Protective Effects of *Digenea simplex* in Glutamate-Challenged Rat Pheochromocytoma Cells and Lipopolysaccharide-Stimulated Microglial Cells

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Abstract—This study examined the *in vitro* effects of a hot-water extract prepared from *Digenea simplex* (DS) in PC12 and BV2 cell models. DS powder (50 g) was extracted with distilled water (800 mL) at 120°C for 3 h, concentrated under reduced pressure, freeze-dried, and reconstituted in distilled water before use. In basal cytotoxicity assays, DS at 1, 10, and 100 μg/mL did not reduce viability in either cell line after 24 h, whereas 200 μg/mL decreased viability to 70 ± 1% in PC12 cells and 82 ± 1% in BV2 cells. In glutamate-challenged PC12 cells, viability increased from 22 ± 2% in the glutamate-only group to 44 ± 3% and 50 ± 2% at 10 and 100 μg/mL DS, respectively ($p<0.001$). Under the same condition, acetylcholine concentration increased from 65 ± 2% to 76 ± 4% and 78 ± 2%, whereas acetylcholinesterase activity decreased from 100% in the glutamate group to 86 ± 3.5% and 81 ± 2% at 10 and 100 μg/mL DS, respectively. In lipopolysaccharide-stimulated BV2 cells, DS reduced nitric oxide production at 1, 10, and 100 μg/mL to 89 ± 3%, 84 ± 2%, and 81 ± 2% of the model group, and reduced prostaglandin E2 production to 86 ± 2% at 100 μg/mL. Representative-blot densitometry normalized to β-actin suggested lower relative values for NF-κB, p38, JNK, caspase-3, and caspase-9 at 100 μg/mL DS. These results indicate that, under the present cell-based conditions, DS extract attenuated glutamate-associated loss of viability in PC12 cells and reduced selected inflammatory readouts in activated BV2 cells. The data do not, by themselves, establish improved cognition or clinical benefit.

Index Terms—BV2 cells, *Digenea simplex*, glutamate, microglia, PC12 cells

I. INTRODUCTION

Excess extracellular glutamate is widely used to model excitotoxic or oxidative neuronal injury in PC12 cells, and previous studies have shown that glutamate exposure decreases viability in association with oxidative stress and glutathione depletion [1], [2]. In parallel, lipopolysaccharide (LPS)-stimulated BV2 microglia are commonly used to evaluate inflammatory readouts such as nitric oxide (NO), prostaglandin E2 (PGE2), and NF-κB/MAPK-associated signaling events [3]-[6]. Cholinergic measures, including acetylcholine (ACh) concentration and acetylcholinesterase (AChE) activity, also remain useful biochemical indicators in glutamate-challenged neuronal models [7], [8].

Marine macroalgae are a promising source of structurally diverse bioactive constituents. *Digenea simplex*, a red alga, has been reported to contain antioxidant components and sulfated polysaccharide fractions with anti-inflammatory and antinociceptive activity [9], [10]. At the same time, *Digenea simplex* is also known to contain kainic-acid-related metabolites, indicating that extraction conditions and dose may strongly influence the net effect of a crude preparation [11], [12].

PC12 cells undergo neuronal differentiation after nerve growth factor treatment and provide a practical model for glutamate-associated injury studies [13]. Based on this framework, the present work evaluated a hot-water extract of *Digenea simplex* (DS) in nerve growth factor-differentiated PC12 cells and LPS-stimulated BV2 cells. The objective was to document basal cytotoxicity, glutamate-associated viability loss, ACh/AChE-related changes, NO/PGE2 production, and selected protein-expression patterns. No claim beyond these cell-based readouts is intended.

II. MATERIALS AND METHODS

A. Materials and extract preparation

Dried *Digenea simplex* powder was obtained from Gulf Specimen Marine Laboratories, Inc., USA, and stored at -20°C until extraction. For preparation of the test extract, 50 g of powder was mixed with 800 mL distilled water and extracted at 120°C for 3 h. The extract was filtered, concentrated with a rotary vacuum evaporator, freeze-dried, and stored at -80°C. Before each experiment, the lyophilized extract was dissolved in distilled water to the required concentration.

B. Reagents and antibodies

Major reagents included GelRed (Sigma, USA), Dulbecco's phosphate-buffered saline (Welgene, Korea), DMEM (Gibco BRL, USA), fetal bovine serum (Invitrogen, USA), lipopolysaccharide (Sigma, USA), CCK-8 assay kit (Dojindo, Korea), nitric oxide detection kit (Intron Biotechnology, Korea), dimethyl sulfoxide (Sigma, USA), penicillin and streptomycin (Hyclone, USA), agarose (FMC, USA), trypan blue (Sigma, USA), a choline/acetylcholine assay kit (Abcam, USA), total RNA prep kit (Intron Biotechnology, Korea), Pro-Prep Protein Extraction Solution (Intron Biotechnology, Korea), Precision Plus Protein Dual Color Standards (Bio-Rad, Japan), LDS Sample Buffer (4×) (Intron Biotechnology, Korea), 30% acrylamide/bis solution 29:1 (Bio-Rad, Japan), skim milk (BD, France), and Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Primary antibodies against NF-κB, p38, JNK, caspase-3, caspase-9, and β-actin, together with anti-rabbit IgG secondary antibody, were obtained from Cell Signaling Technology (Danvers, MA, USA). The PGE2 immunoassay reagent set was from Thermo Fisher Scientific (USA).

C. Cell culture

PC12 cells were maintained in RPMI medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. For neuronal differentiation, PC12 cells were treated with 100 ng/mL 7S nerve growth factor for 9 days. One day before treatment, the cells were transferred to RPMI containing 1% fetal bovine serum. BV2 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin at 37°C in a humidified 5% CO_2 incubator.

D. Basal cytotoxicity assay

PC12 and BV2 cells were seeded in 96-well plates at 1.5×10^5 cells/well and incubated for 24 h. The medium was then replaced, and DS extract was added at 1, 10, 100, or 200 μg/mL. After 24 h, cell viability was determined with the CCK-8 assay according to the working laboratory protocol, and absorbance was measured at 450 nm. Results were expressed as percentages of the untreated control.

E. Glutamate challenge in PC12 cells

Differentiated PC12 cells were seeded in 96-well plates at 2×10^5 cells/well and allowed to attach for 24 h. Cells were then exposed to 20 mM L-glutamate with or without DS extract (1, 10, or 100 μg/mL) for 24 h, a condition broadly consistent with previously reported PC12 injury models [14]. Cell viability was measured by CCK-8 assay at 450 nm. For statistical comparison, DS-treated groups were compared with the glutamate-only group.

F. Acetylcholine concentration and acetylcholinesterase activity

Differentiated PC12 cells were seeded in 12-well plates at 2×10^5 cells/well, incubated for 24 h, and then treated with 20 mM L-glutamate in the absence or presence of DS extract (1, 10, or 100 μg/mL) for 24 h. For acetylcholine measurement, culture supernatants were reacted with hydroxylamine and $FeCl_3$ solution, and absorbance was measured at 540 nm. For AChE activity, the supernatants were assayed using a colorimetric reaction mixture and read at 570 nm.

G. NO and PGE2 assays in BV2 cells

BV2 cells were seeded in 96-well plates at 1.5×10^5 cells/well and incubated for 24 h. The culture medium was replaced, and cells were exposed to 1 μg/mL LPS together with DS extract (1, 10, or 100 μg/mL) for 24 h. NO production in the culture supernatant was measured with the nitric oxide detection kit at 540 nm. PGE2 in the culture supernatant was measured by immunoassay according to the working laboratory protocol. For graphical presentation, the LPS-only group was normalized to 100%, and the untreated control group was plotted separately.

H. Western blot analysis

BV2 cells were treated with 1 μg/mL LPS in the absence or presence of DS extract (1, 10, or 100 μg/mL) for 24 h. After treatment, cells were washed twice with phosphate-buffered saline and lysed on ice. Total protein concentration was determined by BCA assay. Equal amounts of protein (20 μg) were mixed with sample buffer, separated on 12% SDS-PAGE gels, and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk and incubated with antibodies against NF-κB, p38, JNK, caspase-3, caspase-9, and β-actin, followed by anti-rabbit IgG and enhanced chemiluminescence detection. Band intensities from the representative blot were semiquantified with ImageJ after local background subtraction and β-actin normalization; the LPS group was set to 100 for relative comparison [17].

I. Statistical analysis

All experiments were performed three times independently. Data are presented as mean ± standard deviation (SD). Differences among groups were evaluated by one-way analysis of variance (ANOVA), followed by Student's t-test for comparison with the relevant model group. Statistical significance was defined as $p<0.05$, $p<0.01$, or $p<0.001$.

III. RESULTS

A. Basal cytotoxicity of DS extract in PC12 and BV2 cells

DS extract did not reduce basal viability in either cell line at 1, 10, or 100 μg/mL after 24 h. In PC12 cells, viability was 102 ± 3%, 103 ± 2%, and 98 ± 2% at 1, 10, and 100 μg/mL, respectively, whereas 200 μg/mL reduced viability to 70 ± 1%. In BV2 cells, viability was 105 ± 3%, 105 ± 3%, and 99 ± 2% at 1, 10, and 100 μg/mL, respectively, whereas 200 μg/mL reduced viability to 82 ± 1%. Concentrations above 100 μg/mL were therefore excluded from subsequent experiments. The full response profiles are presented in Fig. 1.

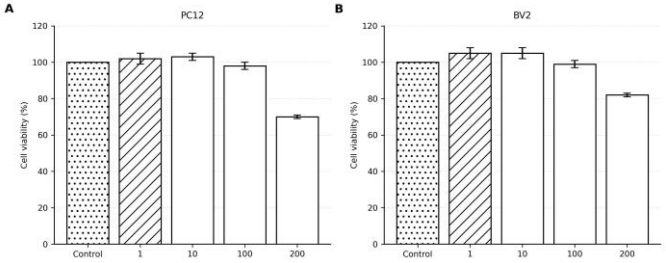

Figure 1. Baseline viability of PC12 cells (A) and BV2 cells (B) after 24 h exposure to DS extract at 1, 10, 100, and 200 μg/mL.

Viability was determined with the CCK-8 assay and is reported as mean ± SD.

B. Effect of DS extract on glutamate-associated loss of viability in PC12 cells

Exposure to 20 mM L-glutamate markedly reduced PC12 cell viability to 22 ± 2%. Co-treatment with DS at 1 μg/mL produced little change (25 ± 2%), whereas 10 and 100 μg/mL significantly increased viability to 44 ± 3% and 50 ± 2%, respectively (both $p<0.001$ vs. glutamate-only group). These data indicate that DS attenuated glutamate-associated loss of viability at 10-100 μg/mL under the present conditions. Representative viability data are shown in Fig. 2.

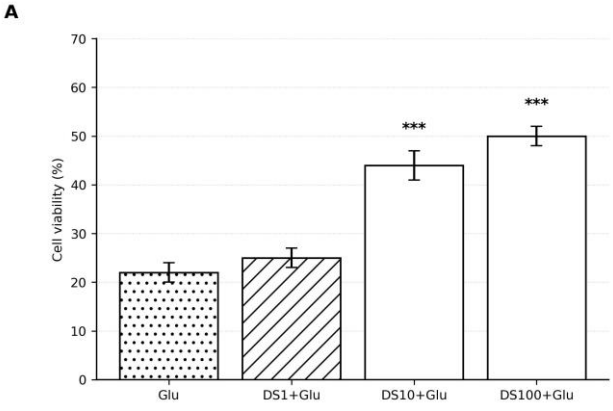

Figure 2. Viability of differentiated PC12 cells exposed to 20 mM L-glutamate in the absence or presence of DS extract (1, 10, or 100 μg/mL) for 24 h. Cell survival was assessed by CCK-8 assay. Values are shown as mean ± SD; ***$p<0.001$ versus the glutamate-only group.

C. Effects of DS extract on ACh concentration and AChE activity in glutamate-treated PC12 cells

For ACh measurement, the untreated control was normalized to 100%, whereas glutamate treatment reduced the signal to 65 ± 2%. DS at 1 μg/mL did not alter this value (65 ± 4%), but 10 and 100 μg/mL increased the relative ACh level to 76 ± 4% ($p<0.05$) and 78 ± 2% ($p<0.01$), respectively. For AChE activity, the glutamate-only group was set to 100%, and the untreated control showed 30 ± 1%. DS at 1 μg/mL yielded 95 ± 3% with no statistical significance, whereas 10 and 100 μg/mL reduced AChE activity to 86 ± 3.5% and 81 ± 2%, respectively. The corresponding measurements are presented in Fig. 3.

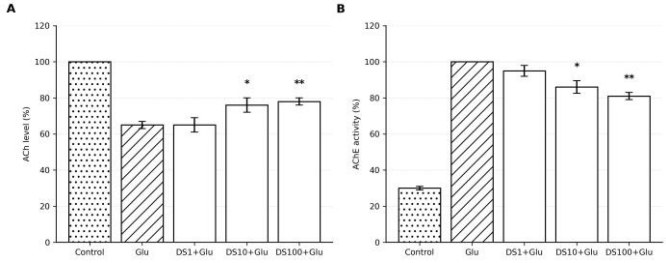

Figure 3. Effects of DS extract on acetylcholine concentration (A) and acetylcholinesterase activity (B) in glutamate-treated PC12 cells. After 24 h co-exposure to glutamate and DS extract, both readouts were measured colorimetrically. Data are presented as mean ± SD; *$p<0.05$ and **$p<0.01$ versus the glutamate-only group.

D. Effects of DS extract on NO and PGE2 production in LPS-stimulated BV2 cells

When the LPS-only group was normalized to 100%, the untreated control showed 24 ± 1% NO production. DS reduced the NO signal to 89 ± 3%, 84 ± 2%, and 81 ± 2% at 1, 10, and 100 μg/mL, respectively, with statistical significance at all three doses ($p<0.05$). For PGE2, the untreated control showed 18 ± 3% relative to the LPS-only group. DS treatment produced 98 ± 1% at 1 μg/mL and 92 ± 2% at 10 μg/mL without statistical significance, whereas 100 μg/mL decreased the level to 86 ± 2% ($p<0.05$). These inflammatory readouts are summarized in Fig. 4.

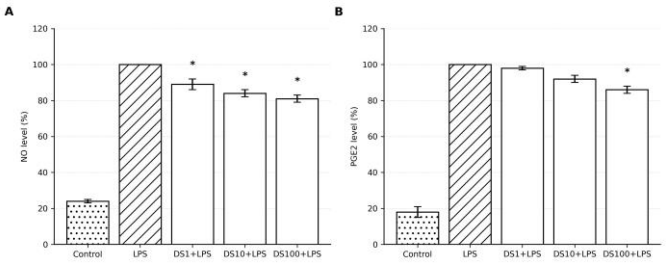

Figure 4. Nitric oxide (A) and prostaglandin E2 (B) production in BV2 microglial cells stimulated with 1 μg/mL LPS for 24 h in the presence of DS extract (1, 10, or 100 μg/mL). NO was measured with a nitric oxide detection kit, and PGE2 was quantified by immunoassay. Results are expressed as mean ± SD; *$p<0.05$ versus the LPS group.

E. Effects of DS extract on protein-expression patterns in LPS-stimulated BV2 cells

Representative Western blot images showed strong band intensity for NF-κB, p38, JNK, caspase-3, and caspase-9 in the LPS-only group compared with the untreated control. ImageJ-based semiquantitative densitometry after β-actin normalization, with the LPS group set to 100, yielded the following relative values for control, DS 1, DS 10, and DS 100, respectively: NF-κB, 89, 129, 101, and 51; p38, 95, 89, 76, and 55; JNK, 21, 84, 81, and 82; caspase-3, 30, 101, 97, and 69; and caspase-9, 104, 94, 84, and 88. On this semiquantitative basis, the clearest reduction relative to LPS was observed at 100 μg/mL for NF-κB, p38, and caspase-3, whereas JNK and caspase-9 showed more modest decreases. Representative blots and densitometric values are shown in Fig. 5.

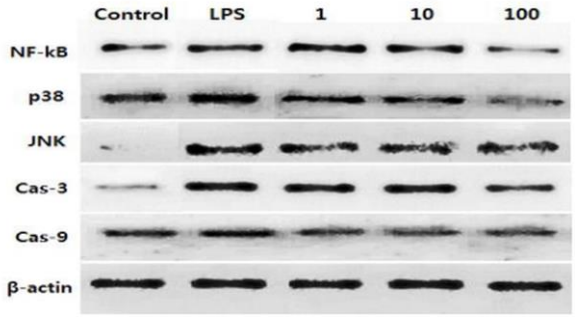

Figure 5. Representative Western blot analysis of NF-κB, p38, JNK, caspase-3, and caspase-9 in BV2 cells treated with 1 μg/mL LPS and DS extract (1, 10, or 100 μg/mL) for 24 h. Lane order was control, LPS, DS 1, DS 10, and DS 100. Semiquantitative ImageJ analysis was performed in ImageJ after β-actin normalization, with the LPS group defined as 100.

IV. DISCUSSION

The present dataset indicates that DS hot-water extract displayed a dose window in which basal cytotoxicity was minimal but measurable changes in cell injury-related readouts were observed. At 1-100 μg/mL, viability remained near the control range in both PC12 and BV2 cells, whereas 200 μg/mL decreased viability in both systems. This concentration-dependent pattern argues against routine use of the highest concentration in future experiments.

In glutamate-challenged PC12 cells, DS improved viability at 10 and 100 μg/mL and partially shifted ACh/AChE-related readouts toward the untreated profile. These changes are consistent with attenuation of glutamate-associated cellular dysfunction, but they should not be interpreted as direct evidence of improved cognition, memory, or disease modification. The current work is limited to cell-based biochemical and viability measurements.

In BV2 cells, DS lowered NO production at all tested concentrations, whereas the effect on PGE2 was more modest and reached significance only at 100 μg/mL. This pattern is compatible with prior BV2 literature in which suppression of NO, PGE2, and NF-κB/MAPK-associated signaling has been used as a marker of reduced inflammatory activation [3]-[6], [15], [16]. In the present representative blot, ImageJ-based semiquantitative densitometry suggested the clearest reduction at 100 μg/mL for NF-κB, p38, and caspase-3, while JNK and caspase-9 changed more modestly relative to the LPS group (=100).

A notable interpretive point is that *Digenea simplex* is chemically complex. Anti-inflammatory polysaccharide fractions have been reported from this species [10], but kainic-acid-related metabolites have also been identified and characterized [11], [12]. Accordingly, the net activity of a crude DS extract may depend strongly on extraction conditions, lot variation, and the balance among constituents. The reduced viability observed at 200 μg/mL may therefore reflect extract complexity rather than a single mechanism.

The major limitations of the present manuscript are the absence of extract standardization data, the lack of a positive control, and the fact that Western blot densitometry was derived from a representative blot rather than from replicate blots quantified independently. All conclusions remain restricted to *in vitro* cell models. Future work should include compositional profiling, batch standardization, positive controls, replicate densitometric analysis, and validation in additional neuronal and microglial systems.

V. CONCLUSION

Under the present experimental conditions, DS hot-water extract was non-cytotoxic in PC12 and BV2 cells up to 100 μg/mL, but reduced viability at 200 μg/mL. In glutamate-treated PC12 cells, DS attenuated loss of viability at 10 and 100 μg/mL and shifted ACh/AChE-related readouts toward the untreated condition. In LPS-stimulated BV2 cells, DS reduced NO production at all tested concentrations, lowered PGE2 production at 100 μg/mL, and semiquantitatively decreased NF-κB-, p38-, JNK-, caspase-3-, and caspase-9-associated protein expression in the representative ImageJ-analyzed blot. These findings support an effect of DS extract on selected cellular injury and inflammatory readouts, but do not establish improved cognition or clinical efficacy.

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