The *in vitro* effect of *Rhodiola quadrifida* and *Rhodiola kirilowii* extracts on pigs blood lymphocyte response to mitogen Concanavalin A

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Abstract

Rhodiola quadrifida (RQ) and Rhodiola kirilowii (RK) are medicinal plants belonging to the Crassulaceae family. Their adaptogenic properties are well known and they are traditionally used in Asiatic medicine as anti-stressors and for many other purposes, including infections, inflammatory diseases and protection of people against cardiopulmonary function problems when moving to high altitude. We previously reported that extracts of these plants influenced some parameters of specific and non-specific cellular immunity in rodents. The aim of this work was to study the in vitro effect of aqueous and hydro-alcoholic extracts of under-ground parts of RQ and RK on Con A – induced activation of lymphocytes isolated from the blood of mammals (pigs). For this study we choose MTT proliferation assay. Aqueous (RKW, RQW) and 50% hydro-alcoholic (RKA, RQA) extracts of underground parts of RQ and RK have influenced response of pigs blood lymphocytes to ConA. Stimulatory effect was observed in cell cultures established in the presence of lower concentrations of Rhodiola extracts (from 1 to 10 µg/ml). This effect was dose-dependent, more pronounced in the cultures containing Rhodiola kirilowii than in the cultures with Rhodiola quadrifida extracts, and more pronounced in the cultures established at the presence of hydro-alcoholic extracts. At the presence of higher, 20 µg/ml extracts dose, stimulatory effect disappeared, and in the highest, 50 µg/ml concentration of all types of extracts , the response of lymphocytes to Con A was highly significantly lower than in the cultures which contained the mitogen alone.

Key words: *Rhodiola quadrifida, Rhodiola kirilowii, pigs, blood lymphocytes, Con A.*

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Introduction

Mounting an efficient immune response depends on the careful regulation of lymphocyte activation. One of the best models for studying the control of mammalian cell growth and proliferation is the response of lymphocytes to polyclonal activators – mitogenic agents, which stimulate growth, DNA synthesis and division. Number of common mitogens are lectins. Lectins are molecules which bind to specific carbohydrate groups. They bind to glycoproteins on the surface of the lymphocytes and cause activation. One of the best known T-cell mitogen is concanavalin A (ConA), a plant lectin from extract of Jack bean. Con A is a protein molecule with two sugar-binding sites, which can interact with sugar moieties on cell surfaces. Internalization of Con A is not required, but Con A must bridge binding sites on the lymphocyte surface to induce lymphocyte activation. Con A can activate cytotoxic effector lymphocytes, helper lymphocytes and suppressor

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lymphocytes, initiating their transformation into large, proliferating blast-like cells [1-5].

Binding of a mitogen to its glycoprotein receptors induce changes in the distribution of microfilaments and in the secondary structure of DNA, causes an increase in [Na+]i and a decrease in [K+]i, due to the Con A – induced increase of permeability caused by increased membrane fluidity. Within minutes of binding of Con A increased phospholipid incorporation into the membrane, increased fluxes of ionic calcium and potassium, increased uptake of nucleosides, sugar and amino acids have been noted. The adenosine 3'5'-monophosphate (cyclic AMP, cAMP) levels rose and fell sharply 10 hours after stimulation with Con A. Both the increase and decrease in cAMP appear to be required for progression of lymphocytes into the S phase of growth. Con A activation of lymphocytes change their homing behavior what is likely connected with a decline in L-selectin expression and an increase in CD11a and ICAM-1 expression on the surface of Con A-treated T cells [6-11].

Our previous studies have shown, that extracts prepared from rhizomes and roots of Asiatic plant adaptogens, *Rhodiola rosea* (RR), *Rhodiola quadrifida* (RQ) and *Rhodiola kirilowii* (RK), influenced various parameters of cellular immunity in rodents (mice and rats) [12-15]. The aim of this work was to study the *in vitro* effect of aqueous and hydro-alcoholic extracts of under-ground parts of RQ and RK on Con A – induced activation of lymphocytes isolated from the blood of mammals (pigs). For this study we choose MTT proliferation assay. In this assay yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a certain wavelength by a spectrophotometer. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable (living) cells.

Material and Methods

Rhizomes and roots of RQ were collected in Altai mountain in Mongolia thanks to dr H. Wiedenfeld, and further processed in Research Institute of Medicinal Plants (RIMP) in Poznań. The Mongolian plant material was identified; voucher specimen was deposited at the herbarium of the Institute of Botany of Mongolian Academy of Science in Ulaanbatar. *Rhodiola kirilowii* roots and rhizomes were cultivated, collected in September 2003 and identified in RIMP. Extracts and their chemical analysis were performed at the RIMP by Mrozikiewicz PM, Mścisz A, Krajewska-Patan A, Mielcarek S, Buchwald W; and at Warsaw Medical University by Zych M and Malinowski M. Sample extractions were performed in the temperature 40-45°C as described before [12]. Briefly: air-dried finely powdered rhizomes were extracted two times with water (aqueous extract) or 50% ethanol (hydro-alcoholic extract), evaporated to dryness in a rotary vacuum evaporator and lyophilized.

Animals

Blood for experiments was collected from the vena cava cranialis of four PWZ piglets, 4-5 month old, 40-50 kg body mass, females. Experiments were approved by Local Ethical Committee.

Cell cultures

Leucocytes were isolated from blood by centrifugation at 2000 g for 30 min at 4°C on the Gradisol L gradient (Aqua-Medica, Poland), washed three times in PBS and resuspended in RPMI 1640 medium (Sigma) supplemented with 10% of FCS (Foetal Calf Serum, Gibco-BRL) at a stock concentration of 2×10^6 cells/ml of medium. Viability of cells was checked by supravital staining with 0.1% w/v trypan blue.

Proliferative response of lymphocytes

Proliferative response of lymphocytes stimulated by mitogen ConA was determined by MTT assay as described before [12-14]. MTT [3-(4,5-Dimethyl thiazol-2-yl) 2,5-diphenyl-tetrazolium bromide] (Sigma) was dissolved in PBS at concentration of 5 mg/ml and filtered. On 96-well culture plates (Costar, USA) 100 µl of blood lymphocytes in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 0.02 mM 2-mercaptoethanol, 1% hepes buffer, penicillin/streptomycin (100U/100 µg/ml) were mixed with 100 µl of RPMI 1640 containing ConA in concentration 5 µg/ml. 3 cultures from each pool of leukocytes were established. After 72 h incubation at 37°C with 5% carbon dioxide atmosphere (Assab Incubator, Sweden), 50 µl of MTT solution were added into each well and plates were incubated for 4 h at 37°C. After incubation the plates were centrifuged (1400 g, 15°C, 5 min).

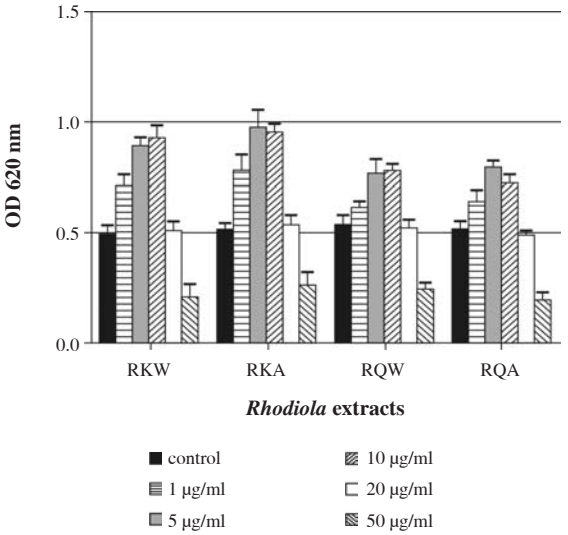

Fig. 1. The *in vitro* effect of *Rhodiola* extracts on the proliferative response of pigs lymphocytes to ConA (mean ± SD)

Table 1. Statistical analysis of the results

Two-way ANOVA				
Source of variation	% of total variation	p value		
Interaction	3.24	0.0003		
Concentration	88.68	< 0.0001		
Drugs	3.44	< 0.0001		
Source of variation	p value summary	significant?		
Interaction	***	yes		
Concentration	***	yes		
Drugs	***	yes		
Bonferroni posttests				
Control vs. 1 µg/ml				
Drugs	Difference	t	p value	Summary
RKW	0.2200	5.365	< 0.001	***
RKA	0.2700	6.585	< 0.001	***
RQW	0.08000	1.951	> 0.05	ns
RQA	0.1300	3.170	< 0.01	**
Control vs. 10 µg/ml				
Drugs	Difference	t	p value	Summary
RKW	0.4300	10.49	< 0.001	***
RKA	0.4400	10.73	< 0.001	***
RQW	0.2500	6.097	< 0.001	***
RQA	0.2100	5.122	< 0.001	***
Control vs. 20 µg/ml				
Drugs	Difference	t	p value	Summary
RKW	0.01000	0.2439	> 0.05	ns
RKA	0.02000	0.4878	> 0.05	ns
RQW	–0.01000	0.2439	> 0.05	ns
RQA	–0.0300	0.7317	> 0.05	ns
Control vs. 50 µg/ml				
Drugs	Difference	t	p value	Summary
RKW	–0.2900	7.073	< 0.001	***
RKA	–0.2500	6.097	< 0.001	***
RQW	–0.2900	7.073	< 0.001	***
RQA	–0.3200	7.804	< 0.001	***

Supernatants were removed and 100 µl of DMSO (Sigma) were added into each well and incubated for 15 min at room temperature. After incubation the solubilized reduced MTT was measured colorimetrically at 620 nm in a plate microreader (MRX 3 Dynatech). All samples were tested in triplicate and the mean value served as the result. *Rhodiola* extracts were present in culture medium during whole cultivation period (72 hours) in concentrations 1, 5, 10, 20 and 50 µg/ml.

Statistical analysis

The results of experiments were analysed by a two-way ANOVA and the significance of differences between groups was verified with a Bonferroni post-test (GraphPadPrism software package).

Results

Aqueous and 50% hydro-alcoholic extracts of underground parts of *Rhodiola quadrifida* and *Rhodiola kirilowii* have influenced response of pigs blood lymphocytes to ConA. Stimulatory effect was observed in cell cultures established in the presence of lower concentrations of *Rhodiola* extracts (from 1 to 10 μg/ml). This effect was dose-dependent, more pronounced in the cultures containing *Rhodiola kirilowii* than in the cultures with *Rhodiola quadrifida* extracts, and more pronounced in the cultures established at the presence of hydro-alcoholic extracts. At the presence of higher, 20 μg/ml extracts dose, stimulatory effect disappeared, and in the highest, 50 μg/ml concentration of all types of extracts, the response of lymphocytes to Con A was highly significantly lower than in the cultures which contained the mitogen alone (Fig. 1 and Table 1 – no statistically significant differences between the concentration of 5 μg/ml and 10 μg/ml).

Discussion

The present study have confirmed the results obtained previously for cultures of rat lymphocytes established in the presence of RQ and RK extracts, for concentrations 1-10 μg/ml of culture medium [13, 14]. This stimulatory effect was also observed in experiments with the extracts prepared from other *Rhodiola* species, *Rhodiola rosea* (RR) [12]. In that work we also observed inhibitory effect of higher RR extract doses, when cultures of pig blood lymphocytes were stimulated with Con A. In the same time, *in vitro* studies performed with rat or pig lymphocyte cultures which contained *Rhodiola* extracts alone (in concentration from 50 to 1000 μg/ml), showed no differences from the control cultures [12-14]. So, inhibition of Con A response by 50 μg/ml concentration of extracts was not connected with their cytotoxicity. The inhibition might be connected with the stronger activation of T suppressor cells, than of remaining T lymphocytes, by Con A in the presence of higher doses of *Rhodiola* extracts in the culture medium. Alternatively, it may be due to the competitive blocking of receptors for mitogen by high concentration of some *Rhodiola* extract compounds containing sugar moieties. Stimulatory and inhibitory effects of various *Rhodiola* concentrations may be due to the presence in the extracts of some polyphenols. It was reported by Sehm [16] that in ConA – stimulated cultures of cows blood leukocytes high concentrations of epigallocatechin gallate (EGCG) were inhibitory and low concentrations were stimulatory on pro-inflammatory cytokines mRNA expression. Stimulatory and inhibitory effects of Rhodiolas on ConA -induced lymphocyte activation may be also connected with their influence on catecholamine release and cAMP level [17]. It was described that while the early activation of type I cAMP- dependent protein kinase may mediate in a positive manner the induction of mitogenic response of lymphocytes to ConA, concomitant activation of type II protein kinase may inhibit this process [18]. It is also possible, that some compounds present in *Rhodiola* extracts behave as adrenergic agonists and may influence ConA-induced proliferation of lymphocytes on this way [19]. The stimulatory and inhibitory effects of Rhodiolas on ConA-induced T cell activation might be secondary to their ability to induction of opioid peptide biosynthesis and ability to induction of opioid receptors. It was described that endogenous opioid peptides are involved in the modulation of several immune functions. It was reported that the adaptogenic, cardiopulmonary protective, and central nervous system activities of *Rhodiola* have been attributed primarily to its ability to influence levels and activity of monoamines and opioid peptides such as beta-endorphins [20-23]. Jiang et al tested the influence of saponosides from the plant *Mimosa tenuiflora* on human and murine lymphocyte growth *in vitro* and they observed synergistic effect of these compounds with Con A on lymphocyte DNA synthesis and proliferation [24].

A detailed explanation of the phenomenon requires further study and becomes the subject of subsequent experience.

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