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The Influence of Water-Soluble Polysaccharides of *Crataegus sanguinea* Pall. on Nitric Oxide Production by Macrophages

A. A. Ligacheva[1], E. Yu. Sherstoboev[1], M. G. Danilets[1], E. S. Trofimova[1], S. V. Krivoshchekov[2], S. R. Khasanova[3], N. V. Kudashkina[3], A. M. Gur'ev[2], and M. V. Belousov[2]

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The *in vitro* addition of water-soluble polysaccharides isolated from the leaves of *Crataegus sanguinea* Pall. to culture of mouse peritoneal macrophages induced classical activation of antigen-presenting cells by increasing NO synthase activity and reducing arginase expression.

Key Words: *plant polysaccharides; NO synthase; arginase; macrophages*

Polysaccharides of higher plants attract much attention due to combination of high biological activity and low toxicity [8]. It is known that plant-derived water-soluble polysaccharides interact with Toll-like receptors of macrophages and trigger MAP-kinases and NF-κB intracellular signaling pathways leading to a significant increase in gene expression of both proinflammatory cytokines (TNFα, IL-6) and inducible NO synthase (iNOS) [9]. NO production depends on the availability of its precursor arginine and activity of various NOS isoforms. Arginase hydrolyzes arginine to ornithine and urea and can potentially control NO production. Thus, activation of iNOS and/or arginase determines the type of inflammatory response in the body [6].

We studied the effect of water-soluble polysaccharides isolated from the leaves of the *Crataegus sanguinea* Pall. (*Rosaceae* family) on the NOS/arginase balance in the experiment.

[1]E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk National Research Medical Center, Russian Academy of Sciences, Tomsk, Russia; [2]Novosibirsk State Medical University, Ministry of the Health of the Russian Federation, Novosibirsk, Russia; [3]Bashkir State Medical University, Ministry of the Health of the Russian Federation, Ufa, Republic of Bashkortostan, Russia. ***Address for correspondence:*** mvb63@mail.ru. M. V. Belousov

MATERIALS AND METHODS

Peritoneal macrophages and lymphoid cells were isolated from certified female C57BL/6 mice (n=40, age 6-8 weeks, body weight 18-22 g) obtained from the Department of Experimental Biological Models, E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine. All experimental manipulations were performed in accordance with the Directive 2010/63/EC of the European Parliament and of the Council (On the Protection of Animals Used for Scientific Purposes; September 22, 2010), and GOST 33216-2014 "Guidelines for the Maintenance and Care of Laboratory Animals".

Water-soluble polysaccharides (WSPS) used in the experiments were isolated at the Department of Pharmaceutical Analysis, Siberian State Medical University from the leaves of the *Crataegus sanguinea* by water extraction, followed by filtration, dialysis and freeze drying. The studied compound is a polysaccharide complex with a protein admixture (9.03±0.11%); the content of uronic acids, in terms of galacturonic acid, according to spectrophotometry data is 17.71±0.23%. WSPS contains major components galactose, glucose, galacturonic acid, mannose, and arabinose and a minor monosaccharide xylose. Analysis of molecular mass distribution by high-performance size-exclusion chro-

matography showed that the complex of the WSPS of *Crataegus sanguinea* leaves has a wide mass distribution of polysaccharides from 1215 kDa (main fraction 60.84%) to 9.5 kDa (23.97%) [2]. The studied WSPS was dissolved in complete culture medium (CCM) of the following composition: RPMI-1640 (Sigma-Aldrich), 10% fetal bovine serum (HyClone), 20 mM HEPES (Sigma-Aldrich), 0.05 mM 2-mercaptoethanol (Sigma-Aldrich), 50 μg/ml gentamicin (Sigma-Aldrich), and 2 mM L-glutamine (Sigma-Aldrich) and added to the cell culture in a concentration of 20 or 40 μg/ml.

Mouse peritoneal macrophages (MP) were isolated by washing the abdominal cavity of mice with ice-cold sodium chloride solution. Mature MP were isolated from the cell suspension using the EasySep Biotin Positive Selection Kit and Anti-Mouse F4/80 Antibody (Stem Cell). Peritoneal MP ($2.5\text{-}3.0\times10^6$ cells/ml) were cultured in CCM in 96-well flat-bottom plates for 48 h at 37°C, 5% CO_2, absolute humidity with the addition of WSPS from *Crataegus sanguinea* leaves in different concentrations or with standard MP activator LPS of *E. coli* (serotype O111:B4, Sigma-Aldrich). When studying the proliferative activity of peritoneal MP, the cells were cultured for 72 h under the above conditions.

Mononuclear cells (MNC) isolated from suspensions of splenic cells on a Histopaque-1077 gradient (Sigma-Aldrich) were cultured in CCM for 72 h in round-bottom plates (2×10^6 cells/ml) at 37°C, 5% CO_2, absolute humidity with the addition of WSPS from *Crataegus sanguinea* leaves in different concentrations, or with the addition of concanavalin A (Con A, 4 μg/ml; Sigma-Aldrich). Then, the proliferative activity of MNC was studied.

NO production was evaluated by the content of nitrites in the supernatants of MP cultures using Grace reagent [3]. The reagent (0.1 ml) was mixed with an equivalent volume of supernatant, the absorption was measured on a Titertek Multiskan MCC multichannel spectrophotometer (LabSystems) at 540 nm. The concentration of nitrites was determined by a calibration curve constructed using standard solutions of sodium nitrite.

Arginase activity in lysate of peritoneal MP was assayed by the method of M. Munder, *et al.*, (1998) in our modification by measuring urea concentration Urea-450 test system (Bio-LA-Test) on a Titertek Multiskan MCC multichannel spectrophotometer at 540 nm [1,5]. The amount of arginase catalyzing the formation of 1 μM urea per minute was taken as 1 unit of activity (U) of the enzyme.

To detect possible endotoxin impurity in the studied WSPS samples, the polysaccharide samples or 1 μg/ml LPS (as a method control) and polymyxin B (InvivoGen) with a concentration of 10 μg/ml were placed in a 96-well flat-bottom plate, and incubated in CCM at 37°C, 5% CO_2, and absolute humidity for 1 h. Then, peritoneal MP ($2.5\text{-}3.0\times10^6$ cells/ml) were added to the wells, cultured for 48 h under the above conditions, the supernatant was collected from the wells and the concentration of nitrites was measured.

The proliferation of peritoneal MP and MNC was evaluated by the colorimetric MTT method (Sigma-Aldrich); the sediment was dissolved with DMSO (Sigma-Aldrich) after incubation cells with the studied substances [4]. The absorption of the resulting solutions was measured on a Titertek Multiskan MCC multichannel spectrophotometer at 540 nm. Proliferative activity of cells was expressed in optical density units.

The experimental data were processed using Statistica 8.0 software (StatSoft, Inc.). For each sample, the arithmetic mean (X), error of the mean (m), and mean deviation (σ) were calculated. The normality of the distribution was checked using the Shapiro—Wilk test. Comparison of sample means was carried out according to the Dunnett's test for comparing several experimental samples with one control in case of a normal distribution or according to the Kruskal—Wallis test for k-unrelated samples ($k>2$) and Dunn's criterion in case of distribution that differs from the normal.

RESULTS

The effect of WSPS from leaves of *Crataegus sanguinea* Pall. on the NOS/arginase balance was assessed using two concentrations of polysaccharides: 20 and 40 μg/ml (Table 1). LPS, a standard activator of MP (control 2), increased NO production by 17.3 times. The studied WSPS in concentrations of 20 and 40 μg/ml increased nitrite production by 9.5 and 17 times, respectively. However, the revealed stimulating effect of WSPS in the lowest dose (20 μg/ml) was inferior to LPS activation of MP. Arginase activity in peritoneal MP of experimental animals after addition of LPS and WSPS significantly decreased (Table 1). However, arginase activity after incubation with WSPS was significantly higher than after LPS stimulation.

It is known that plant polysaccharides often include impurities of endotoxin LPS, a structural component of the membranes of gram-negative bacteria [7]. In general, endotoxin is not toxic, but its presence in injectable drugs is extremely undesirable, because it can trigger of a cascade reaction, endotoxic shock, and even death. Therefore, the proven absence of such impurities in pharmacological substances gives significant advantages for the further study and development of drugs.

To identify the endotoxin impurity in WSPS of *Crataegus sanguinea* Pall., it was used in a concen-

TABLE 1. Effect of WSPS Isolated from *Crataegus sanguinea* Pall. on NO Production and Arginase Activity in Peritoneal MP of Intact C57BL/6 mice (*X*±*m*; *n*=6)

Test substance	Concentration, μg/ml	Concentration of nitrites, μM	Arginase activity, activity units
CCM (control 1)	—	2.17±0.13	31.08±0.38
LPS (control 2)	1	37.49±1.18*	6.05±0.18*
WSPS	20	20.70±0.69*+	8.09±0.37*+
	40	36.96±0.99*	—

Note. $p<0.05$ in comparison with *control 1, +control 2.

TABLE 2. Effect of WSPS Isolated from *Crataegus sanguinea* Pall. on NO Production by Peritoneal MP of Intact C57BL/6 Mice in the Absence and Presence of Polymyxin B (*X*±*m*; *n*=6)

Test substance	Concentration, μg/ml	Concentration of nitrites, μM	
		without polymyxin B	with polymyxin B
CCM (control 1)	—	2.17±0.13	2.17±0.11
LPS (control 2)	1	37.49±1.18*	17.05±0.74*o
WSPS	20	20.70±0.69*+	18.26±1.05*

Note. $p<0.05$ in comparison with *control 1, +control 2, owithout polymyxin B.

TABLE 3. Effect of WSPS Isolated from *Crataegus sanguinea* Pall. on the Proliferation of Immunocompetent Cells of Intact C57BL/6 Mice (*X*±*m*; *n*=6)

Test substance	Concentration, μg/ml	Proliferation, optical density units	
		MP	MNC
CCM (control 1)	—	0.438±0.009	—
LPS (control 2)	1	0.396±0.006	—
WSPS	20	0.342±0.011*	—
	40	0.319±0.015*+	—
CCM (control 1)	—	—	0.159±0.007
Con A (control 2 MNC)	4	—	0.264±0.002*
WSPS	20	—	0.196±0.110*+
	40	—	0.194±0.021*+

Note. $p<0.05$ in comparison with *control 1, +control 2.

tration of 20 μg/ml. Addition of polymyxin B did not affect intact cells and significantly (by 2.2 times) reduced NO production when stimulating MP by LPS (Table 2). At the same time, the use of polymyxin B did not affect the NO-producing properties of WSPS isolated from the *Crataegus sanguinea* Pall.

The addition of the WSPS in concentrations of 20 and 40 μg/ml significantly reduced proliferation of peritoneal MP. Incubation with 40 μg/ml WSPS significantly decreased proliferative activity of MP relative to LPS-stimulated control (Table 3).

After culturing with WSPS isolated from *Crataegus sanguinea* Pall. in both concentrations, proliferation of MNC significantly increased relative to the intact control, but remained lower than in the group with Con A stimulation (Table 3).

Thus, the studied WSPS suppressed proliferation of MP, but stimulated division of MNC, but less efficiently than the standard mitogen.

Our screening study of immunotropic activity of WSPS isolated from the leaves of the *Crataegus sanguinea* Pall. revealed their significant NO-stimulating properties that did not depend on endotoxin impurities. This effect also did not depend on increased proliferation of MP, but was due to activation of nitrite secretion, probably due to increased production of

Th1 cytokines. Hence, the revealed effects indicate the ability of WSPS isolated from the *Crataegus sanguinea* Pall. to activate antigen-presenting cells by the classical pathway due to stimulation of NOS activity and decrease in arginase expression.

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