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In-vitro Effects of Bacterial Melanin in Macrophage "RAW 264.7" Cell Culture

T.R. Petrosyan[a,*], A.S. Hovsepyan[b], S.V. Avetisyan[b] and N.K. Kurian[c]
[a]*Yerevan State Medical University, Yerevan, Armenia*
[b]*SPC "Armbiotechnology" SNPO NAS, Republic of Armenia*
[c]*Cochin University of Science and Technology, Cochin, India*

Abstract. Biotechnologically obtained bacterial melanin (BM) has been extensively studied as a potential pharmacological preparation with neuroprotective and anti-inflammatory effects. Anti-inflammatory action of BM was tested in animal model of induced encephalomyelitis. The goal of presented research was to examine anti-inflammatory potential of BM in lipopolysaccharide-stimulated murine RAW 264.7 cells. The cells were treated with different concentrations of BM (from 6 mg/ml to 4, 2 and 1 mg/ml) and incubated for 20 hours. Results were compared with data obtained from vehicle control treated cells. Two tailed *t*-test was used to evaluate the results. The obtained data showed that BM reduces the production of nitric oxide and prostaglandin E2. The substance suppresses production of pro-inflammatory cytokines including interleukin (IL)-6 and IL-1b.

The results revealed that BM has anti-inflammatory activity and has a potential to suppress neuroinflammation. Brain macrophages-are the only cells that mediate brain inflammation. Extracellular neuromelanin can activate the CNS microglia inducing neuroinflammation and neurodegeneration. The water-soluble biotechnological melanin does not activate microglia – the resident brain macrophages.

Keywords: Bacterial melanin, inflammation, level of cytokines, nitric oxide, cyclooxygenase

INTRODUCTION

Treatment strategies for the neurodegenerative disorders that are used by clinicians apply a number of neuroprotective agents, with different structure and different mechanism of action. Majority of the offered strategies are aimed to support the cell survival, accelerate posttraumatic recovery of CNS functions and suppress neuroimflammation. Inflammation is the response of the body against cell injury caused by irritants and pathogens. Macrophages and T cells are involved in the inflammation process, as well as inflammatory mediators including prostaglandins (PG), pro-inflammatory cytokines and nitric oxide (NO) [1]. Persisting chronic influence of agents or irritants may result in inflammatory diseases. Activated macrophages up-regulate inducible nitric oxide production (iNOS) and cyclooxygenase 2 (COX-2) that results in increased NO and PGE2 production [2, 3]. Macrophages have a main role in immune response initiation against different agents, and using different pathways.

A group of studies has confirmed neuroprotective action of melanocyte-stimulating-hormone on locomotor recovery following CNS lesion [4–6]. Currently melanins of different origin are being thoroughly studied and used as medicinal or cosmetic preparations.

Melanins are multicolored pigments that have a polymer structure. They inhibit free radical chain

*Correspondence to: T.R. Petrosyan, 6 Orbeli Street, 88. Yerevan, Armenia. Tel.: +374 93734579; E-mail: tigpetrosyan@mail.ru.

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reactions and possess a significant antioxidant action. These unique properties of melanin explain its role in tissues and organs connected with energy transmission, including skin, retina, inner ear and nervous system. Disorders of melanin metabolism can be involved in the etiology of Parkinson's disease, senile macular degeneration, and senile deafness [7, 8]. The disturbances in melanin metabolism are also relevant to the well-known association between pigmentary abnormalities and deafness (Warrensburg's and Usher's syndromes). Research evidence indicates that the Alzheimer disease and Down syndrome are accompanied with pathochemical changes in melanin metabolism [9].

The majority of synthetic and known natural melanins are macromolecules and insoluble in water making significantly difficult the production of pharmacological and cosmetic preparations. Obtaining a soluble natural melanin with a low cost of production can essentially stimulate and accelerate the implementation of melanin in pharmacotherapy, cosmetology and other fields.

For the first time melanin-synthesizing strain with high level of pigment synthesis – Bacillus thuringiensis was obtained. The ecologically safe technology of biosynthesis, isolation and purification of the bacterial melanin (BM) has been elaborated. High biological activity of melanin was shown both on animals and plants [10–15]. BM and its metabolites cross the blood–brain barrier [16]. BM shows higher Cmax after intramuscular (i/m) injection, while a long retention was registered after intraperitoneal (i/p) injection. In the experiments on laboratory animals (white rats) with brain surgical trauma it was shown that BM facilitated the recovery of instrumental conditioned reflexes after unilateral ablation of sensorimotor cortex that resulted in paresis of limbs. Low doses of BM stimulate the recovery of motor functions lost because of nervous tissue damage [14].

In previously conducted *in-vivo* study we have demonstrated the ani-inflammatory action of BM in artificially induced rat model of encephalomyelitis [17]. The purpose of the present study is to analyze effects of water soluble bacterial melanin on the level of pro-inflammatory markers in macrophage cell culture. In the experimental model of autoimmune encephalomyelitis BM was shown to have an anti-inflammatory action, proved by faster motor recovery registered in melanin treated rats. The selected concentration (6 mg/ml, calculated as 170 mg/kg) has been successfully applied in all previous studies with animal models to stimulate the recovery of altered motor functions in rats after destruction of various CNS structures that are responsible for motor behavior [18, 19].

MATERIALS AND METHODS

Cell culture

RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (Himedia, India) supplemented with 10% fetal bovine serum (Himedia, India), 100 μg/mL streptomycin, 100 U/mL penicillin (Himedia, India) at 37°C and 5% CO2/95% humidified air in a CO_2 incubator (Eppendorf, Germany).

Bacterial melanin solution

The melanin-synthesizing strain Bacillus thuringiensis subsp. galleriae K1 (Deposited number INMIA 11212) was obtained from the strain B. thuringiensis 69-6 as a result of a chemically induced mutagenesis and multistage selection [20]. Bacterial melanin was purified to prevent protein contamination [21]. Four different concentrations of bacterial melanin were used in the study: 1, 2, 4 and 6 mg/ml. The highest concentration – 6 mg/ml (at the rate of 170μg/gr) has been applied in all *in-vivo* studies of the BM project. For the treatment of the cell culture we have applied the same principle of dosage calculation, splitting the concentration from 6 mg/ml to 4, 2 and 1 mg/ml, as the bioavailability of BM in the CNS is significantly high [16]. Dimethylsulfoxide (DMSO) was used as a vehicle for BM.

Cytotoxicity assay

RAW 264.7 cells were placed in 96-well plate at a density of 5×104 cells/100 μl per well and were treated with 1, 2, 4 and 6 μg/mL BM for 4 h and were rinsed with phosphate buffered saline (PBS). After rinsing, cells were incubated with and without LPS (1 μg/mL). After 20 h, 10 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich, USA) was added and the plates were incubated for 5 h at 37 C in a 5% CO_2 incubator. After 100 μl of 0.04 N HCl in isopropanol were added to dissolve formazan crystals. The optical density was measured at 540 nm to quantify viable cells.

Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells were placed in 24-well plate at a density of 3 9 105 cells/500 μl per well and were treated with BM, followed by 1μg/mL LPS stimulation. The collected culture supernatant was used to measure the production of IL- 6, IL-1b, and TNF-a. Each microplate well was coated overnight at 4°C, with purified rat anti-mouse IL-6 antibody (Himedia, India), purified Armenian hamster anti-mouse/rat IL-1b antibody (Himedia, India), and purified Armenian hamster anti-mouse/rat TNF-a antibody (Himedia, India) in coating buffer. Samples were loaded in each well after washing and blocking with PBS containing 3% bovine serum albumin (Himedia, India), and incubated overnight at 4°C. The plate was washed and incubated with biotinylated anti-mouse IL-6 antibody (Himedia, India), IL-1b antibody (Himedia, India), and TNF-a antibody (Himedia, India) for 30 min at room temperature (RT). After incubation, the plate was washed and incubated with streptavidin–alkaline phosphatase (Himedia, India) for 20 min at room temperature. After the plate was washed and phosphatase substrate, p-nitrophenyl phosphate (Sigma-Aldrich) was added. The plate was incubated for 10 min incubation and the optical density of contents was measured at 405 nm. The level of cytokine production was quantified using a standard reference curve based on recombinant murine IL-6 (Peprotech, Rocky Hill, NJ, USA), IL-1b, and TNF-a. To measure PGE2 production, PGE2 ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA) was applied according to the manufacturer's instructions.

Immunoblotting analysis

RAW 264.7 cells were placed in 6-well plate at a density of 2×106 cells/2 mL per well and were treated with BM for 4 h, followed by 1 μg/ml LPS stimulation for 15 min. To test the activation STAT3, cells were stimulated with 10 ng/mL IL-6 for 30 min. Cells were lysed in RIPA lysis and extraction buffer (Sigma-Aldrich, USA) and subsequently incubated on ice for 15 min. Samples were centrifuged at 12,000 rpm for 15 min at 4°C and obtained supernatants were placed into clean microtubes. Protein concentration was measured by Bicinchoninic Acid Kit for Protein Determination (Sigma-Aldrich, USA) following the manufacturer's instructions. The obtained cell lysates were transferred to a 10% sodium dodecyl sulfate–polyacrylamide gel and placed on Immune-Blot_PVDF Membrane for Protein Blotting (Sigma-Aldrich, USA). Tris-buffered saline containing 0.1% Tween 20 and 5% BSA, was used to block the Membrane. After blocking, the membrane was incubated overnight at 4°C with primary antibody and with horse radish peroxidase conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich, USA) for 1 h at room temperature. The bands of proteins were visualized by West-Q Pico ECL solution (GenDEPOT, Barker, TX, USA), and detected by a densitometer. Quantity One software (Bio-Rad) was used to quantify the proteins.

Statistical analysis

Results are presented in the form of means and standard deviations (S.D.). Two-tailed Student's *t* test was used to assess differences between experimental conditions. Results are considered significantly different when $p < 0.05$.

RESULTS

Cytotoxicity of Bacterial melanin

MTT assay was used to evaluate the cytotoxic influence of BM in RAW 264.7 cells. The BM showed no cytotoxic action at concentrations up to six μg/ml when compared to DMSO control both in the presence or absence of LPS (Fig. 1). The 6μg/mL was the concentration used in all previous *in vivo* studies. However none of the 4 tested concentrations of BM demonstrated cytotoxic effects in RAW 264.7 cells, irrespective of LPS. Therefore, the same range of concentrations (1, 2, 4, 6) was used for all testings of BM included in subsequent experiments.

Anti-inflammatory activity of bacterial melanin

Inhibition of iNOS expression

iNOS expression in macrophages during inflammatory process results in the production of NO, the major mediator of inflammation [3]. Western blot analysis was performed to measure iNOS protein expression, respectively. The results showed that BM markedly reduced the level of iNOS upon LPS-stimulation in RAW264.7 cells in a dose dependent manner (Fig. 2).

Inhibition of COX-2 and PGE2 production

COX-2 induced by LPS or cytokine stimulation, synthesizes PG from arachidonic acid. PGE2 is a

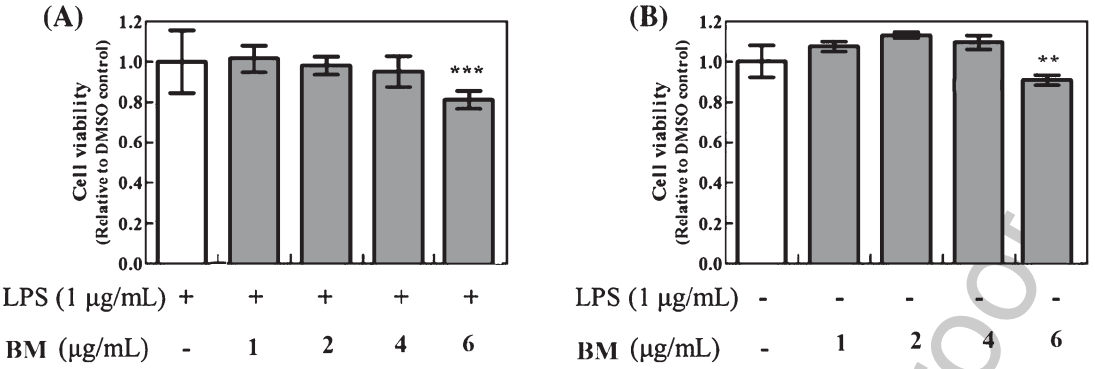

Fig. 1. Cytotoxicity of BM. RAW 264.7 cells were treated with 1, 2, 4, and 6 μg/mL BM for 4 h and then stimulated with (A) or without (B) LPS (1 μg/mL) for 20 h.5 Cell viability was measured by MTT assay, and presented compared to DMSO control. The data are representative of three experiments with similar results, and expressed as mean ± S.D. $^{**}p < 0.01$, and $^{***}p < 0.001$.

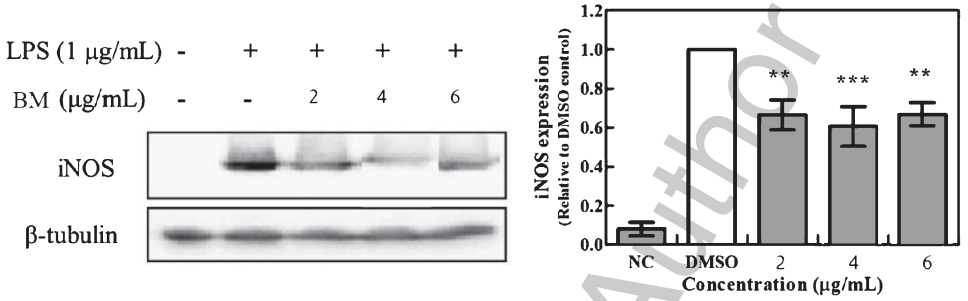

Fig. 2. Inhibition of iNOS expression. RAW 264.7 cells were treated with BM for 4 h, and incubated with BM for 20 h. Total cell lysates were subjected to western blot analysis. Expression level of iNOS protein was normalized by b-tubulin. The data are presented as the mean ± S.D. of three representative experiments with similar results. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

potent mediator of inflammation and is well known to induce fever [22]. The Fig. 3 presents the BM influence on COX-2 production. The treatment of cells with concentration of BM - 4μlg/mL significantly inhibited the expression of COX-2 protein levels compared to DMSO control. Consequently, PGE2 production was also decreased by BM treatment at a concentration of 4 μg/mL (Fig. 4).

LPS influence stimulated the production of IL-6, IL-1b, and TNF-a, and the increase in IL-6 and IL-1b was repressed by BM in a dose dependent manner. The highest efficacy had the concentration 4 mg/mL (Fig. 4). TNF-a production was slightly affected by BM and only the highest concentration of 6 mg/m significantly suppressed the TNF-a activity (Fig. 3C).

Inhibitory effect of BM on ERK and STAT3 signaling pathways

To test the inhibitory influence of BM on intracellular signaling pathways, phosphorylation of MAPKs: extracellular signal-regulated kinase (ERK), p38, and STAT3, (essential mediator of inflammatory signaling pathway) was evaluated in BM treated RAW 264.7 cells. The Fig. 5 shows that the ratios of phosphorylated ERK (p-ERK) to ERK was decreased by BM treatment, indicating the anti-inflammatory effect of BM due to suppression of ERK phosphorylation following the LPS stimulation. BM treatment did not affect the phosphorylation of p38, (Fig. 5 A), BM reduced the phosphorylation of STAT3 (Fig. 5B) without changing the total STAT3 protein levels in response to stimulation with IL-6. The data suggest that immunomodulatory action of BM is due to inhibition of MAPK activity (ERK and STAT3).

Decrease in COX-2 and PGE2 expression

RAW 264.7 cells were treated with BM for 4 h, and incubated with LPS. After 20 h incubation COX-2 protein expression (B), and PGE2 production (C) were tested. Experimental values were presented as the relative value to that of DMSO control. COX-2 protein was normalized by b-tubulin. Each data is representative of three experiments with similar results.

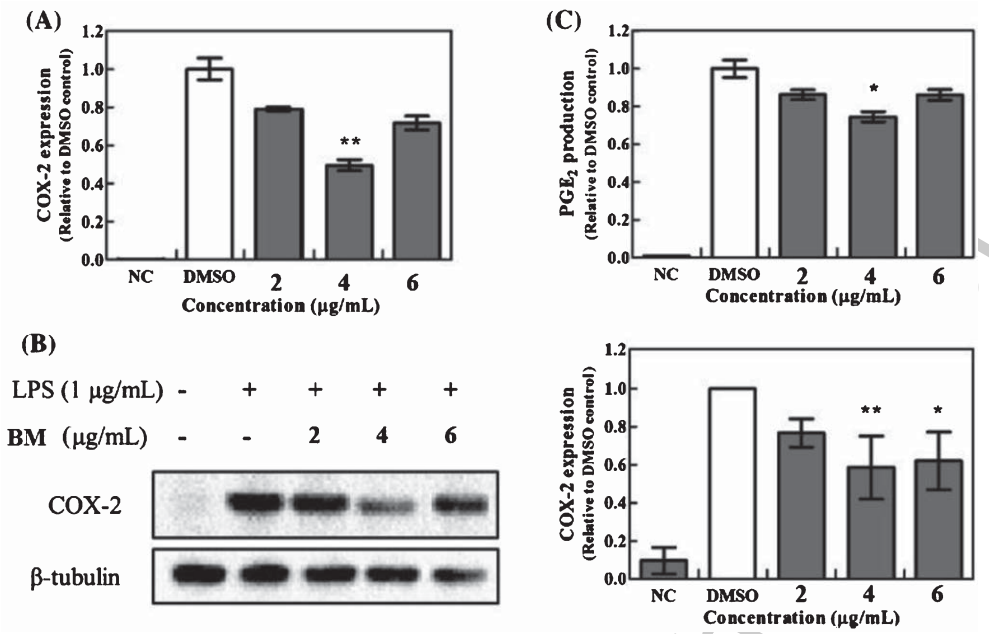

Fig. 3. Decrease in COX-2 and PGE2 expression. RAW 264.7 cells were treated with BM for 4 h, and incubated with LPS. After 20 h incubation, COX-2 mRNA expression (A), COX-2 protein expression (B), and PGE2 production (C) were determined. Experimental values were presented as the relative value to that of DMSO control. Expression level of COX-2 mRNA was normalized by b-actin and COX-2 protein was normalized by b-tubulin. Each data is representative of three experiments with similar results. Data are expressed as the mean ± S.D. $*p < 0.05$ and $**p < 0.01$.

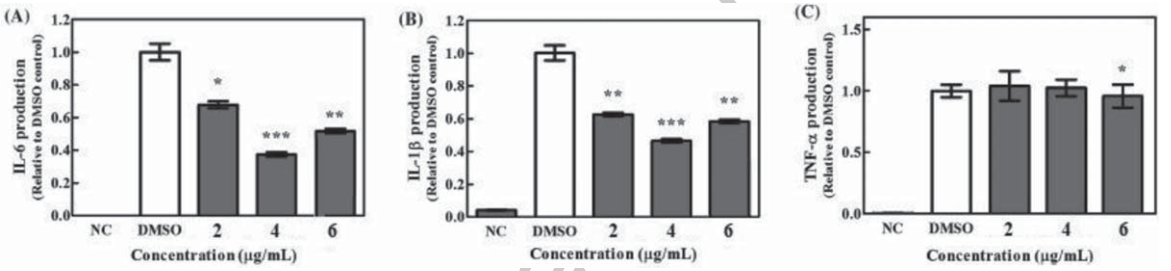

Fig. 4. Inhibition of pro-inflammatory cytokine in RAW 264.7 cells treated with BM for 4 h, and incubated with LPS for 20 h. The production of IL-6 (A), IL-1b, (B) and TNF-a (C) in the supernatant was measured by ELISA. Data are representative of four experiments with similar results, and expressed as the mean ± S.D. $*p < 05$, $**p < 0.01$, and $***p < 0.001$.

Data are expressed as the mean and standard deviation ($*p < 0.05$ and $**p < 01$).

DISCUSSION

In inflammation process activation of macrophage is initiated by a group of transcription factors mediated by MAPK pathways [23]. In macrophages activation of TLR is via the external stimuli such as LPS. The main MAPK pathways are the ERK and p38 pathways. Each of these pathways is activated through sequential phosphorylation following the external stimulation. Activated MAPKs are able phosphorylate downstream targets such as protein kinases and transcription factors, facilitating the transcription of MAPK-regulated genes. This chain leads to the release of pro-inflammatory mediators (NO, PGE2, TNF-a, IL-6, and IL-1b) in activated macrophages [24, 25]. These pro-inflammatory cytokines have specific role in the signaling process and initiating the inflammation. Excessive production of cytokines can cause cell injury and cancer, representing new stimuli for the inflammatory process [26].

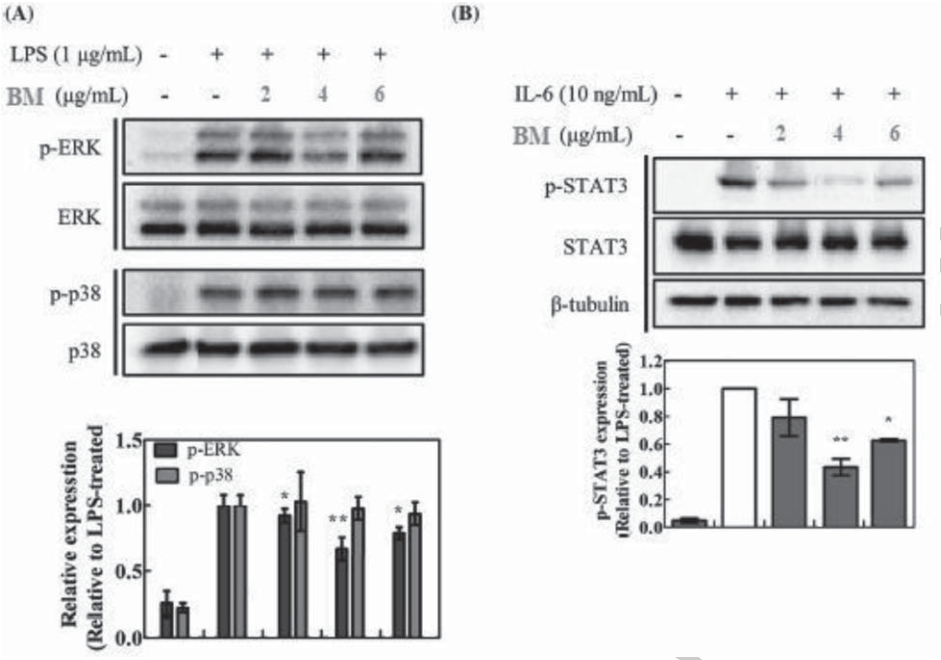

Fig. 5. Effect of BM on the activation of ERK and STAT3. RAW 264.7 cells were treated with 2, 4, and 6 lg/mL BM for 4 h, and incubated with one μg/mL of LPS for 15 minutes. Total cell lysates were subjected to immunoblotting analysis. For STAT3 analysis, cells were treated with 10 ng/mL of IL-6 for 30 min and cell lysates were prepared. p-ERK, ERK, p-p38, p38, and p-STAT3, STAT3 levels were determined by western blot analysis. Expression of p-STAT3 was normalized by total MAPK and total STAT3 expression level. Three independent experiments were performed. The quantified intensity of protein bands from western blots is presented as bar graphs. Data are expressed as the mean ± S.D. $*p < 0.05$.

In the presented study, BM suppressed LPS-induced iNOS and COX-2 protein expression in RAW 264.7 cells resulting in decreased NO and PGE2 production. The production of IL-6 and IL-1b was also decreased by BM treatment, showing insignificant suppression of TNF-a activity.

The immunoblotting analysis revealed that BM suppressed STAT3 phosphorylation, decreased expression of iNOS, IL-6, and IL-1b. The possible explanation for the almost unchanged level of TNF-a may be due to research data confirming that simultaneous blockade of ERK and p38 is required for suppression of TNF-a production in RAW 264.7 cells [27]. In our study BM only inhibited the ERK pathway and not the p38 pathway, and the TNF-a production was not reduced. Additionally, differential regulation of TNF-a activity by ERK signaling depends on the cell type. This could be the potential cause of BM influence on TNF-a level. The research evidence indicates that ERK inhibitor blocks the expression of LPS-induced TNF-a gene only in alveolar macrophages, but not in other types of macrophages [28].

The RAW 264.7 cells derive from murine ascites and treatment with may not be able to inhibit TNF-a production despite the decrease in ERK phosphorylation. Bacterial melanin has a complex structure. In our studies the infrared spectroscopy has revealed COOH, OH and other groups in BM structure. BM contains monomers such as indol, chinol and others. The structure of all melanin is amorphous and it cannot be overlooked that BM may contain components which counteract the anti-inflammatory effects via the ERK signaling pathway and specifically stimulate the production of TNF-a. STAT3 is an essential mediator of inflammatory signaling pathway induced by LPS [29]. Activated STAT3 translocates to the nucleus and modulates the transcription of inflammation-related genes [30]. Stimulating action of LPS on macrophages results in the increased IL-6 production followed by significant activation of STAT3 through IL-6 signaling pathway, and the activated STAT3, in turn, upregulates IL-6 production [31]. In our study BM downregulates IL-6 production by inhibiting ERK phosphorylation in LPS-stimulated RAW 264.7 cells, and also represses

STAT3 phosphorylation upon IL-6 stimulation, indicating that BM exerts dual effects on IL-6 regulation.

The study showed that BM decreases IL-6 production by inhibiting ERK phosphorylation in LPS-stimulated RAW 264.7 cells, and also suppresses STAT3 phosphorylation by IL-6 stimulation. BM induces overall anti-inflammatory activity in LPS-induced murine RAW264.7 cells, reducing the production of NO, PGE2, and pro-inflammatory cytokines via the downregulation of ERK and STAT3 phosphorylation.

Brain inflammation has been the therapeutic target of efforts to treat several brain diseases, including ischemia, trauma, and certain neurodegenerative diseases. The prevailing view has been that microglia-resident brain macrophages-are the only cells that mediate brain inflammation. A possible side effect of melanin application as a pharmacological agent for immunomodulation is immune system overactivation. It has been shown that extracellular neuromelanin can activate the CNS microglia inducing neuroinflammation and consequently neurodegeneration [32, 33]. The water-soluble biotechnological melanin does not activate microglia – the resident brain macrophages.

CONFLICT OF INTEREST

The authors declare not to have a conflict of interest.

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