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LPS로 자극한 RAW 264.7 세포에서 중국 연변에 자생하는 약용 식물 에탄올 추출물의 항염증 효과 연구

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Anti-inflammatory Effects of Ethanol Extract of Chinese Medicinal Plants in Yanjin on LPS-stimulated RAW 264.7 Macrophages

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ABSTRACT

Objectives : This study was fulfilled to investigate nominee materials as anti-inflammatory agent from ethanol extract of Chinese medicinal plants in Yanjin. Among the 20 candidates, we selected most effective one, the ethanol extract of *Cicuta virosa* L. (CVL). The mechanism underlying the anti-inflammatory effects of CVL is not clearly identified as yet. Accordingly, we clarified the anti-inflammatory effects of CVL and its underlying molecular mechanisms in LPS-stimulated RAW 264.7 macrophages.

Methods : RAW264.7 macrophages were incubated with CVL (12.5, 25, or 50 μM) and/or lipopolysaccharide (LPS) (1 ㎍/㎖). Cytotoxicity was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay and the level of nitric oxide (NO) production was measured with Griess reagent. The prostaglandin E_2 (PGE_2) production was measured with enzyme immunoassay kits and the protein expression of inducible nitric oxide synthase (iNOS) was determined using Western blot analysis.

Results : Among the 20 ethanol extract of Chinese medicinal plants of Yanjin tested, CVL significantly reduced the production of NO in a dose-dependent manner via inhibition the protein expressions of iNOS without cytotoxicity on the LPS-stimulated RAW 264.7 macrophages. In addition, CVL also effectively declined the production of PGE_2 in LPS-simulated RAW 264.7 macrophages.

Conclusions : Taken together, these data presented in this study demonstrate that CVL possesses anti- inflammatory activity by suppressing the production of pro-inflammatory mediators NO and PGE_2, and pro- inflammatory protein iNOS expression in LPS-stimulated RAW 264.7 macrophages.

Key words : *Cicuta virosa* L., lipopolysaccharide, nitric oxide, prostaglandin E_2, inducible nitric oxide synthase

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Ⅰ. Introduction

Inflammation is part of a multiple biological response of the body to noxious stimuli (e.g., damaged cells, pathogens, or irritants)[1]. Despite its necessary roles, the excessive or aberrant inflammation can lead to various acute and chronic human diseases, such as rheumatic disease, atherosclerotic lesions, and type Ⅱ diabetes[2]. The activation of pro-inflammatory cells, mainly macrophages and monocytes, plays a crucial function in inflammatory response[3]. In addition, macrophages serve as host defenses about harmful substances and are concerned in diverse disease processes including infections, autoimmune diseases, and inflammatory disorders[4]. Macrophages sense and react to some pathogen through pattern-recognition receptors (PRRs) including toll-like receptors (TLRs) and consequently regulate the inflammatory response[5].

Lipopolysaccharide (LPS), which represents an outer membrane structure and an vital virulence factor of the cell wall of Gram-negative bacteria, acts an essential role in metabolic processes via linkage to the pathogen- sensing system, inducing release of a large number of inflammatory cytokines[6]. LPS activates macrophages by binding to TLR4[1] and activated macrophages produce various pro-inflammatory molecules, including nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α(TNF-α), and interleukines[7]. For that reason, many researchers have been used the LPS-stimulated macrophages on experiments of inflammation.

Korean is an absolute poor country for biological resources. There are about 1.5 million species of biological resources worldwide, but in Korea, there are about 30,000 species of biological resources. Therefore, we conducted this study using the Chinese plants, which have not yet been studied a lot. Among the ethanol extract of Chinese plants, *Cicuta virosa* L. (CVL) is a member of the *Apiaceae* family and the study on anti-inflammatory effect of CVL is not clearly identified as yet. Therefore, in this current study, we explored the anti-inflammatory effects of CVL in RAW 264.7 macrophages, which can be activated with LPS to mimic the situation of inflammation.

Ⅱ. Materials and Methods

1. Chemicals and Reagents

Ethanol extract of Chinese plants was supplied by Foreign Plant Extract Bank (Daejeon, Republic of Korea). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Life Technologies Inc. (Grand Island, NY, USA). LPS (*Escherichia coli*, serotype 055:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliu m bromide (MTT), N6-(1-Iminoethyl)lysine (NIL), NS- 398, and Griess reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The enzyme immunoassay (EIA) kits for prostaglandin E_2 (PGE_2) were obtained from RD Systems. Dimethyl sulfoxide (DMSO) was purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). iNOS and β-actin monoclonal antibodies were purchased Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2. Cell Culture and Sample Preparation

The RAW 264.7 macrophage cell line was obtained from Korea Cell Line Bank (KCLB, Seoul, Republic of Korea). The cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 ㎍/㎖) in a 37℃ and humidified atmosphere of 5% CO_2. The plants were collected in Antu fengxingcun (安图), China in 2012 and authenticated by Yanbian University (YU), Renbo-An. Briefly, the dried and refined Chinese plants were extracted with 1000 ㎖ of 95% (v/v) ethanol for 2 h, twice. The extract was percolated with filter paper (3 ㎜; Whatman PLC, Kent, UK), condensed using a rotary evaporator (Buchi, Swiss), and lypophilized using a freeze dryer (Christ, Germany). The powder was dissolved in DMSO and filtered using Acrodisc® Syringe Filters 0.2 ㎛ Supor® Membrane (Rall Life Sciences, MI, USA).

3. MTT Assay for Cell Viability

Cell viability was assessed using the MTT assay. Briefly, RAW 264.7 cells were seeded into a 96-well plate at a density of 1×10^4 cells per well, and then treated with various concentrations (0, 15.63, 31.25, 62.5, 125, 250, or 500 ㎍/㎖) of ethanol extract of plants for 24 h. After treatment, the extract-treated cells were incubated with MTT solution (5 ㎎/㎖) for 4 h at 37℃. After discarding the supernatant, the formazan product that formed in the cell was dissolved in DMSO. Cell viability was measured at 570 nm using an Epoch microplate spectrometer (Biotek, Winooski, VT, USA).

4. NO Assay

NO content was analyzed indirectly by measuring the supernatants of cultured RAW 264.7 cells for nitrite

using the Griess reagent (1% sulfanilamide in 5% phosphoric acid, 1% α −naphthylamide in H_2O). RAW 264.7 cells were seeded into a 24−well plate at a density of 5 × 10^5 cells per well, and then treated with various concentrations of ethanol extract of plants for 1 h. After pre−incubation of the extract, the cells were stimulated with LPS (1 ㎍/㎖) for 48 h. A 50 ㎕ amount of cell culture media was mixed with 50 ㎕ of Griess reagent in a 96−well plate, incubated at room temperature for 15 min, and then measured at 540 nm using an Epoch microplate spectrometer (Biotek, Winooski, VT, USA).

5. PGE_2 Assay

RAW 264.7 cells were seeded into a 24−well plate at a density of 5 × 10^5 cells per well, and then treated with various concentrations of CVL for 1 h. After pre−incubation of CVL, the cells were stimulated with LPS (1 ㎍/㎖) for 24 h. The release of PGE_2 in the cultured media was measured by ELISA kit according to the manufacturer's instructions.

6. Western Blot Analysis

The cells were suspended in PRO−PREP™ protein extraction solution (Intron Biotechnology, Seoul, Republic of Korea). The suspension was incubated on the ice for 20 min and then centrifuged 11,000 *g* for 30 min. The protein concentration was determined using the Bio−Rad protein assay reagent according to the manufacturer's instructions (Bio−Rad, Hercules, CA, USA). Equal amounts (30 ㎍) of protein sample were separated on a sodium dodecyl sulfate (SDS) polyacrylamide gel, followed by transferred onto a polyvinylidene fluoride (PVDF) membrane. Membranes were incubated for 1 h with 5% skim milk at room temperature and then incubated overnight with a 1:1000 dilution of primary antibody at 4℃. The blots were washed three times with Tween 20/Tris−buffered saline (T/TBS) and incubated with a 1:2500 dilution of horseradish peroxidase− conjugated secondary antibody for 2 h at room temperature. The blots were again washed three times with T/TBS and then visualized by enhanced chemiluminescence (GE Healthcare, Waukesha, WI, USA).

7. Statistical Analysis

Each result is expressed as the mean ± standard deviation (SD) of triplicate experiments. Statistical analysis was fulfilled using SPSS statistical analysis software (version 19.0; International Business Machines, Armonk, NY, USA). Statistically significant differences were determined using analysis of variance and Dunnett's post hoc test, and P−values of less than 0.05 were considered statistically significant.

Ⅲ. Results

1. Anti−inflammatory Effect of Ethanol Extract of Chinese Medicinal Plants in Yanjin

To select candidate of anti−inflammation agents, we investigated the effect of ethanol extract of Chinese plants on cell viability and LPS−induced NO production in RAW264.7 macrophages. As shown in Table 1, among the ethanol extract of 20 plants, 8 plants (*Delphinium maackianum* Regel; DMR, *Arabis pendula* L.; APL, *Lactuca indica* L.; LIL, *Athyrium sinense* Rupr.; ASR, *Polygonum posumbu* Buch.−Ham.; PPBH, *Lychnis cognata* Maxim; LCM, *Tephroseris flammea* (Turcz. Ex DC.); TF, and *Artemisia feddei* H. Lév. & Vaniot; AFH) had cytotoxicity at concentrations of less than 62.5 ㎍/㎖ as determined by the MTT assay. Thus, there were only 12 non−cytotoxicity plants at concentrations of more than 62.5 ㎍/㎖, and we selected these 12 plants for NO screening. In table 2, 'IC50' is the concentration of the individual plants required to inhibit 50% of NO production and the 'inhibitory ratio' is measured to assess the inhibition effects in maximum concentration of individual plants. The data showed that only 4 plants (*Cirsium viassovianum* Fisch.ex DC.; CVF, *Carex neurocarpa* Maxim.; CNM, *Cicuta virosa* L.; CVL, and *Circaea caulescens* Kom.; CCK) had the inhibitory ratio of more than 30% on NO production. In addition, *Cicuta virosa* L. (CVL) provoked inhibitory effect of NO production about 70%. Based on these data, we selected CVL for the subsequent experiments.

2. Effect of CVL on the viability of RAW 264.7 macrophages

Cytotoxicity of CVL in RAW 264.7 cells was performed using MTT assay. As shown in Figure 1, RAW 264.7 cells were treated with different concentrations of CVL (15.63, 31.25, 62.5, 125, 250, or 500 ㎍/㎖) for 24 h. The cell viability was not affected by CVL up to 62.5 ㎍/㎖. Accordingly, we investigated the anti−inflammatory effects of CVL with concentration 12.5, 25, and 50 ㎍/㎖ in LPS−stimulated RAW 264.7 macrophages.

Table 1. Effect of Ethanol extract of Chinese medicinal plants in Yanjin on the cell viability in RAW 264.7 macrophages.

No.	Scientific name	IC50(㎍/㎖)
1	*Cirsium viassovianum* Fisch.ex DC.	293.9
2	*Delphinium maackianum* Regel	62.22
3	*Arabis pendula* L.	34.15
4	*Lactuca indica* L.	62.42
5	*Athyrium sinense* Rupr.	57.44
6	*Euphrasia tatrica* Fisch. Ex Spr.	197.2
7	*Thlaspi arvense* L.	458.1
8	*Polygonum posumbu* Buch.-Ham.	34.95
9	*Lilium distichum* Nakai	102.9
10	Lychnis cognata Maxim	27.03
11	*Echinochloa crusgalli* var. *submutica* (Mey.) Kitag.	153.4
12	*Tephroseris flammea* (Turcz. Ex DC.)	24.57
13	*Asyneuma japonicum* (Miq.) Briq	63.51
14	*Carex neurocarpa* Maxim.	234.8
15	*Phragmites australis* (Clav.) Trin.	276.8
16	*Callistephus chinensis* (L.) Ness	135.9
17	*Artemisia feddei* H. Lév. & Vaniot	61.42
18	*Cicuta virosa* L.	73.48
19	*Aconitum panicligeru* (Nakai) S.H. Li	158.1
20	*Circaea caulescens* Kom.	70.73

Each value represents the mean ± SD (n=3).

Table 2. Effect of Ethanol extract of Chinese medicinal plants in Yanjin on LPS-stimulated NO production level in RAW 264.7 macrophages.

No.	Scientific name	Inhibition ratio(%)	IC50(㎍/㎖)
1	*Cirsium viassovianum* Fisch.ex DC.	39.40 ± 0.80	126.02
2	*Euphrasia tatrica* Fisch. Ex Spr.	12.77 ± 1.58	97.77
3	*Thlaspi arvense* L.	26.92 ± 0.26	193.05
4	*Lilium distichum* Nakai	17.31 ± 0.44	102.53
5	*Echinochloa crusgalli var. submutica* (Mey.) Kitag.	13.93 ± 0.71	171.43
6	*Asyneuma japonicum* (Miq.) Briq	7.76 ± 1.63	〉500
7	*Carex neurocarpa* Maxim.	40.96 ± 0.97	63.26
8	*Phragmites australis* (Clav.) Trin.	22.66 ± 0.90	249.56
9	*Callistephus chinensis* (L.) Ness	19.17 ± 0.37	167.68
10	*Cicuta virosa* L.	71.63 ± 0.29	36.55
11	*Aconitum panicligeru* (Nakai) S.H. Li	7.63 ± 0.93	117.14
12	*Circaea caulescens* Kom.	38.75 ± 0.62	33.75

Each value represents the mean ± SD (n=3).

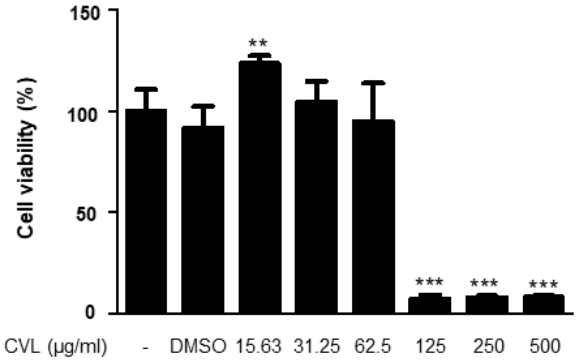

Figure 1. Effect of CVL on the viability of RAW 264.7 cells. The cells were treated with different concentrations of CVL for 24 h, and their viability were determined using MTT assay. Values represent mean ± SD of three independent experiments. The values are represented as mean ± S.D. (n = 4). **P < 0.01, ***P < 0.001 compared to DMSO.

3. Effect of CVL on LPS-stimulated NO production in RAW 264.7 macrophages

To further estimate the inhibition of CVL on LPS-stimulated NO production in RAW 264.7 macrophages, the cells were pre-treated with or without CVL (1.5, 3.1, 6.2, 12.5, 25, or 50 ㎍/㎖) for 1 h before LPS stimulation (1 ㎍/㎖). The LPS-stimulated NO production was significantly decreased by CVL in a dose-dependent manner. In addition, high concentration of CVL (50 ㎍/㎖) showed elevated inhibitory effect on NO production than NIL used as a positive control.

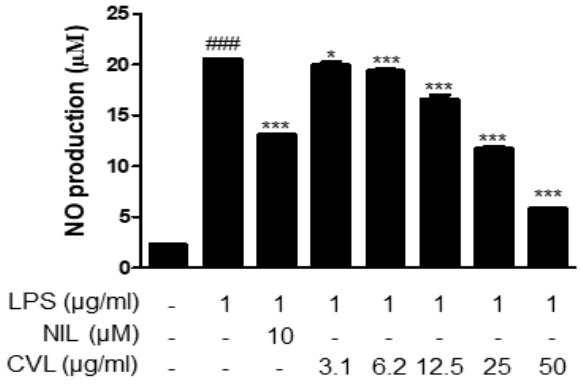

Figure 2. Effect of CVL on production of NO in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated indicated concentrations of CVL (1.5, 3.1, 6.2, 12.5, 25, or 50 ㎍/㎖) for 1 h, and followed by LPS stimulation (1 ㎍/㎖) for 48 h. The nitric oxide (NO) production was determined using Griess reagent. The values are represented as mean ± SD (n = 6). ###P<0.001, compared to CON; *P<0.05 and ***P<0.001 compared to LPS.

4. Effect of CVL on LPS-stimulated PGE_2 production in RAW 264.7 macrophages

Next, we examined the effect of CVL on LPS-stimulated PGE_2 production in RAW 264.7 macrophages. The cells were treated with or without CVL (12.5, 25, or 50 ㎍/㎖) for 1 h and then treated with LPS (1 ㎍/㎖) for 24 h. As shown in Figure 3, LPS-stimulated PGE_2 production was significantly diminished by CVL. Our data revealed that CVL exhibits suppressive activity on PGE_2 production as well as NO production in LPS- stimulated RAW 264.7 macrophages.

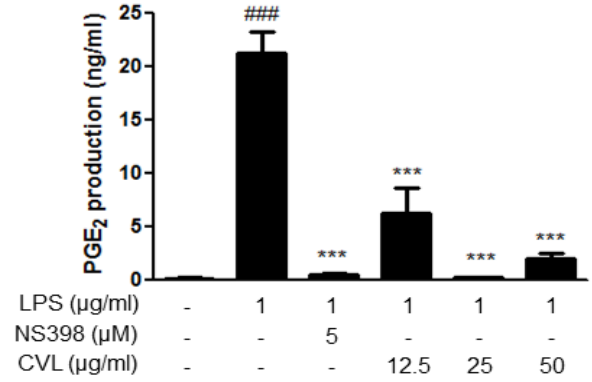

Figure 3. Effect of CVL on production of PGE_2 in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated indicated concentrations of CVL (12.5, 25, or 50 ㎍/㎖) for 1 h, and followed by LPS stimulation (1 ㎍/㎖) for 24 h. The prostaglandin-E_2 (PGE_2) levels in the culture media was measured by ELISA kit. The values are represented as mean ± SD (n=6). ###P<0.001, compared to CON; ***P<0.001 compared to LPS.

5. Effect of CVL on LPS-stimulated iNOS protein expression in RAW 264.7 macrophages

Previous reports suggested that LPS effectively activated iNOS transcription leading to the overproductions of NO in RAW 264.7 macrophages[8]. We explored whether CVL could inhibit the iNOS protein expression using western blot analysis. The expression of iNOS protein was strongly increased after stimulation with LPS in RAW 264.7 macrophages. However, pre-treated cells with CVL were significantly abolished the expression of iNOS protein (Figure 4). We suggest that inhibition of iNOS expression may be related to the inactivation of NO production by CVL in LPS-stimulated RAW 264.7 cells.

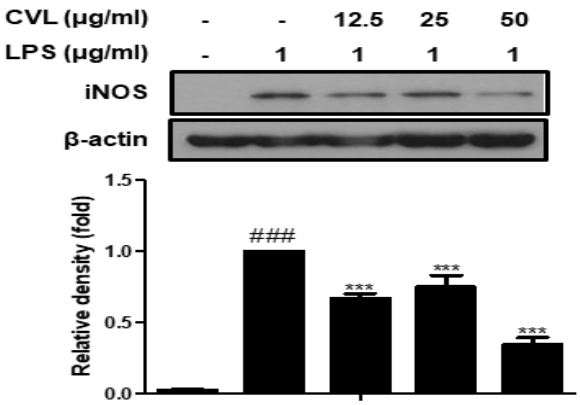

Figure 4. Effect of CVL on iNOS protein expression in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated indicated concentrations of CVL (12.5, 25, or 50 ㎍/㎖) for 1 h, and followed by LPS stimulation (1 ㎍/㎖) for 24 h. The values are represented as mean ± SD (n = 6). ###P < 0.001, compared to CON; ***P < 0.001 compared to LPS.

Ⅳ. Discussion

Normal inflammation has been considered a protective reaction to the external pathogens or internal injury[9]. Acute inflammation is an adaptive host defense mechanism against infection or injury whereas chronic inflammation causes various disorders, such as arthritis, diabetes, and cancer[10]. During the inflammatory process, macrophages were activated and produced a vast amount of cytokines, including pro- and anti-inflammatory cytokines[11]. As the major pro-inflammatory cells, macrophages are responsible for invading pathogens by releasing a great amounts of pro-inflammatory mediators including NO, PGE_2 as well as a variety of pro-inflammatory cytokines[12]. In addition, the excessive and chronic activation of macrophages brings about systemic inflammatory diseases, such as asthma, rheumatoid arthritis, sepsis, and Alzheimer's disease[13]. In the inflammation research, LPS is generally used to induce inflammation as it can stimulate the secretion of pro-inflammatory cytokines in macrophages[14]. Thus, the inhibition of activated macrophages by LPS as a strategy for treating inflammatory diseases is considered an important target.

We first examined the cytotoxicity of ethanol extract of Chinese medicinal plants in RAW 264.7 cells using MTT assay (Table 1). Among twenty plants, 8 plants caused significant cytotoxicity at concentrations of less than 61.25 ㎍/㎖. In contrast, the other twelve plants of them showed that cell viability at more than 61.25 ㎍/㎖ was not different from that in control group. When macrophages are activated in the presence of LPS, the macrophages produce a naturally high level of pro-inflammatory molecules, such as cytokines, and NO[15]. NO has been reported to play an critical role in the pathogenesis of inflammatory disorders under abnormal conditions[16]. Therefore, screening of anti-inflammatory materials from natural products through estimation of their suppression effect on NO production has been extensively performed[17]. Our results indicated that four plants (*Cirsium viassovianum* Fisch.ex DC.; CVF, *Carex neurocarpa* Maxim.; CNM, *Cicuta virosa* L.; CVL, and *Circaea caulescens* Kom.; CCK) of them showed strong inhibitory ratio (more than 30%) on NO production (Table 2). Moreover, we selected the ethanol extract of CVL, a most effective one, as anti-inflammatory candidate according to these results of Table 1 and 2. Furthermore, we conducted the inhibitory effect of CVL on LPS-stimulated NO production in RAW 264.7 macrophages using six concentrations (1.5, 3.1, 6.2, 12.5, 25, or 50 ㎍/㎖). The six concentrations of CVL simultaneously inhibited LPS-stimulated NO production and these results indicated that high concentration (50 ㎍/㎖) of CVL is capable of decreasing NO production in RAW 264.7 macrophages better than NIL. Because NO is synthesized by the iNOS[18], the iNOS has been also considered as promising targets for testing the capability of molecules against inflammatory disorders[19]. In these regards, we investigated the effect of CVL on the expression of iNOS protein in LPS-stimulated RAW 264.7 macrophages. In the current study, CVL significantly decreased the expression of iNOS protein (Figure 4) and this result revealed that CVL reduced LPS-stimulated NO production by suppressing iNOS expression. The PGE_2, a prostanoid synthesized from arachidonic acid, can induce fever, increase vascular permeability, maintain hyperalgesic responses, and play a function in muscle regeneration[20]. PGE_2 is also a bioactive lipid with diverse biological activities involved in inflammation[21]. It was observed that CVL treatment significantly inhibited the PGE_2 production in LPS-stimulated RAW 264.7 macrophages (Figure 3). A COX_2 inhibitor, NS 398, was used as positive control to inhibit de novo synthesis of PGE_2.

Taken together, treatment of CVL in RAW 264.7 cells inhibited LPS-stimulated NO, PGE_2 production and iNOS expression. Therefore, our research indicate that CVL extract represents potential natural source that will be useful for treatment of inflammatory conditions.

Ⅴ. Conclusion

In conclusion, the data presented in this study demonstrate that CVL possesses anti-inflammatory activity via suppression on production of pro-inflammatory mediators NO and PGE_2 and expression of pro-inflammatory protein iNOS in LPS-stimulated RAW 264.7 macrophages.

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