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Study of Immunotropic Properties of Water-Soluble Polysaccharides Isolated from *Conium maculatum* Grass

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Addition of water-soluble polysaccharides isolated from *Conium maculatum* L. to the mouse peritoneal macrophage culture induces classical activation of antigen-presenting cells due to an increase in NO synthase activity and a decrease in arginase expression.

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

Polysaccharides of the bacterial wall can bind to pattern-recognizing structures of macrophages [7]. It has been shown that polysaccharides of microorganisms, fungi, and higher plants exhibit immunomodulating properties due to their ability to change the functional state of antigen-presenting cells (macrophages and dendritic cells) [3,4,6]. The immunomodulatory effect of plant polysaccharides on macrophages mainly consists in stimulation of ROS generation, cytokine secretion, cell proliferation, and phagocytic activity [11,14]. Nitric oxide (NO) is a neurotransmitter involved in numerous physiological and pathological processes of various systems, including immune responses and inflammation [15]. In the immune system, stimulation and activation of macrophages is accompanied by massive release of NO that can kill microorganisms, parasites, and tumor cells; it can also cause inflammatory reactions and protect the body from adverse external factors [12,13].

Our aim was to study the effect of water-soluble polysaccharides isolated from *Conium maculatum* L. grass of the *Apiaceae* family on NO production by peritoneal macrophages (MP).

MATERIALS AND METHODS

Peritoneal MP and lymphoid cells were isolated from female C57BL/6 mice (n=40) aged 6-8 weeks weighing 18-22 g (certified 1st category mice; Department of Experimental Biological Models, E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine). All manipulations were carried out in accordance with the Directive 2010/63/EU of the European Parliament and Council (On the Protection of Animals used for Scientific Purposes; September 22, 2010) as well as GOST 33216-2014 Guidelines for the Maintenance and Care of Laboratory Animals.

We used water-soluble polysaccharides (WSPS) isolated from *Conium maculatum* L. grass at the Department of Pharmaceutical Analysis of the Siberian State Medical University by water extraction, followed by filtration, dialysis, and freeze drying. The studied WSPS represented a polysaccharide complex with an admixture of proteins (5.74±0.06%); the content of uronic acids, in terms of galacturonic acid, according to the spectrophotometry results was 20.5±0.3%. WSPS included major components — galactose, glucose and arabinose and a minor monosaccharide — mannose. Analysis of the molecular mass distribution by size-exclusion HPLS showed that the WSPS com-

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plex of *Conium maculatum* L. consists of two polysaccharide fractions with molecular weights of 1359 and 9.5 kDa with a relative content of 59.34 and 39.79%, respectively [1].

WSPS of the *Conium maculatum* L. grass was dissolved in complete culture medium (CCM) consisting of RPMI-1640 (Sigma) 10% fetal calf serum (HyClone), 20 mM HEPES (Sigma), 0.05 mM 2-mercaptoethanol (Sigma), 50 μg/ml gentamicin (Sigma), and 2 mM L-glutamine (Sigma) and added to the cell culture in concentrations of 2, 20, and 40 μg/ml.

Peritoneal MP were isolated by washing the abdominal cavity with ice-cold NaCl solution. Mature MP were isolated from the resulting cell suspension using EasySep Biotin Positive Selection Kit and antibodies specific to mouse F4/80 MP receptor (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, and absolute humidity in the presence of different concentrations of WSPS or standard MP activator LPS of *E. coli* (serotype O111: B4, Sigma). When studying proliferative activity of peritoneal MP, the cells were cultured for 72 h under the specified conditions.

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

NO production was assessed by the content of nitrites in MP supernatants using Grace reagent [5]. The reagent (0.1 ml) was mixed with an equivalent volume of the supernatant and absorption was measured on a Titertek Multiskan MCC multichannel spectrophotometer (LabSystems) at λ=540 nm. The concentration of nitrites was determined by a calibration curve constructed using standard solutions of sodium nitrite.

Arginase activity was measured by the method [9] in our modification [2] in the lysate of peritoneal MP by the concentration of urea using Urea-450 test system (Bio-LA-Test) according to the protocol attached to the test system using a Titertek Multiskan multichannel spectrophotometer MCC (LabSystems) at λ=540 nm [2,9]. The amount of arginase catalyzing the formation of 1 μmol urea per minute was taken as 1 unit of enzyme activity (U).

To detect possible endotoxin impurity in the studied polysaccharide samples, the polysaccharide samples or 1 μg/ml LPS (control of the method) and polymyxin B (10 μg/ml, InvivoGen) were placed in a 96-well flat-bottom plate, 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, and the concentration of nitrites in the supernatant collected from the wells was measured.

Proliferation of peritoneal MP and mononuclear cells was evaluated using the colorimetric method with MTT (Sigma) by dissolving the precipitate in DMSO (Sigma) after incubation of cells with the studied substances [8]. Absorption was measured on a Titertek Multiskan MCC multichannel spectrophotometer (LabSystems) at λ=540 nm. The values of the proliferative activity of cells were expressed in of optical density units.

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

RESULTS

The NO-activating properties of WSPS of the *Conium maculatum* L. grass were evaluated in two series of experiments using 3 polysaccharide concentrations — 2, 20, and 40 μg/ml (Fig. 1). LPS, a standard activator of MP, increased NO production by 6.4 and 17.3 times. WSPS in concentrations of 2, 20, and 40 μg/ml increased nitrites production by 3.8, 8.8, and 14 times, respectively. However, their stimulating effect was significantly lower than LPS-induced activation of MP.

It is known that PS of plant origin often include impurities of endotoxin — LPS, a structural component of the bacterial wall of gram-negative bacteria

TABLE 1. Effect of WSPS Isolated from *Conium maculatum* L. Grass on the Production of NO by Peritoneal MP of Intact C57BL/6 Mice in the Absence and Presence of Polymyxin B (X±*m*)

Experimental conditions	Concentration of nitrites, μM	
	without polymyxin B	with polymyxin B
CCM	4.37±0.15	3.13±0.13
LSP, 1 μg/ml	28.17±0.47*	7.45±0.28*°
WSPS, 20 μg/ml	41.25±0.59*+	43.57±0.52*+

Note. $p<0.05$ in comparison with *CCM, +LPS, and °samples without polymyxin B.

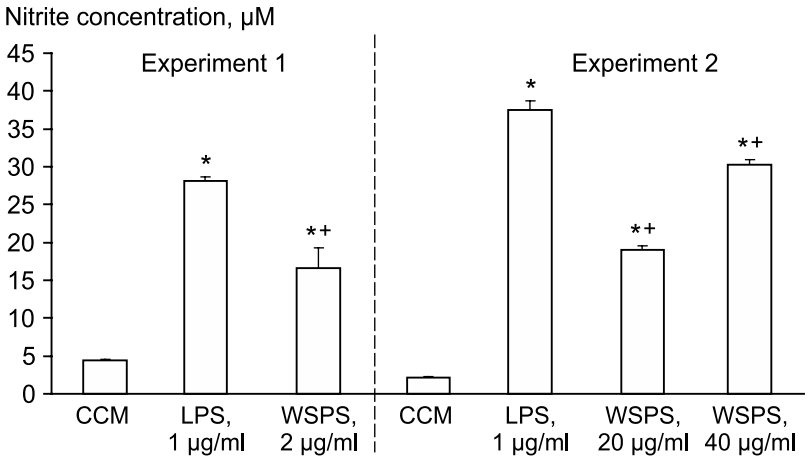

Fig. 1. Effect of WSPS isolated from *Conium maculatum* L. grass on NO production by peritoneal MP of intact C57BL/6 mice. $p<0.05$ in comparison with *CCM, $^{+}$LPS. $n=6$ in all cases.

TABLE 2. Effect of WSPS Isolated from *Conium maculatum* L. Grass on Arginine Metabolism in Peritoneal MP of Intact C57BL/6 Mice (X±*m*)

Experimental conditions	Concentration of nitrites, μM	Arginase activity, U
CCM	1.64±0.09	31.08±0.38
LPS, 1 μg/ml	3.98±0.13*	6.05±0.18*
WSPS, 40 μg/ml	5.70±0.11*+	5.43±0.38*

Note. $p<0.05$ in comparison with *CCM, $^{+}$LPS. $n=6$ (NO) and $n=12$ (arginase).

[10]. In general, endotoxin is not a toxic substance, but its presence in injectable drugs is extremely undesirable, because it can lead to the development of a cascade reaction — endotoxic shock and even death. Therefore, the proven absence of such impurities during analysis of various pharmacological effects provides significant advantages for the further study and development of drugs.

To identify endotoxin impurity in the polysaccharide complexes of *Conium maculatum* L., the mean working concentration of WSPS was used. The addition of polymyxin B did not affect intact cells and significantly (by 3.8 times) reduced NO production by LPS-stimulated MP (Table 1). At the same time, polymyxin B did not affect NO-production by WSPS-stimulated cells.

In supernatants of intact peritoneal MP, low concentration of NO was detected, while cell lysates showed high arginase activity (Table 2). Incubation of MP with LPS and WSPS led to a significant increase in NO production and a decrease in arginase activity in comparison with intact MP by 5.1 and 5.7 times, respectively.

Addition of WSPS in a concentration of 20 μg/ml did not affect proliferation of peritoneal MP. A significant increase of proliferative activity of MP (by 1.2 times relative to the LPS-stimulated control) was observed after addition of WSPS in the lower concentration (2 μg/ml).

Culturing of mononuclear leukocytes with WSPS in a concentration of 20 μg/ml significantly (by 1.2 times) increased proliferation of mononuclear cells relative to the intact control, that, however, was not detected during incubation with WSPS in the higher concentration (40 μg/ml) (Table 3). At the same time, proliferative activity of lymphoid cells in the presence of WSPS in a dose of 20 and 40 μg/ml was statistically lower than in the presence of Con A (by 1.3 and 1.7 times, respectively). Thus, the studied polysaccharides produced no toxic effects on the immune system cells.

Our screening study of the immunotropic activity of water-soluble polysaccharides from *Conium maculatum* L. grass revealed their significant NO-stimulating properties that did not depend on endotoxin

TABLE 3. Effect of WSPS Isolated from *Conium maculatum* L. Grass on Proliferation of Immunocompetent Cells of Intact C57BL/6 Mice ($n=6$; $X±m$)

Experimental conditions	Proliferation (optical density), arb. units	
	MP	mononuclears
CCM	0.458±0.005	
LPS, 1 μg/ml	0.416±0.010	
WSPS, 2 μg/ml	0.498±0.018+	
WSPS, 20 μg/ml	0.431±0.015	
CCM		0.159±0.007
Con A A, 4 μg/ml		0.264±0.002*
WSPS, 20 μg/ml		0.197±0.008*o
WSPS, 40 μg/ml		0.158±0.008o

Note. $p<0.05$ in comparison with *CCM, $^{+}$LPS, and ^{o}Con A.

admixture. This effect did not depend on activation of cell proliferation, but was due to activation of nitrite secretion.

Thus, WSPS isolated from the *Conium maculatum* L. can induce activation of antigen-presenting cells by the classical pathway due to stimulation of NO-synthase activity and decrease in arginase expression.

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