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Protective Effect of *Acanthopanax senticosus* Extract on Alloxan-induced β-cell Damage

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Abstract

The protective effect of *Acanthopanax senticosus* (AS) extract on alloxan-induced pancreatic β-cell damage was investigated in HIT T-15 cells, a Syrian hamster pancreatic β-cell line. Alloxan caused the pancreatic β-cell damage through the generation of reactive oxygen free radicals, increased DNA fragmentation, and decreased cellular NAD^+ levels. The β-cell damage was significantly prevented by the pretreatment with water soluble extract of AS roots. These results suggest that the protective effect of AS extract, on alloxan-induced β-cell damage, is primarily due to the inhibition of the generation of reactive oxygen free radical species (ROS) by alloxan.

Key words: *Acanthopanax senticosus* (AS), protective effect, alloxan, oxygen free radicals

INTRODUCTION

Alloxan, a cyclic urea derivative, has been used to induce diabetes mellitus in experimental animals by causing a selective cytotoxicity on the insulin-producing pancreatic β-cells (1,2). Although the precise diabetogenic mechanism of alloxan is not fully understood, there is evidence indicating that the tissue damage induced by alloxan is mediated through the formation of ROS such as superoxide anion radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) (3-6).

Alloxan is easily reduced to dialuric acid by a variety of biological reducing agents including cysteine, reduced glutathione (GSH) and ascorbate (5). In the presence of oxygen, the autoxidation of dialuric acid is to alloxan-generating ROS (4). There are many reports that the scavengers of ROS can reduce alloxan toxicity *in vitro* (7-10) and *in vivo* (11-14). Okamoto (15) has proposed that the primary target of ROS produced by alloxan is genomic DNA of pancreatic β-cells. We previously reported that alloxan caused an increase in intracellular Ca^{2+} levels, which played an important role in the diabetogenenicity of alloxan (16,17).

Acanthopanax senticosus (AS) is an oriental herb and has been reported to have immunomodulatory (18), hypoglycemic (19), antiviral (20), and antitumour activities (21). The active components in AS have been classified as phenylpropane compounds, lignans, coumarins, polysaccharides and other components (22).

Therefore, the present study was attempted to investigate the protective effect of AS extract on alloxan-induced β-cell damage using HIT-T 15 cell, a Syrian hamster β-cell line.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents used in this study were analytical grade and were obtained from commercial suppliers.

Preparation of AS extracts

AS were cultivated at the medicinal garden of Panax Co. Ltd. (Jeonju, Korea). The roots were collected, and identified by Dr. Kang-Seop Lee (Panax-Bio Institute, Panax Co. Ltd., Jeonju, Korea). A voucher specimen was obtained from the Herbarium of Panaxia Co. Ltd.

The water-soluble components of AS were extracted from the dried and powdered form of roots by boiling for 5 hours in distilled water. The extract was filtered and vacuum dried.

Cell culture

HIT-T 15 cells were obtained from Korea Cell Bank (College of Medicine, Seoul National University, Korea). HIT-T 15 cells were cultured in RPMI-1640 medium containing 11 mM glucose supplemented with 10% fetal

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bovine serum, 100 units/mL of penicillin, 0.1 μg/mL streptomycin, and 2.5 μg/mL amphotericin B at 37°C in 5% CO_2 and 95% O_2.

Cell viability assay

Cell viability was estimated by the trypan blue dye exclusion method (23). Cells were cultured in RPMI medium for 3~4 days prior to experiments and preincubated for 1 hour with Krebs-Ringer (KR) buffer containing 119 mM NaCl, 4.74 mM KCl, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 2.54 mM $CaCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES, and 0.1% bovine serum albumin at pH 7.4. Cells were then incubated in KR buffer supplemented with various drugs. After experimental treatment, the cells were incubated briefly with 0.05% trypan blue. The number of cells excluding trypan blue was counted on a hemocytometer. Cell viability was expressed as percentage of unstained (surviving) cells.

DNA fragmentation assay

The cellular DNA fragmentation of HIT-T 15 cells was determined using a Cellular DNA Fragmentation ELISA kit (Boehringer Mannheim GmbH, Germany). HIT-T 15 cells were incubated with 10 μM bromodeoxy uridine (BrdU) overnight at 37°C, centrifuged at 250×g for 10 min, adjusted to 1×10^5 cells/mL in RPMI-1640 medium and plated in a 96-well plate (2×10^4 cells/well). After treatment with alloxan for 30 min, DNA fragmentation of cells was determined by an ELISA kit using peroxidase-conjugated anti-BrdU antibody solution. The absorbance was measured at 450 nm against substrate solution as a blank. The reference wavelength was 690 nm.

Detection of the intracellular ROS

The generation of intracellular ROS was detected using a ROS sensitive fluorescent dye and a chemiluminogenic agent (24). Dihydroethidium (DHE, Sigma, St. Louis, USA) was used for the detection of superoxide anion and 2′-7′-dichlorofluorescein diacetate (DCFH-DA, Molecular Probes, Oregon, USA) for hydrogen peroxide. DHE is freely permeable into cells and, in the presence of superoxide anion, is oxidized to ethidium bromide, which in turn, binds to DNA in the nucleus and emits red fluorescence (25). DCFH-DA penetrating the cell is initially converted into DCFH by cellular esterase, and then DCFH is in turn oxidized to DCF in the presence of hydrogen peroxide, which emit green fluorescence (26). HIT T-15 cells were incubated with KR buffer at 37°C for 1 hour and then, cells were treated with 1 μM DHE or 30 μM DCFH-DA at 37°C. After 20 min, cells were treated with alloxan (2 mM) in the presence of fluorescent dye and incubated for another 10 min. After washing with KR buffer, the generation of ROS was detected by fluorescence under a confocal fluorescence microscope (Olympus, Japan).

Determination of cellular NAD^+

HIT-T 15 cells were lysed with 0.6 M perchloric acid, and the extract was adjusted to pH 5.0 with 3 N KOH. Nicotinamide-adenine dinucleotide (NAD^+) content in the cell extract was determined as described by Bernofsky & Swan (27).

Statistical analysis

Statistical analysis of the data was performed with Student's *t*-test and ANOVA. Differences with $p<0.05$ were considered statistically significant.

RESULTS

Cytotoxic effect of alloxan on β-cell viability

To study the cytotoxic effect of alloxan on HIT T-15 cells, cells were exposed to various concentrations of alloxan. After 30 min, the cell viability was determined by trypan blue dye exclusion test. Alloxan induced cell death in a dose dependent manner, with a median lethal dose (LD_{50}) of alloxan to HIT T-15 cells of about 2 mM (Fig. 1).

Protective effects of AS extracts on alloxan-induced β-cell cytotoxicity

To investigate the protective effect of AS extract on alloxan-induced β-cell death, HIT T-15 cells were pretreated with AS extract at 37°C for 48 hours prior to

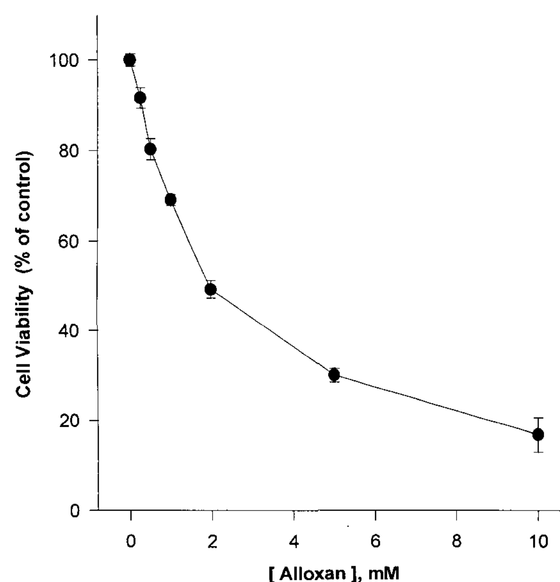

Fig. 1. Effect of alloxan on the viability of HIT T-15 cells. Cells were treated with various concentrations of alloxan at 37°C for 30 min. Cell viability was determined by the ability to exclude trypan blue dye. Each value denotes the mean± SE of five separate experiments.

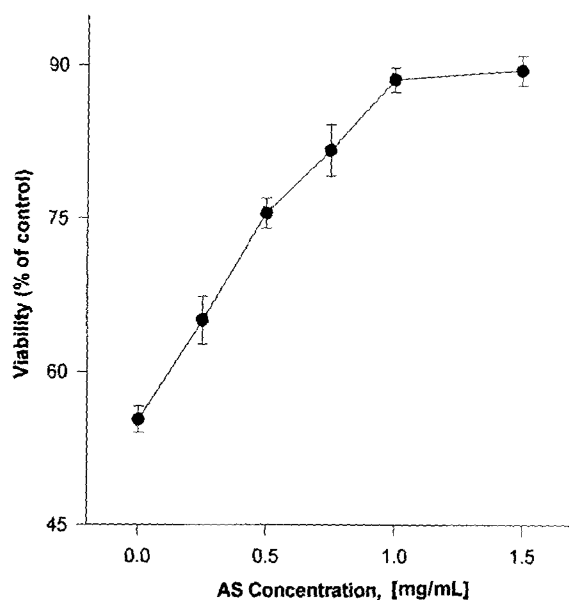

Fig. 2. Protective effect of AS extract against cytotoxicity induced by alloxan. HIT T-15 cells were pretreated at 37°C for 48 hours with various concentrations of AS extract. After washing with KR buffer, the cells were incubated with 2 mM alloxan at 37°C. After 30 min, cell viability was determined by trypan blue exclusion assay. Data were given as mean ± SE for five determinations.

alloxan (2 mM), and the cell viability determined. As shown in Fig. 2, AS extract significantly protected against alloxan-induced β-cell death in a dose dependent manner, and only 10% of HIT T-15 cells were dead at the concentration of 1 mg/mL AS.

The protective effect of AS extract (1 mg/mL) on alloxan-induced β-cell death was increased with the incubation time, reaching nearly to plateau when cells were pretreated for 48 hours with AS before alloxan treatment (Fig. 3).

Effect of AS extract on the generation of ROS induced by alloxan

Alloxan induced the increase of red and green fluorescence (Fig. 4), which indicated that alloxan induced

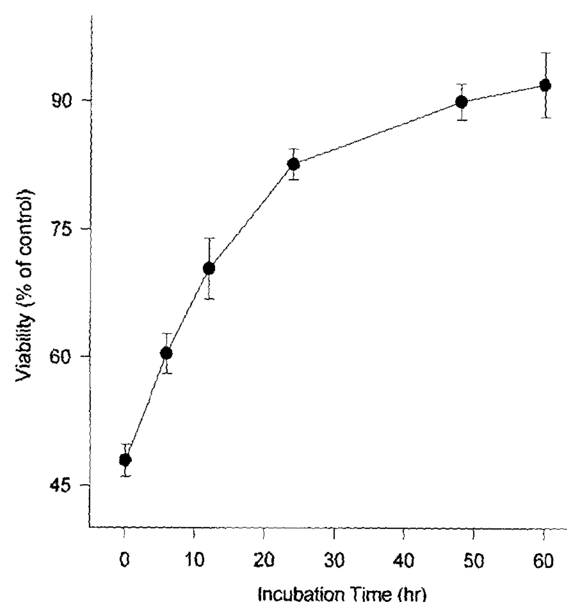

Fig. 3. Effect of AS extract on alloxan-induced β-cell death as a function of incubation time. HIT T-15 cells were preincubated with 1 mg/mL AS extract for the indicated time before alloxan. After treatment with 2 mM alloxan for 30 min, cell viability was determined. Values represent mean ± SE obtained from five separate experiments.

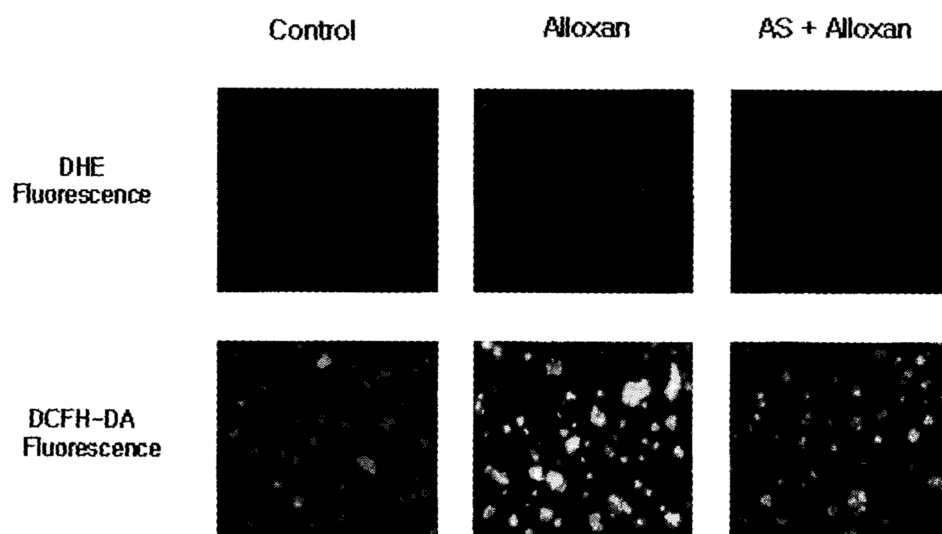

Fig. 4. Inhibitory effect of AS extract on the generation of ROS by alloxan. The generation of ROS by alloxan was detected using two fluorescent dyes; superoxide-sensitive dye, HHE (red fluorescence) and hydrogen-peroxide-sensitive dye, DCFH-DA (green fluorescence). HIT T-15 cells were incubated with DHE (1 μM) or DCFH-DA (30 μM) at 37°C. After 20 min, cells were treated with alloxan (2 mM) in the presence of fluorescent dye and incubated for another 10 min. After washing with KR buffer, the red or green fluorescence was observed under confocal fluorescence microscopy.

the generation of ROS such as superoxide anion radicals and hydrogen peroxides. The increase of red and green fluorescence by alloxan was significantly inhibited by pretreatment with AS extract (Fig. 4).

Effect of AS extract on the alloxan-induced DNA fragmentation

Okamoto (15) reported that oxygen free radicals formed by alloxan induced the cellular DNA strand breaks. As shown in Fig. 5, alloxan caused about a 2-fold increase in DNA fragmentation as compared with that of control cells. But this increase in DNA fragmentation by alloxan was significantly reduced by pretreatment with AS extract (63% of alloxan control).

Effect of AS extract on the depletion of cellular NAD^+ by alloxan

It is known that poly (ADP-ribose) synthetase is activated when DNA is fragmented (15,28). Taken as a whole, the cellular level of NAD^+, the substrate for poly (ADP-ribose) synthetase, was significantly decreased when DNA was fragmented. The cellular NAD^+ level of alloxan-treated HIT T-15 cells was markedly reduced (Fig. 6). However, the decreased level of NAD^+ was significantly attenuated by pretreatment with AS extract in a dose-dependent manner. On the other hand, AS extract did not cause any change in the cellular level of NAD^+ by itself.

DISCUSSION

Alloxan induces diabetes in experimental animals through the selective damage of the pancreatic β-cells. It is generally accepted that β-cell damage induced by alloxan occurs through the noxious reactive oxygen free radicals (5,28,29). Even though the exact subcellular site for the initial attack by alloxan is not very clear, evidence linking it to damage to plasma membrane (30), mitochondria (31) and nuclei (15,28) has been reported.

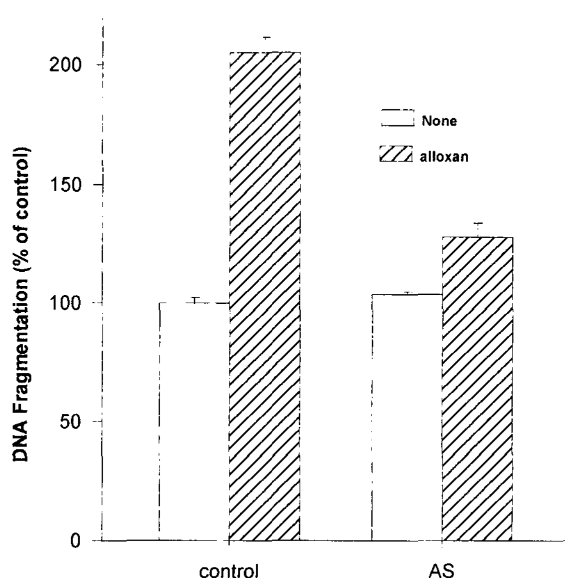

Fig. 5. Effect of AS extract on alloxan-induced DNA fragmentation. HIT T-15 cells were pretreated with AS extract (1 mg/mL) for 48 hours before 2 mM alloxan, and the amount of DNA fragmentation was measured by ELISA. Values represent the mean ± SE from five determinations.

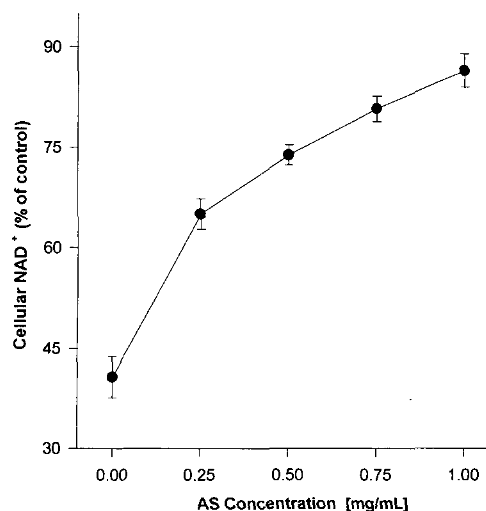

Fig. 6. Effect of AS extract on the depletion of cellular NAD^+ by alloxan. After preincubation of HIT T-15 cells at 37°C for 48 hours with various concentrations of AS extract, cells were treated with 2 mM alloxan at 37°C. After 30 min, the cellular NAD^+ level of cells was determined. Data were given as the mean ± SE of five separate experiments.

Okamoto (15) proposed that reactive oxygen species produced by alloxan causes DNA strand breaks, and that the damaged DNA activates nuclear poly (ADP-ribose) synthetase, which depletes the cellular pool of NAD^+, resulting in β-cell damage. We previously reported that alloxan caused an increase in cytosolic free Ca^{2+} in rat pancreatic β-cells, and that pretreatment of rats with Ca^{2+}-antagonists completely prevented hyperglycemia induced by alloxan (16,17). The results suggested that Ca^{2+} plays an important role in the diabetogenic mechanism of alloxan.

HIT-T 15 cells were used to investigate the protective effect of AS extract on alloxan-induced β-cell damage. HIT-T 15 cells have similar characteristics to normal pancreatic islets, in respect to the capacity of glucose-stimulated insulin secretion (32,33), and sensitivity to alloxan.

Acanthopanax senticosus (AS) has been used in traditional oriental medicine. In the present study, we investigated the protective effect of AS extract on alloxan-induced β-cell damage. Firstly, AS extract significantly protected against alloxan-induced β-cell damage, including the inhibition of the generation of ROS, the increase in DNA fragmentation, and the decrease in the

cellular NAD^+ level. According to Okamoto's model, the generation of ROS induced by alloxan initiates the β-cell damage. Therefore, the protective effect of AS extract on alloxan-induced β-cell damage is due to the significant inhibition of the generation of ROS, such as superoxide anion radicals, hydrogen peroxides, and hydroxyl radicals. Malaisse et al. (34) reported that an exquisite sensitivity of the insulin producing pancreatic β-cell to alloxan results from low levels of intracellular antioxidant enzyme activities, such as glutathione peroxidase and catalase. Secondly, AS extract has no direct scavenger activity toward ROS by itself (data not shown). Finally, the results suggest that the protective effect of AS extract is due to the induction of ROS scavenging enzymes, such as superoxide dismutase, catalase, or glutathione peroxidase etc. Sui et al. (19) reported that the saponin isolated from leaves of AS has a hypoglycemic activity. The active components of AS root extract which protect against alloxan-induced β-cell damage, were not determined.

In conclusion, pretreatment of HIT T-15 cells with the water soluble extract of AS from roots significantly protected against alloxan-induced β-cell damage. This protective effect may be due to the inhibition of the generation of ROS by alloxan. Further studies are needed to define the possible active components and to elucidate the protective mechanism of AS extract.

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