drugs can be explained by the presence of flavonoid compounds (6).

Among flavonoids, quercetin (3,5,7,3',4'-pentahydroxyflavone) is the major representative of the flavonoid subclass. In general, quercetin prevents oxidant injury and cell death (7) by several mechanisms, such as scavenging oxygen radicals (8, 9) protecting against lipid peroxidation (10–12), but as a prooxidant it is capable of reducing free Fe³⁺ to Fe²⁺, which reacts

with H₂O₂ forming a hydroxyl radical (13). Epidemiological studies showed that consumption of certain flavonoids containing quercetin is correlated with a decreasing risk of coronary artery disease mortality (14, 15).

The immune system is strongly affected by a variety of environmental, physical, psychological and essentially by chemical stressors (16). The interplay between oxidative stressors as generators of reactive oxygen species and exogenous, as well as endogenous antioxidants is crucial for proliferation and differentiation of immune cells (17).

The present study, as part of a complex research, included investigation of the influence of flavonoid quercetin excess on the hematological parameters and the immune biomarkers of lymphocytes in mice.

MATERIALS AND METHODS

Animals and maintenance. Male BALB/c mice (10 weeks old) were obtained from the vivarium of the Institute of Immunology (Vilnius, Lithuania). After acclimatization (2 weeks) the animals were housed in solid-bottomed cages containing bedding of wood shavings and were allowed food and water *ad libitum*. The room temperature was maintained at 21–

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24 °C and a 12/12 h light/dark cycle was employed. The mice were divided at random into two equal groups of 10 animals each. During the experimental period of 4 weeks, the control group (group A) was supplied with pure drinking water and the experimental group (group B) received quercetin solution 100 mg/l (as quercetin dihydrate, ROTH, Germany). Quercetin consumption was recorded twice a week. At the end of the experiment, the mice were sacrificed by cervical dislocation and their organs were weighed. Approval of the Lithuanian Ethic Committee for Laboratory Animal Use was obtained prior to commencement of the experiments.

Body and organ weight. At the beginning of the experiment the average body weight of mouse was 24.90 ± 1.87 g (group A) and 24.78 ± 1.89 g (group B). The body and organ (liver, spleen, heart, lung, kidney) weight were determined by analytical balance at the end of the experiment.

Hematological parameters.

Blood was collected from the heart in syringes, stabilized with EDTA (Sigma, USA), and 0.1 ml of blood of each mouse was analyzed with a HEMAVET /850S hematological counter (CDC Technologies Inc., Oxford, Connecticut, USA).

Flow cytometry analysis. Blood and spleen white cells were isolated by a routine method using ammonium chloride lysing solution, and were processed for flow cytometry testing by the standard direct labelling method (18). Cells, suspended to a concentration of 1×10^6 /ml, were stained in the dark for 30 min with 1 µg of each appropriately labelled monoclonal antibodies: Cy-Chrome-antiCD3e, FITC-antiCD4, PE-antiCD8a, FITC-antiNK (NK-1.1⁺), PE-antiCD19 (BD Pharmingen, Germany). Isotypical control was performed with the appropriately labelled irrelevant immunoglobulines and the Fc Block was used as required. Multiparameter flow cytometric analysis was performed on a FACSCalibur™ flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA). Data were acquired using CELLQuest software. We investigated the CD3⁺, CD19⁺, NK-1.1⁺ populations and the CD4⁺CD8⁻, CD8⁺CD4⁻, CD4⁺CD8⁺, NK-1.1⁺CD3⁺ subpopulations of lymphocytes.

Data analysis. Statistical analysis was performed using the Microsoft Excel Ver. 7.0 computer program. The data were expressed as mean values (M) ± standard deviation (SD). Statistical differences between the groups were assessed by Student's t test. The results were considered significant at $P < 0.05$.

RESULTS

An average quercetin intake calculated on the basis of liquid consumption was 20 mg/kg body weight/day. The body and organ weight analysis showed that at the end of the experiment the mean weight of body, liver, spleen, heart and kidney did not differ in groups A and B, except the tendency of lung weight increment in group B mice ($0.1 > P > 0.05$) by 20.7%. The lung weight in group A mice was 174.8 ± 16.3 mg.

Table. **Hematological parameters of control and quercetin-supplemented groups of mice**

Parameters	Group A	Group B	Standard limit
Leukocytes (K/µl)	7.32 ± 1.62	7.05 ± 1.47	1.8–10.7
Lymphocytes (K/µl)	5.69 ± 1.86	5.77 ± 0.89	0.9–9.3
Neutrophils (K/µl)	0.87 ± 0.21	0.93 ± 0.26	0.1–2.4
Monocytes (K/µl)	0.31 ± 0.08	0.32 ± 0.13	0.0–0.4
Eosinophils (K/µl)	0.055 ± 0.04	0.027 ± 0.03	0.0–0.2
Basophils (K/µl)	0.008 ± 0.006	0.005 ± 0.004	0.0–0.2
Erythrocytes (M/µl)	8.14 ± 0.95	8.89 ± 0.64	6.4–9.4
Hemoglobin (g/dl)	12.84 ± 0.61	$15.17 \pm 0.54^*$	11.0–15.1

* $P < 0.05$.

Hematological analysis showed that the excess of quercetin did not significantly influence the hematological parameters (Table). We detected only a tendency of decrement of eosinophil, basophil amounts and an increment of hemoglobin (11.8%, $P < 0.05$) in quercetin-supplemented mouse group in comparison with the control group.

Using flow cytometry multiparameter analysis, we identified three (CD3⁺, CD19⁺, NK-1.1⁺) populations and four (CD4⁺CD8⁻, CD8⁺CD4⁻, CD4⁺CD8⁺, NK-1.1⁺ CD3⁺) subpopulations of lymphocytes (blood and spleen) and to quantify their relative proportions. The main population of lymphocytes in the blood of both groups of mice comprised T cells with the CD3⁺ marker, but in the spleen the main population of lymphocytes was with the CD19⁺ marker (Fig. 1). These populations were not influenced in quercetin-supplemented mice. The population of NK-1.1⁺ cells in the spleen was also increased in mice administered quercetin. The content of NK-1.1⁺ cells in blood was 1.3 times ($P < 0.05$) below the average of that of the control group.

Subpopulation analysis showed that CD4⁺CD8⁻ (helper/inducer) and CD8⁺CD4⁻ (cytotoxic/suppressor) cells in blood and spleen were $26.48 \pm 2.82\%$, $15.08 \pm 2.15\%$, and $22.20 \pm 3.02\%$, $12.47 \pm 1.96\%$, respectively (Fig. 2A). In quercetin-supplemented mice these cell subpopulations were not altered. Double-positive lymphocytes CD4⁺CD8⁺ were found in the blood and spleen of all mice in very small quantities, and administration of quercetin increased this popu-

lation in blood 1.6 times ($P < 0.05$), but in the spleen there was only a tendency to an increment of this subpopulation. The immunoregulatory index (II), the ratio of $CD4^+CD8^-/CD8^+CD4^-$ lymphocytes in the blood and spleen did not change after quercetin supplementation (Fig. 2B). NK T cells ($NK-1.1^+ CD3^+$) were found in a small quantity in the blood and spleen, and administration of quercetin significantly suppressed (2.1-fold, $P < 0.05$) this subpopulation in the blood, while in the spleen it was significantly increased (1.9-fold, $P < 0.05$).

DISCUSSION

The body and organ weight analysis showed only a tendency of lung weight increment in mice of group B ($0.1 > P > 0.05$) by 20.7% after a four-week quercetin supplementation at a dose of 20 mg/kg bw/day. This dose is higher (1.4–1.5 fold) than the upper limit of the usual dose in human diet (1).

Hematological analysis showed that quercetin excess did not significantly influence the hematological parameters. We detected only a tendency of eosinophil, basophil decrement and hemoglobin increment (11.8%, $P < 0.05$) in quercetin-supplemented group of mice in comparison with the control group. The quercetin as a potent antioxidant for outer cell membrane structures is a stabilizing factor for erythrocyte membrane integrity and possibly prolongs its life and functioning.

Using multiparameter analysis by flow cytometry, we identified three populations and four subpopulations of lymphocytes (blood and spleen) as well as quantified their relative proportions. The main population of lymphocytes in the blood of both groups of mice comprised T cells with the $CD3^+$ marker, but in the spleen the main population of lymphocytes possessed the $CD19^+$ marker. These populations

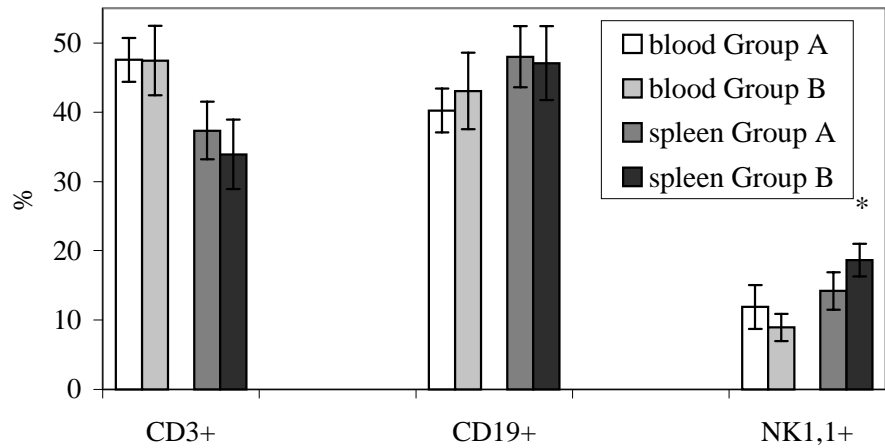


Fig. 1. Distribution of lymphocyte populations in mice

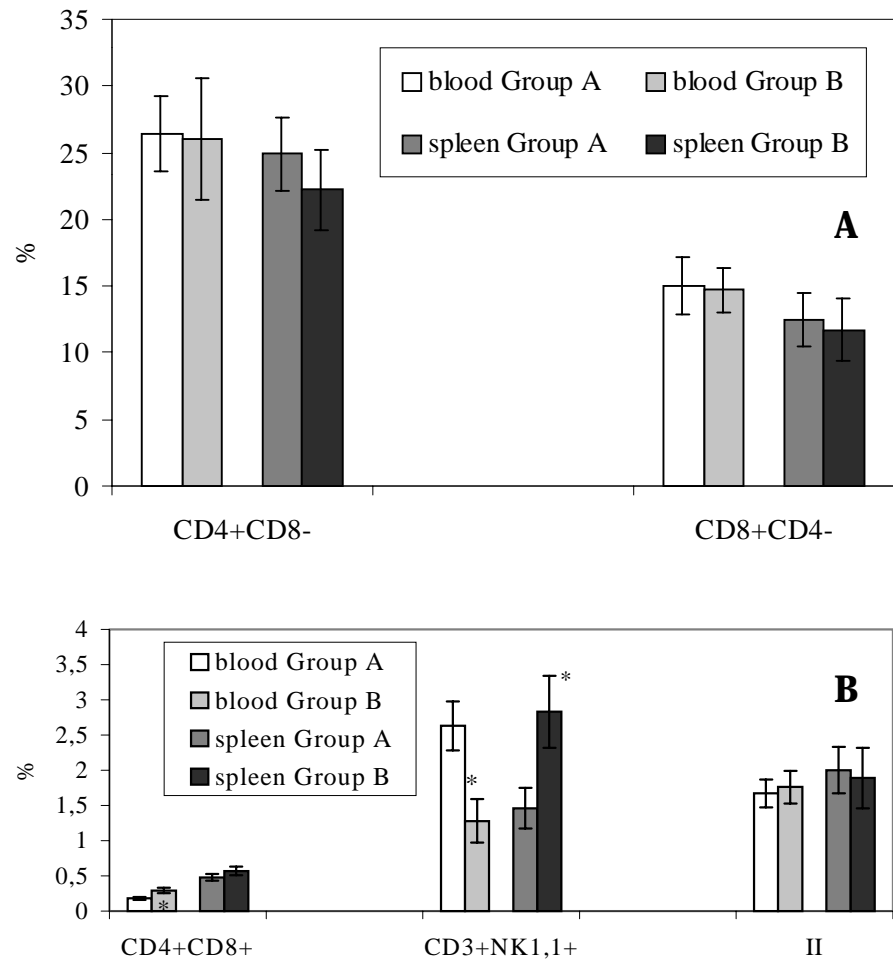


Fig. 2. Distribution of main (A) and minor (B) lymphocyte subpopulations and immunoregulatory index (II) in mice

were not influenced in quercetin-supplemented mice. The spleen population of $NK-1.1^+$ cells was increased in mice that received quercetin. In their blood, the content of $NK-1.1^+$ cells was 1.3 times ($P < 0.05$) lower than in the blood of the control group.

Quercetin administration did not alter the proportions of helper/inducer ($CD4^+CD8^-$), cytotoxic/suppressor ($CD8^+CD4^-$) cells and the immunoregulatory index ($CD4^+CD8^-/CD8^+CD4^-$ ratio) in the blood

and spleen. The amount of double-positive lymphocytes CD4⁺CD8⁺ (the progenitors of T cells) was found in the blood and spleen of all mice to be very low, and administration of quercetin increased this population in the blood 1.6 times ($P < 0.05$), but in the spleen only a tendency of increment of this subpopulation was observed. Possibly quercetin stimulates proliferation of these cells *in vivo*. During the process of T maturation in the thymus CD4⁺CD8⁻ T cells became CD4⁺CD8⁺ and then differentiated into mature CD4⁺ CD8⁻ or CD8⁺ CD4⁻ T cells (19, 20).

It is well known that some organs, particularly the liver, contain a unique phenotype (NK⁺CD3⁺) distribution compared to blood (21). We have found that after quercetin administration this population decreased in blood (2.1-fold, $P < 0.05$) but increased in the spleen (1.9-fold, $P < 0.05$), possibly indicating the clonal proliferation or efflux of this lineage cells. NK CD3⁺ cells express both cytolytic and regulatory activities during virus infections or an inflammatory process (22). It was demonstrated that NK T cells, as effective cytotoxic cells of the organism, were derived from CD4⁺CD8⁺ thymocytes and migrated into the blood stream escaping apoptosis in the thymus (23).

New findings *in vitro* and *ex vivo* provide evidence that quercetin is able to protect against chemically induced DNA damage in human lymphocytes, which may underlie its suggested anticarcinogenic properties (7). Recent studies of the bee product propolis containing the flavonoids hesperidin and quercetin showed that it may be considered as an anti-inflammatory agent influencing different types of immunoresponses probably via immunoregulatory T cells (24).

Our experiment with supplementation of quercetin to mice revealed that the immune system is a relatively sensitive system, and a prolonged quercetin intake at high doses initiates disturbances in immune homeostasis. The ensuing consequences of such disturbances are transformed proportions of lymphocyte subpopulations in blood as well as in spleen.

CONCLUSIONS

1. Prolonged quercetin supplement to mice (equivalent of 1.4–1.5 g/day for humans) did not alter the main hematological parameters and lymphocyte populations substantially.

2. Increment of hemoglobin in the blood and the tendency of increment of lung weight were recorded after quercetin treatment.

3. The percentage of minor lymphocyte subpopulations CD4⁺CD8⁺ and NK1.1⁺CD3⁺ in the blood and spleen of mice was affected by quercetin treatment *in vivo*.

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KVERCETINO POVEIKIS PELIØ IMUNOKOMPETENTINÈMS LÅSTELÈMS

Santrauka

Darbo tikslas – ištirti kvercetino poveikà *in vivo* pelèms pagal hematologinius rodiklius, kraujo ir bluþnies limfocitø pavirðinius þymenis. Tai yra flavonoidø poveikio *in vivo* ir *in vitro* kompleksinio tyrimo dalis.

Metodai. Naudota ilgalaikė (4 sav.) kvercetino dieta BALB/c pelèms. Kontrolinė grupė girdyta vandeniu, o eksperimentinė – kvercetino (100 mg/L) tirpalu. Kūno, organø masės ir hematologinė analizės atliktos bandymo pabaigoje. Kraujo ir bluþnies pavyzdþiai analizuoti tÈkmės citometru atitinkamai papymėjus limfocitus (CD3⁺, CD19⁺, NK-1.1⁺, CD4⁺CD8⁻, CD8⁺CD4⁻, CD4⁺CD8⁺, NK-1.1⁺CD3⁺).

Rezultatai. Nustatyta plauėiø svorio didėjimo tendencija (0,1 > P > 0,05) peliø bandomojoje grupėje. Kvercetino perteklius padidino hemoglobino kiekà kraujyje (11,8%, P < 0,05). Daugiaparetrinės tÈkmės citometrijos metodu nustatyta NK-1.1⁺ (1,3 karto, P < 0,05), NK-1.1⁺CD3⁺ (1,9 karto, P < 0,05) lãsteliø skaiėiaus padidėjimas bluþnyje ir CD4⁺CD8⁺ – kraujyje (1,6 karto, P < 0,05), taip pat NK-1.1⁺CD3⁺ lãsteliø skaiėiaus sumapėjimas kraujyje (2,0 kartus, P < 0,05).

Išvados. Ilgalaikis kvercetino perteklius padidina hemoglobino kiekà peliø kraujyje ir turi átakos plauėiø svorio didėjimui (tendencija). Kvercetinas nedideles CD4⁺CD8⁺ ir NK-1.1⁺CD3⁺ limfocitø subpopuliacijas kraujyje ir bluþnyje veikia skirtingai, stebimas jø kiekiø persiskirstymas.

Raktaþodþiai: kvercetinas, limfocitai, tÈkmės citometrija, pelė