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**Effects of Asian sand dust particles on the respiratory and immune system**

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Running title : ASD affect the respiratory and immune system

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## **Abstract**

Epidemiologic studies have reported that Asian sand dust particles (ASD) can affect respiratory health, however, the mechanisms remain unclear. We investigated the effects of ASD on airway epithelial cells and immune cells, and their contributing factors to the effects. Human airway epithelial cells were exposed to ASD collected on 1-3 May (ASD1), on 12-14 May (ASD2), 2011 in Japan and heat-treated ASD1 for excluding heat-sensitive substances (H-ASD) at a concentration of 0, 3, 30, or 90  $\mu\text{g}/\text{mL}$  for 4 or 24 h. Furthermore, bone marrow-derived dendritic cells (BMDC) from atopic prone mice were differentiated by culture with GM-CSF then these BMDC were exposed to the ASD for 24 h. Also splenocytes as mixture of immune cells were exposed to the ASD for 72 h. All ASD dose-dependently reduced viability of airway epithelial cells. Non-heated ASD showed a dose-dependent increase in the protein release of IL-6 and IL-8. The raises induced by ASD1 were higher than those by ASD2. ASD1 and ASD2 also elevated ICAM-1 at the levels of mRNA, cell surface protein and soluble protein in culture medium. In contrast, H-ASD did not change most of these biomarkers. Non-heated ASD showed enhancement in the protein expression of DEC205 on BMDC and in the proliferation of splenocytes, whereas H-ASD did not. These results suggest that ASD affect airway epithelial cells and immune cells such as BMDC and splenocytes. Moreover, the difference in ASD events and components adhered to ASD can contribute to the health effects.

**Key Words:** Airway epithelial cells, Asian sand dust particles, Bone-marrow-derived dendritic cells, Respiratory and immune system, Splenocytes

## **Short Abstract for Table of Contents**

We investigated the effects of Asian sand dust particles (ASD) on respiratory and immune system *in vitro*. ASD enhanced the production of IL-6, IL-8 and ICAM-1 from human airway epithelial cells, as well as the expression of DEC205 on bone marrow-derived dendritic cells and the proliferation of splenocytes. Moreover, these effects differed partly by ASD events or by components adhered to ASD. These results suggest that ASD can contribute to the respiratory and immune health.

## Introduction

Asian sand dust particles (ASD) frequently spread over large areas, including East China, the Korean peninsula, Taiwan, and Japan especially in the spring season. ASD themselves are mainly composed of silicon, aluminum, calcium, and iron. Occurring in the Gobi Desert and the Ocher Plateau, ASD mix various chemical and biological materials such as sulfate ( $\text{SO}_4^{2-}$ ), nitrate ( $\text{NO}_3^-$ ), polycyclic aromatic hydrocarbons (PAH), pollen, bacteria, and fungi (Ho *et al.*, 2005; Lee *et al.*, 2009; Maki *et al.*, 2008, 2010; Mori *et al.*, 2002, 2003; Tamamura *et al.*, 2006; Wu *et al.*, 2004; Yamada *et al.*, 2012; Yeo and Kim 2002). The characteristic of ASD can change during its long-range transport, and it is concerned that not only ASD themselves but also materials adhered to ASD cause adverse health effects (Ichinose *et al.*, 2005; Naota *et al.*, 2010, 2012; Yanagisawa *et al.*, 2007). Indeed, several epidemiological studies have suggested that exposure to ASD may increase daily mortality, cardiovascular, and respiratory problems in Seoul and Taipei (Chen *et al.*, 2004; Kwon *et al.*, 2002).

Airway epithelial cells are the important target because they are the first barriers to inhaled ASD and are capable of releasing mediators including cytokines known to be associated with health problems such as asthma. Epidemiologically, events of ASD have been associated with worsening of asthma (Kanatani *et al.*, 2010; Watanabe *et al.*, 2011). Previous studies have demonstrated that repeated airway exposure of mice to ASD induces lung inflammation in the presence or absence of allergen such as OVA, Dp and pollen (Ichinose *et al.*, 2005, 2006, 2009; Hiyoshi *et al.*, 2005). Therefore, ASD have a significant influence directly and/or indirectly to respiratory health. ASD are one of the risk factors for the development/exacerbation of asthma, however, the main mechanism remains unclear.

Damages of airway epithelial cells and pro-inflammatory responses are key events in the invasion and recognition of inhaled allergens which activates the network of dendritic cells (DC) and subsequent immune responses. DC has the ability to present allergens to naive T cells. When allergens enter the airways, DC in the airways capture allergens and migrate to secondary lymphoid tissues such as local lymph nodes. Activation of naive T cells initiates differentiation into effector T cells via an interaction with DC. Activation of allergen presentation by DC can lead to amplification of the allergen-related immunoglobulin production and allergic inflammation through the proliferation/activation of lymphocytes. On the other hand, asthma is characterized by accumulation of eosinophils, mast cells and  $\text{CD4}^+$  T lymphocytes producing interleukin-4 (Lambrecht and Hammad, 2012). Accordingly, DC and lymphocytes play important roles in the possible mechanisms of the development/exacerbation of asthma.

In the present study, we focused on the effects of ASD on airway epithelial cells which are the primary site of entry for inhaled xenobiotics in the respiratory system. Moreover, we examined their effects of ASD on bone marrow derived DC (BMDC) and

splenocytes from atopic prone NC/Nga mice.

## **Materials and methods**

### *Cell culture*

The BEAS-2B cell line, derived from human bronchial epithelial cells transformed by an adenovirus 12-SV40 hybrid virus, was purchased from European Collection of Cell Cultures (Salisbury, Wiltshire, UK). Airway epithelial cells were seeded in 96 well or 12 well collagen I coated plates and incubated for 72 h to reach semi-confluence in serum free-medium LHC-9 (Life technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

### *Animals and preparation of bone marrow cells and splenocytes*

Ten-week-old SPF NC/NgaTndCrlj male mice were purchased from Charles River Japan (Osaka, Japan). They were housed in an animal facility that was maintained at 24–26°C and a 12 h light/dark cycle under conventional conditions. The procedures of all animal studies were approved by the Animal Research Committee at Kyoto University. Mice were sacrificed by cervical dislocation, and exsanguinated from the cut abdominal aorta and vein. After removing the surrounding muscle tissue, the bones were left in 70% ethanol for 3 min and washed with RPMI1640 medium (Life technologies). Both ends of the bones were cut and then the marrow was flushed with RPMI1640 using a syringe with 24G needle. The marrow suspension was passed through sterile 250 nylon mesh to remove small pieces of bone and debris and red blood cells were lysed with BD PharmLyse (Becton dickinson, NJ, USA). Spleen was pushed through a sterile 200 mesh stainless steel sheet and red blood cells were also lysed with BD PharmLyse. The cells were centrifuged at 400×g for 5 min at 20°C. After washing with RPMI 1640, the cells were resuspended in culture medium R10, which was RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, MP Biomedicals Inc., Eschwege, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, St. Louis, MO, USA), and 50 µM 2-mercaptoethanol (Life technologies). The numbers of viable cells were determined by the trypan blue (Life technologies) exclusion method.

### *Differentiation of BMDC*

BMDC were differentiated using a modified protocol of Lutz *et al.* (1999). Bone marrow cells ( $4 \times 10^5$  /ml) were cultured in R10 medium containing 20 ng/ml GM-CSF. At day 3, another same volume of the medium containing 20 ng/ml GM-CSF was added to the culture. At day 6, half the culture medium was replaced with fresh medium. At day 8, non-adherent and loosely adherent cells were collected by gentle pipetting. The differentiated BMDC were centrifuged at 400×g for 5 min at 20°C and were resuspended in fresh medium. The numbers of viable cells were determined by the trypan blue (Life technologies) exclusion

method.

#### *Preparation of ASD*

ASD were collected on 1-3 May (ASD1) and on 12-14 May (ASD2), 2011 at Kitakyushu in Japan using an Andersen air sampler (AH-600, Shibata scientific technology LTD., Saitama, Japan) at a flow rate of 770 L/min. These ASD were sieved to a diameter of less than 6.8  $\mu\text{m}$  on teflon filter. A part of ASD1 was treated by heat at 360°C for 30 min (H-ASD) for excluding adhered substances which are sensitive to heat. The mean distribution peak of ASD1 and ASD2 diameter were observed at 3.8 or 2.3  $\mu\text{m}$ , respectively.

#### *Analysis of LPS and $\beta$ -Glucan in ASD.*

The contents of LPS and  $\beta$ -glucan in each particle sample were measured by the kinetic assay using Endospec ES-24S set (Seikagaku Corp., Tokyo, Japan) for LPS activity and Glucatell Kit (Associates of Cape Cod. Inc., MA, USA) for  $\beta$ -glucan activity, according to the manufacturer's protocol. In brief, approximately 2.5 mg of each particle sample was suspended in 1 mL water (LPS and  $\beta$ -glucan free; Seikagaku Corp., Tokyo, Japan) for 1 h and was placed on the bench top at room temperature for 2 h. The supernatants then were recovered and tested for LPS and  $\beta$ -glucan concentrations using PyroColor-MP:Chromogenic Diazo-Coupling Kit (Associates of Cape Cod. Inc., MA, USA) for endpoint-colorimetry. The detection limits for LPS and  $\beta$ -glucan were, 0.001 EU/mL, and 2 pg/mL, respectively.

#### *Experimental protocol*

After airway epithelial cells grew to semi-confluence in LHC-9, cells were exposed to ASD at a dose of 0, 3, 30, or 90  $\mu\text{g}/\text{mL}$  for 4 or 24 h. The cell viability, release of interleukin (IL)-6, IL-8, soluble intercellular adhesion molecule-1 (sICAM-1) in the culture supernatants, ICAM-1 mRNA expression, and ICAM-1 protein expression on cell surface were evaluated by WST-1 assay, enzyme-linked immunosorbent assay (ELISA), real-time RT-PCR, and fluorescence-activated cell sorter (FACS), respectively.

BMDC from NC/Nga mice were exposed to ASD at a dose of 0, 3, 30, or 90  $\mu\text{g}/\text{mL}$  for 24 h. The DEC205 protein expression on cell surface was evaluated by FACS.

Splenocytes from NC/Nga mice were exposed to ASD at a dose of 0, 3, 30, or 90  $\mu\text{g}/\text{mL}$  for 72 h. The cell proliferation was evaluated by 5-bromo-2'-deoxyuridine (BrdU) ELISA.

#### *Cell viability*

Cell viability was measured by WST-1 assay using Premix WST-1 Cell Proliferation Assay System (TaKaRa Bio Inc., Shiga, Japan). In brief, WST-1 reagent was added to each well of 96 well plate and mixed well by gently rocking the plate. Airway epithelial cells were

incubated with WST-1 reagent at 37°C for 3 h. After incubation, absorbance was measured on iMarkMicroplate Absorbance Reader (Bio-Rad Laboratories, Hercules, CA, USA) with wavelength at 450 nm and a reference wavelength at 630 nm. Results were expressed as the percentage of viable cells compared with untreated cells (0 µg/mL).

#### *Quantitation of inflammatory proteins in the culture supernatants*

After exposure to ASD, medium was harvested and centrifuged at 300 or 400×g for 5 min to remove floating cells. The final supernatants were stored at -80°C until analysis. The levels of IL-6, IL-8 (Thermo Scientific, Waltham, MA, USA) and sICAM-1 (eBioscience, San Diego, CA, USA) in the culture medium were measured by ELISA according to the manufacturer's instructions. Absorbance was measured on iMark Microplate Absorbance Reader (Bio-Rad Laboratories) with wavelength at 450 nm and a reference wavelength at 550 or 630 nm. The detection limits of IL-6, IL-8, and sICAM-1 assay were less than 1 pg/mL, 2 pg/mL, and 0.06 ng/mL, respectively.

#### *Extraction of RNA and quantitative RT-PCR analysis*

Total RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. This RNA was treated with DNase by RNase-Free DNase Set (Qiagen). Total RNA was reverse transcribed to cDNA using High Capacity RNA-to-cDNA kit according to the manufacturer's instructions (Life technologies). In brief, reverse transcription was carried out at 37°C for 60 min and at 95°C for 5 min in 20 µL reaction of RT Buffer Mix and RT Enzyme Mix. The mixture was then cooled to 4°C and stored at -20°C until use. The quantitation of mRNA expression was carried out using the ABI Prism 7000 Sequence Detection System (Life technologies). The PCR amplification was performed as described previously (Yanagisawa *et al.*, 2007). In brief, the PCR amplification performed with 50 µL final reaction mixture consisting of 25 µL TaqMan Gene Expression Master Mix (Life technologies), 2.5 µL TaqMan Gene Expression Assay contained with TaqMan probe and pair primers (Life technologies), 20.5 µL DNase/RNase-Free Distilled Water (Life technologies) and 2 µL cDNA. The cDNAs were amplified according to the thermal profile of 50°C for 2 min and then 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The relative intensity was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control gene. TaqMan probes and pair primers for ICAM-1 and GAPDH were designed by Life technologies, which did not disclose these sequences.

#### *FACS analysis*

For FACS analysis, the following monoclonal antibodies were used: ICAM-1 (HCD54, PE-conjugated, Biolegend, San Diego, CA, USA); DEC205 (NLDC-145,

PE-conjugated, Milteny Biotec GmbH, Gladbach Germany). After ASD exposure, the cells were resuspended in 50  $\mu$ L PBS with 0.3% bovine serum albumin and 0.05% sodium azide (Wako Pure Chemical Industries, Osaka, Japan) and were incubated with 0.1-1  $\mu$ g amount of each antibody for 10-45 min at 4°C. After incubation, the cells were washed, and the fluorescence was measured by a FACS Calibur (Becton Dickinson). For each sample, fluorescence data from 10,000 cells were collected, and positive cells expressed % events or mean fluorescence intensity (MFI) were calculated.

#### *Cell proliferation*

Cell proliferation was measured with a Cell-Proliferation-ELISA Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. This technique is based on the incorporation of the pyrimidine analogue BrdU instead of thymidine into the DNA of proliferating cells. BrdU incorporated into DNA is measured by a sandwich-type enzyme immunoassay using monoclonal anti-BrdU antibodies. Splenocytes were exposed to ASD for 72 h, and cell proliferation was measured by adding BrdU to each well 20 h before the measurement. Absorbance was measured on iMark Microplate Absorbance Reader (Bio-Rad Laboratories) with wavelength at 450 nm and a reference wavelength at 630 nm.

#### *Statistical analysis*

Data were represented as mean  $\pm$  standard error of the mean (S.E.M.) for each experimental group (n=4). Differences among groups were analyzed using Tukey multiple comparison test (Excel Statistics 2010, Social Survey Research Information Co. Ltd., Tokyo, Japan). A *p* value < 0.05 was considered to indicate a significant difference.

## **Results**

#### *Concentration of LPS and $\beta$ -glucan in ASD.*

The contents of LPS in ASD1, ASD2, and H-ASD were 0.2864, 0.9993, and 0.0038 EU/mg, respectively. The contents of  $\beta$ -glucan in ASD1, ASD2, and H-ASD were 0.3549, 28.1611 ng/mg, and not detected, respectively.

#### *Effects of ASD on viability of airway epithelial cells*

We investigated the effect of ASD on the viability of airway epithelial cells after exposure to ASD for 24 h. All ASD reduced cell viability in a dose-related manner (Fig.1; *p* < 0.05 vs. 0  $\mu$ g/mL). All ASD at a dose of 90  $\mu$ g/mL induced approximate 10-15% decrease in cellular viability.

#### *Effects of ASD on cytokine production of airway epithelial cells*



To evaluate whether ASD have pro-inflammatory effects on airway epithelial cells, we examined the production of IL-6 and IL-8 after exposure to ASD for 24 h (Figs 2a and 2b). ASD1 and ASD2 dose-dependently increased protein release of IL-6 and IL-8 ( $p < 0.05$  vs. 0  $\mu\text{g/mL}$ ), and the levels of IL-6 and IL-8 released by ASD1 at a dose of 90  $\mu\text{g/mL}$  were higher than those released by ASD2 at the same dose ( $p < 0.05$  vs. ASD1), whereas H-ASD did not release the proteins.

#### *Effects of ASD on ICAM-1 expression of airway epithelial cells*

Expression of ICAM-1 in airway epithelial cells was examined after exposure to ASD for 4 or 24 h. As a result of ASD exposure for 4 h, ASD1 and ASD2 increased ICAM-1 mRNA level in a dose-related manner (Fig. 3a;  $p < 0.05$  vs. 0  $\mu\text{g/mL}$ ). Their enhancing effect on ICAM-1 mRNA at a dose of 90  $\mu\text{g/mL}$  was much greater with ASD1 than with ASD2 (Fig. 3a;  $p < 0.05$  vs. ASD1), whereas H-ASD showed no changes. Next, we measured ICAM-1 protein expression on cell surface after ASD exposure for 24 h by FACS analysis. ASD1 and ASD2 at a dose of 90  $\mu\text{g/mL}$  increased ICAM-1 expression (Fig. 3b;  $p < 0.05$  vs. 0  $\mu\text{g/mL}$ ). The levels of ICAM-1 induced by ASD1 at doses of 3, 30  $\mu\text{g/mL}$  were higher than those induced by ASD2 at the same doses (Fig 3b;  $p < 0.05$  vs. ASD1), whereas H-ASD did not increase them. Subsequently to FACS analysis, we determined the release of sICAM-1 in the culture supernatants. ASD1 and ASD2 exposure for 24 h increased sICAM-1 at a dose of 90  $\mu\text{g/mL}$  (Fig. 3c;  $p < 0.05$  vs. 0  $\mu\text{g/mL}$ ). ASD1 exposure tended to show more inducible sICAM-1 level than ASD2 exposure. In contrast, ICAM-1 expression in airway epithelial cells caused by H-ASD showed no change.

#### *Effects of ASD on DEC205 expression of BMDC*

To determine the effect of exposure to ASD for 24 h on the surface expression of DEC205, we analyzed the expression patterns on BMDC (Fig 4). Overall, ASD1 and ASD2 increased the surface expression of DEC205 molecule in a dose-dependent manner ( $p < 0.05$  vs. 0  $\mu\text{g/mL}$ ). On the other hand, H-ASD did not elevate the expression of DEC205.

#### *Effects of ASD on proliferation of splenocytes*

Proliferation of splenocytes was also examined after exposure to ASD for 72 h. ASD1 and ASD2 enhanced proliferation of splenocytes (Fig. 5;  $p < 0.05$  vs. 0  $\mu\text{g/mL}$ ). On the other hand, H-ASD did not induce proliferation.

## **Discussion**

Exposure of airway epithelial cells to non-heated ASD facilitated cellular viability and pro-inflammatory responses, via the expression of IL-6, IL-8 and ICAM-1. Furthermore, exposure of immune cells to ASD also activated the network of DC and subsequent immune

response, at least via the expression of DEC205 on BMDC and the proliferation of splenocytes. However, H-ASD did not affect them. Changes in some biomarkers of pro-inflammatory responses showed different profile between events of ASD.

Previous experimental reports have indicated that ASD exhibit cytotoxic effect *in vitro* (Choi *et al.*, 2011; Kim *et al.*, 2003; Yamada *et al.*, 2012) and *in vivo* (Lei *et al.*, 2004; Naota *et al.*, 2010; Ichinose *et al.*, 2005). On the basis of these reports, ASD themselves which are free from chemical and biological pollutants and components adhered to ASD have possibility to cause damage in several cell population. We also observed that ASD decreased cell viability in airway epithelial cells in the present study. ASD1 and H-ASD indicated similar action from the point of view of cellular viability in airway epithelial cells. In short, physical stimulation caused by ASD, components of ASD themselves or heat-resistance substances adhered to ASD may contribute to cellular viability. Although the mechanism by which ASD reduce cellular viability remains unclear, some factors such as reactive oxygen species, Fenton activity and reactive nitrogen species might be related with ASD-induced cytotoxicity (Kim *et al.*, 2003).

Airway epithelial cells, which play important roles in physical barrier and immunological responses against dust, are source of cytokines such as IL-6 and IL-8. IL-6 and IL-8 are pro-inflammatory cytokines induced by response to environmental insults and play important roles in acute inflammation in respiratory system by stimulating lymphocytes, inducing neutrophils recruitment and up-regulating mucin secretion (Bautista *et al.*, 2009; Chen *et al.*, 2003; Levine *et al.*, 1993; Thacker *et al.*, 2006). Indeed, various chemicals and allergens, including diesel exhaust particles, polycyclic aromatic hydrocarbons and dust mite stimulate IL-6 or IL-8 production from airway epithelial cells (King *et al.*, 1998; Park *et al.*, 2009; Totlandsdal *et al.*, 2012) as well as human primary airway epithelial cells (Bayam *et al.*, 1998a; Lordan *et al.*, 2002; Ohtoshi *et al.*, 1998). Moreover, Bayram *et al.* (1998b) have reported that release of inflammatory mediators including IL-8 from bronchial epithelial cells of asthmatic subjects to diesel exhaust particles may possibly result in exacerbation of their symptoms. Also in the present study, ASD significantly elevated IL-6 and IL-8 protein release. It has been reported that ASD which are obtained from different storm events elevate IL-6 and IL-8 mRNA in human epidermal keratinocytes, and that the enhancing effects differ by the day of the ASD events (Choi *et al.*, 2011). Kim *et al.* (2011) have also suggested that ASD elevate IL-8 release and mucin gene expression in human primary nasal polyp epithelial cells. In an animal study, ASD have increased IL-6 protein in bronchoalveolar lavage fluids of rats (Lei *et al.*, 2004). Accordingly, the present results are consistent with the previous studies and support that ASD induce pro-inflammatory cytokines from airway epithelial cells. The effects differ by the day of storm events, which may be due to difference in dust sources, transfer pathways, and in chemical and biological compositions.

ICAM-1 is a transmembrane glycoprotein of the immunoglobulin superfamily of

adhesion molecules, and is constitutive on many cell types. Moreover, ICAM-1 is an inducible ligand for lymphocyte function associated antigen (LFA-1) and implicated in leucocyte recruitment toward target tissues. Finally, soluble ICAM-1 is released from cell-surface ICAM-1 (Lawson and Wolf 2009). It has been reported that increased level of serum sICAM-1 is related to severity of bronchial asthma, in brief, sICAM-1 is released into the serum of asthmatic patients in response to inflammatory mediators (Shiota *et al.*, 1996). Previous reports have suggested that diesel exhaust particles up-regulate expression of ICAM-1 in airway epithelial cells (Takizawa *et al.*, 2000), which indicates that ICAM-1 is a marker of inflammatory responses against air pollutants. We investigated ICAM-1 expression on airway epithelial cells after ASD exposure. ASD1 and ASD2 elevated ICAM-1 mRNA, cell surface protein and soluble protein in culture medium. In contrast, H-ASD did not increase them. To our knowledge, this is the first experimental demonstration of ASD's enhancing effects on ICAM-1 expression. It may underlie intracellular mechanisms, such as the involvement of NF- $\kappa$ B and MAPK signal transduction pathways which are important in the transcription of ICAM-1 (Lawson and Wolf 2009).

Several studies have identified a critical role for DC in allergens-related airway inflammation. After detecting environmental stimuli, airway epithelial cells secrete endogenous signals such as thymic stromal lymphopoietin (TSLP), thereby activate dendritic cells and subsequent immune responses (Lambrecht *et al.*, 2012). Bleck *et al.* (2006) have showed that diesel exhaust particles induce monocyte-derived DC maturation via primary cultures of human airway epithelial cells-DC interactions. Although it has been reported that ASD can enhance chemical mediator release in basophilic cells (Yamada *et al.*, 2012), the effects of ASD on immune cells such as DC have been less studied. We first discovered that ASD could potentiate immune activation in BMDC and splenocytes from atopy-prone mice. ASD significantly increased the surface expression of molecules related to DC maturation/activations as DEC205. DEC205 is a member of the macrophage mannose receptor family. This molecule is known to mediate the capture and internalization of ligands for subsequent processing and presentation by dendritic cells (Jiang *et al.*, 1995). Koike *et al.* (2008, 2009) have reported that carbon black nanoparticles and di-(2-ethylhexyl) phthalate can promote the maturation/activation and function of DEC205 on BMDC, which could be related to their enhancing effects on allergic diseases or responses. The present study also demonstrated ASD1 and ASD2 activate function of BMDC, whereas H-ASD did not.

Previous reports have indicated that various environmental pollutants activate splenocytes (Koike *et al.*, 2009, 2010; Kobayashi *et al.*, 2012). Our result showed ASD1 and ASD2 increased proliferation of splenocytes, although its detailed cellular contribution has not been established. We examined the expression of T cell receptor (TCR) and cytokine production of splenocytes after ASD exposure. ASD1 and ASD2 exposure for 24 and 72 h did not increase TCR positive cell (%) and IL-4 release (data not shown). T cell activation

may be related with proliferation of splenocytes a little. Exposure to ASD1 and ASD2 for 72 h tended to increase IL-2 release, although their levels were low (the levels detected in ASD1, ASD2 and H-ASD at a dose of 90  $\mu\text{g/mL}$  were  $26.5 \pm 5.7$ ,  $24.4 \pm 2.9$   $\text{pg/mL}$ , not reliably detectable, respectively. The level in control (0  $\mu\text{g/mL}$ ) was not detected). Further investigations are needed to understand the main factor which contributes to the proliferation of splenocytes.

Many studies have reported that ASD provide various chemical and biological materials (Ho *et al.*, 2005; Lee *et al.*, 2009; Maki *et al.*, 2008, 2010; Mori *et al.*, 2002, 2003; Tamamura *et al.*, 2006; Wu *et al.*, 2004; Yamada *et al.*, 2012; Yeo and Kim 2002). These materials mostly disappeared by heating at 360°C for 30 min, or might form another product in part. Endotoxin and  $\beta$ -glucan are components of the outer membrane of gram-negative bacteria and fungi walls, respectively, which are associated with an increase of respiratory disease (Douwes *et al.*, 2003). The contents of LPS in ASD1, ASD2, and H-ASD were 0.2864, 0.9993, and 0.0038 EU/mg. The contents of  $\beta$ -glucan in ASD1, ASD2, and H-ASD1 were 0.3549, 28.1611 ng/mg, and not detected. The levels of LPS and  $\beta$ -glucan in H-ASD were extremely low compared with non-heated ASD. It has been suggested that crude ASD cause bronchitis and alveolitis, whereas heated-ASD cause very slight pathological changes in murine lungs. Moreover, microbial materials adhered to ASD may induce immune response via the activation of Toll-like receptor 2 using RAW264.7 cells (He *et al.*, 2011; Ichinose *et al.*, 2008). The present study also showed that there was significant difference in particle behaviour by heat treatment, and was in accordance with the previous studies. However, LPS and  $\beta$ -glucan levels in ASD1 were lower than those in ASD2, and activating effects in respiratory system by ASD1 were higher than those by ASD2. Apart from LPS and  $\beta$ -glucan, previous studies have indicated that co-exposure of  $\text{SO}_4^{2-}$  or constituent of cedar pollen and ASD enhances inflammatory responses (Ichinose *et al.*, 2005, 2009; Hiyoshi *et al.*, 2005; Yamada *et al.*, 2012). PAH and  $\text{NO}_2$  which can be mixed into the ASD may also epidemiologically have adverse respiratory effects (Delfino RJ, 2002). Hodgkins *et al.* (2010) have suggested that  $\text{NO}_2$  inhalation induces maturation of pulmonary  $\text{CD11c}^+$  cells (including DC) which promote antigen specific  $\text{CD4}^+$  T cell polarization. Therefore, in addition to biological materials, the toxic chemical materials which are sensitive to heat treatment during transport may strongly influence human respiratory health problems.

Exposure of airway epithelial cells to ASD reduced cellular viability and facilitated pro-inflammatory responses. Furthermore, exposure of immune cells to ASD also activated the network of DC and subsequent immune response. However, heat-treated ASD did not affect those biomarkers. The present study provides evidence that ASD can contribute to exacerbation of respiratory and immunological diseases, and the difference in the events of ASD and in heat-sensitive components adhered to ASD can enhance the health effects.

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## Figure legend

**Figure 1.** Effects of ASD on the viability of airway epithelial cells. Cell viability was assessed by WST-1 assays. Cells were treated with the indicated concentrations of ASD for 24 h. Data are presented as the percentage of the viability of the control. Data are mean  $\pm$  S.E.M. of four individual cultures. \*  $p < 0.05$  versus 0  $\mu\text{g/mL}$ . #  $p < 0.05$  versus ASD1.

**Figure 2.** IL-6 and IL-8 production from airway epithelial cells in response to ASD. The levels of (a) IL-6 and (b) IL-8 in the culture supernatant were measured by ELISA. Data are mean  $\pm$  S.E.M. of four individual cultures. \*  $p < 0.05$  versus 0  $\mu\text{g/mL}$ . #  $p < 0.05$  versus ASD1.

**Figure 3.** Effects of ASD on the ICAM-1 level of airway epithelial cells. (a) Expression of ICAM-1 mRNA in airway epithelial cells was examined using real-time RT-PCR after ASD exposure for 4 h. (b) Expression of ICAM-1 on airway epithelial cells was analyzed by FACS after ASD exposure for 24 h. (c) The level of sICAM-1 in the culture supernatant was measured by ELISA after ASD exposure for 24 h. \*  $p < 0.05$  versus 0  $\mu\text{g/mL}$ . #  $p < 0.05$  versus ASD1.

**Figure 4.** Effects of ASD on DEC205 expression of BMDC. BMDC from NC/Nga mice were treated with the indicated concentrations of ASD for 24 h, and were analyzed by FACS. Data are presented as positive cells expressed % events. Data are mean  $\pm$  S.E.M. of four individual cultures. \*  $p < 0.05$  versus 0  $\mu\text{g/mL}$ . #  $p < 0.05$  versus ASD1.

**Figure 5.** Effect of ASD on the proliferation of splenocytes from NC/Nga mice. Cells were treated with the indicated concentrations of ASD for 72 h. Data are presented as the percentage of the proliferation of the control. Data are mean  $\pm$  S.E.M. of four individual cultures. \*  $p < 0.05$  versus 0  $\mu\text{g/mL}$ . #  $p < 0.05$  versus ASD1.

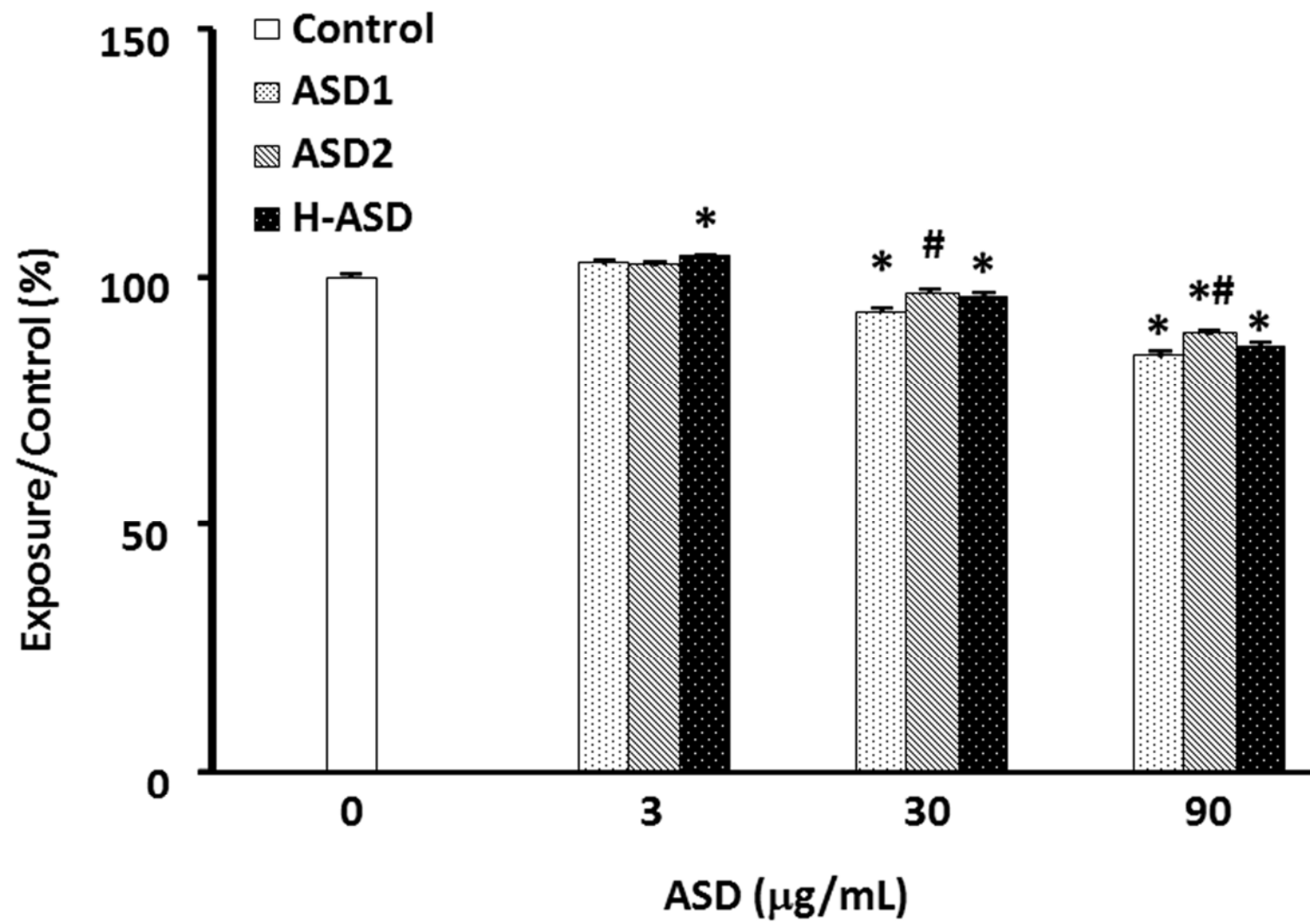


Figure 1

**a**

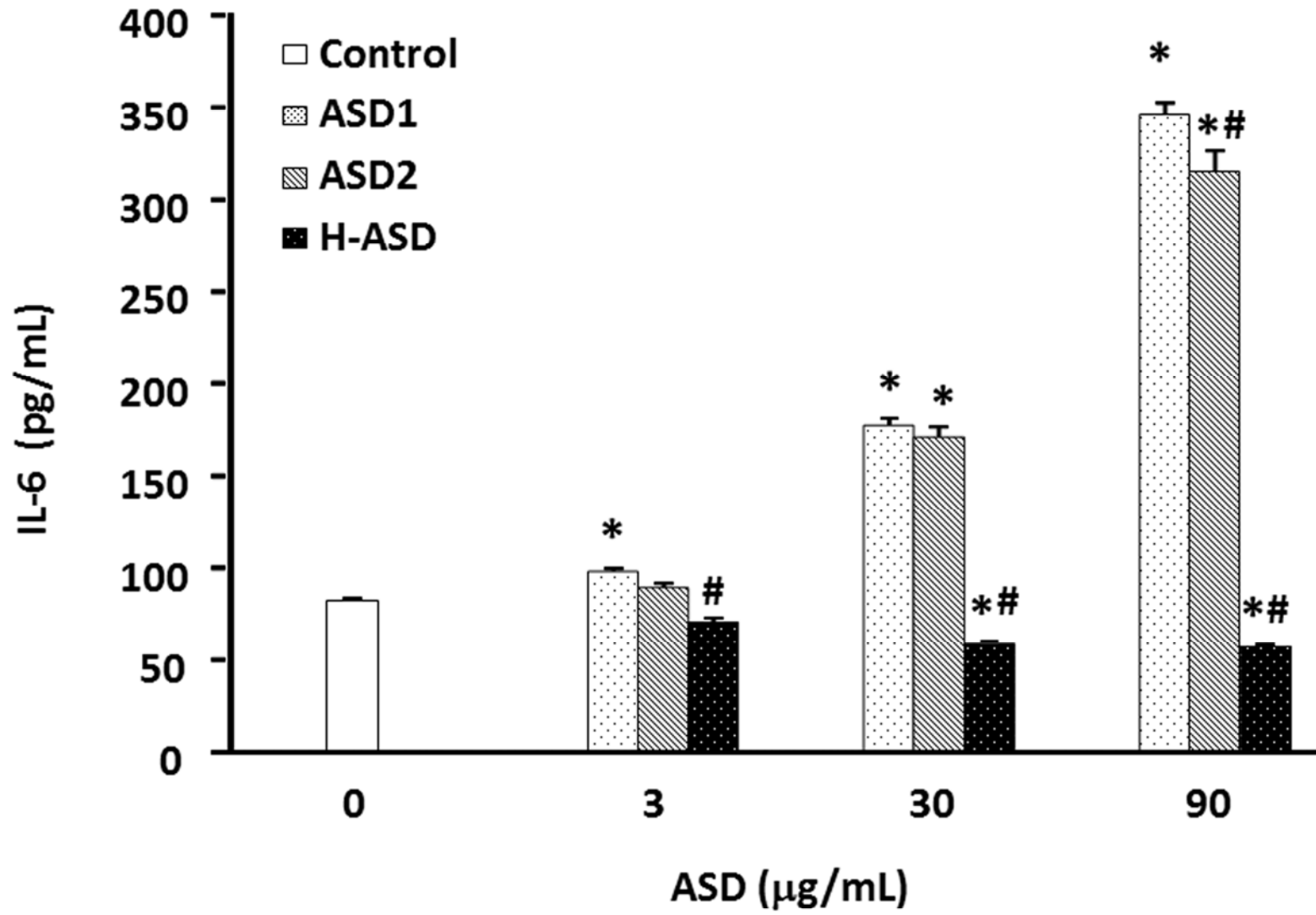


Figure 2(a)

**b**

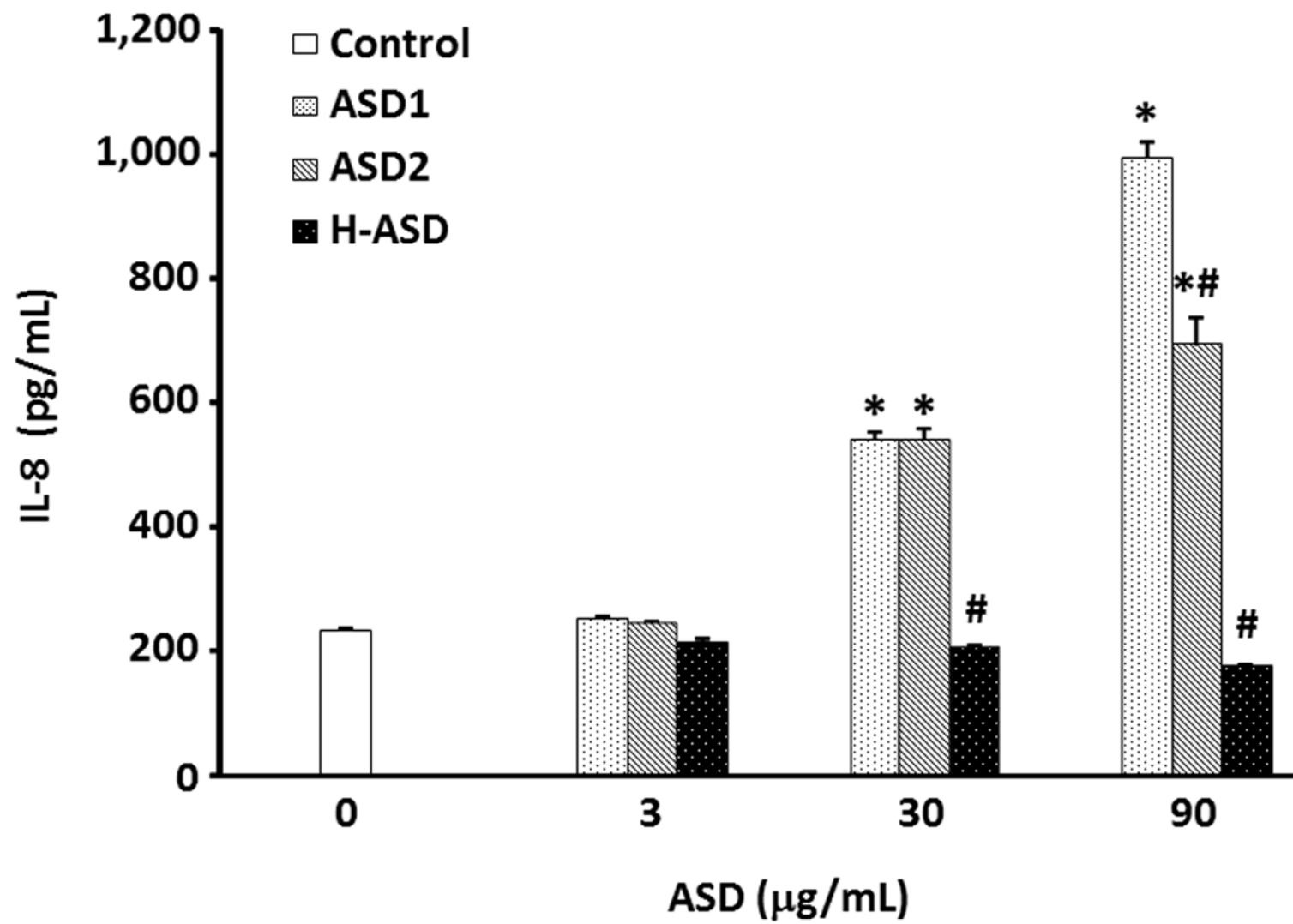


Figure 2(b)

**a**

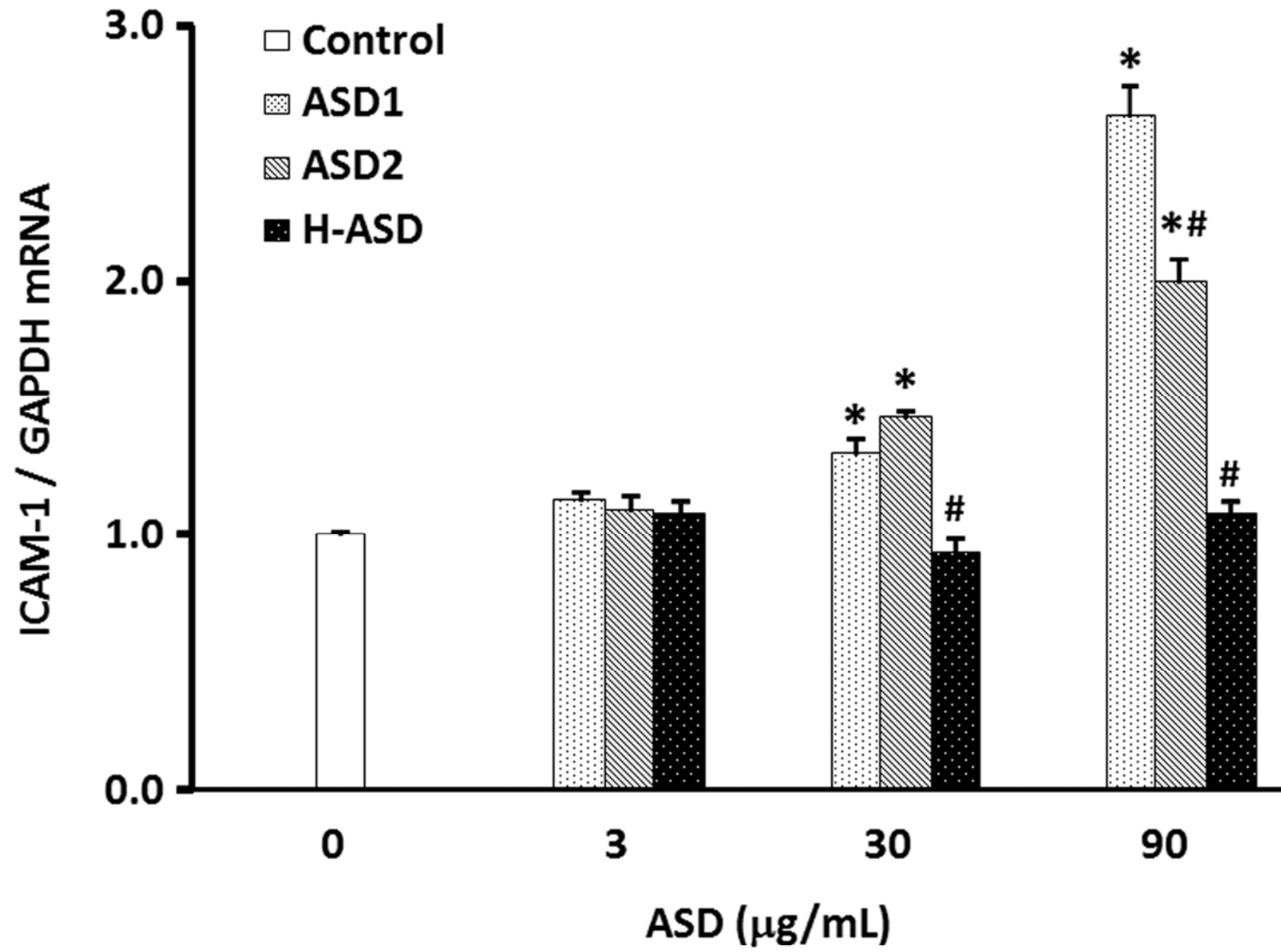


Figure 3(a)

**b**

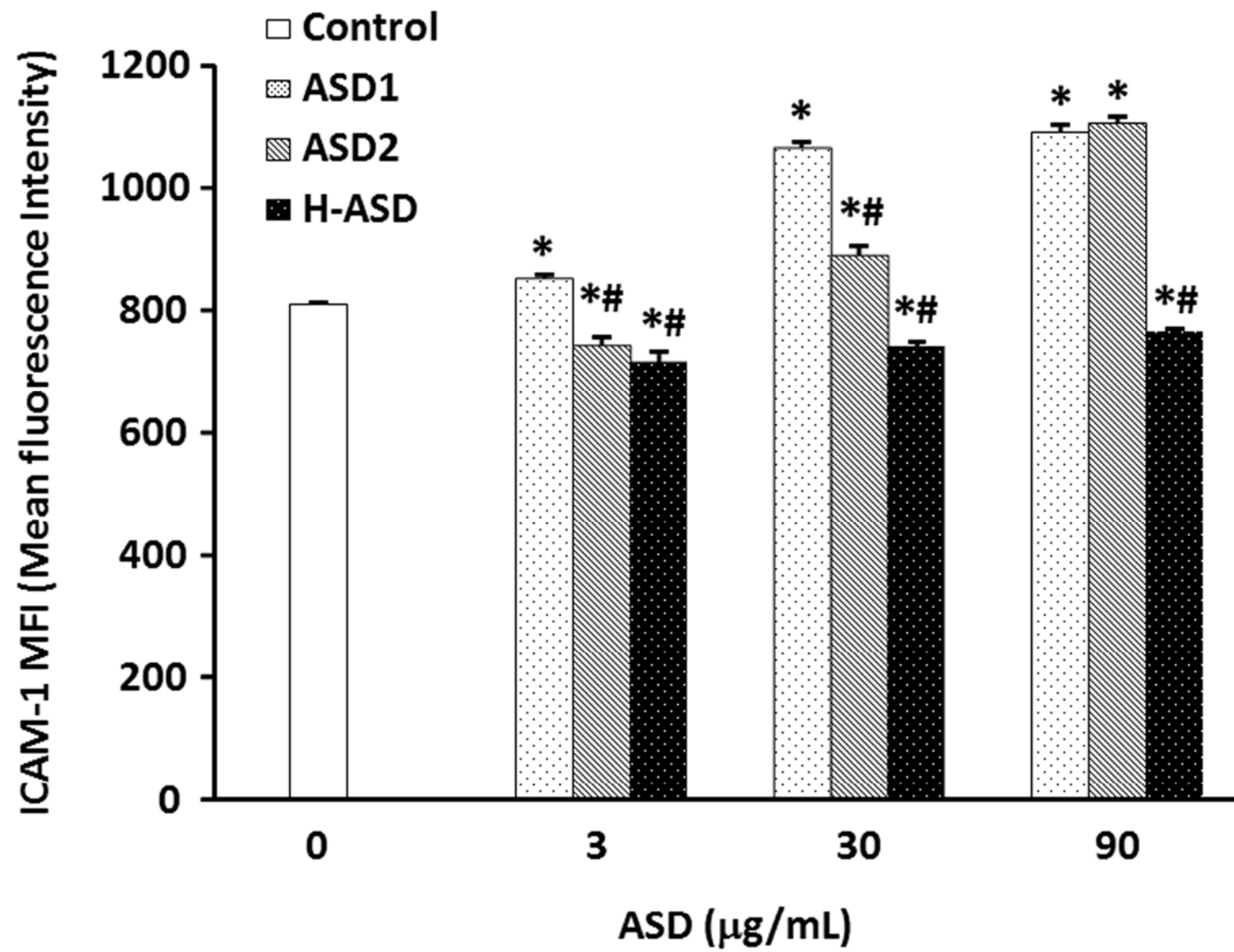


Figure 3(b)



**C**

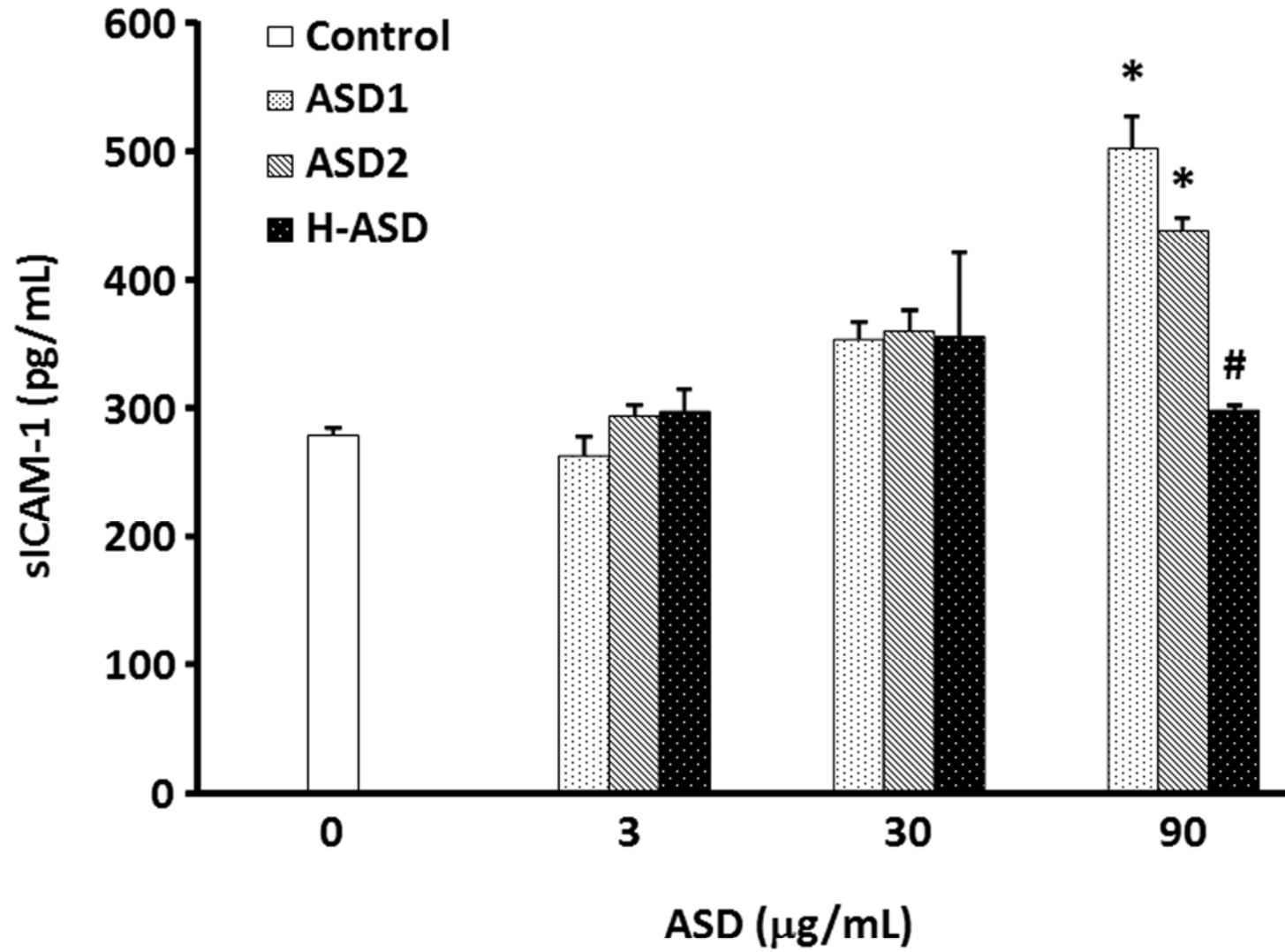


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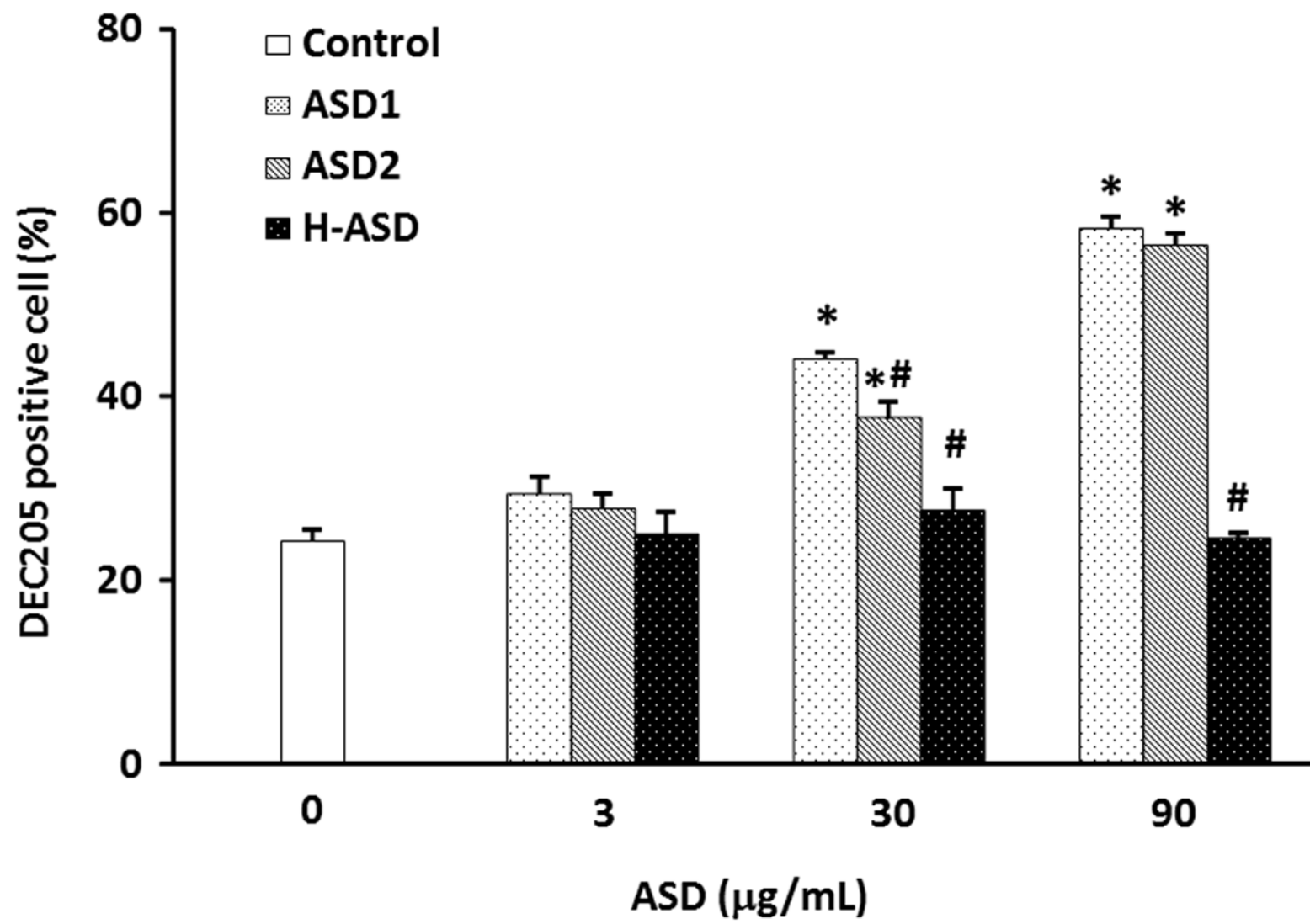


Figure 4

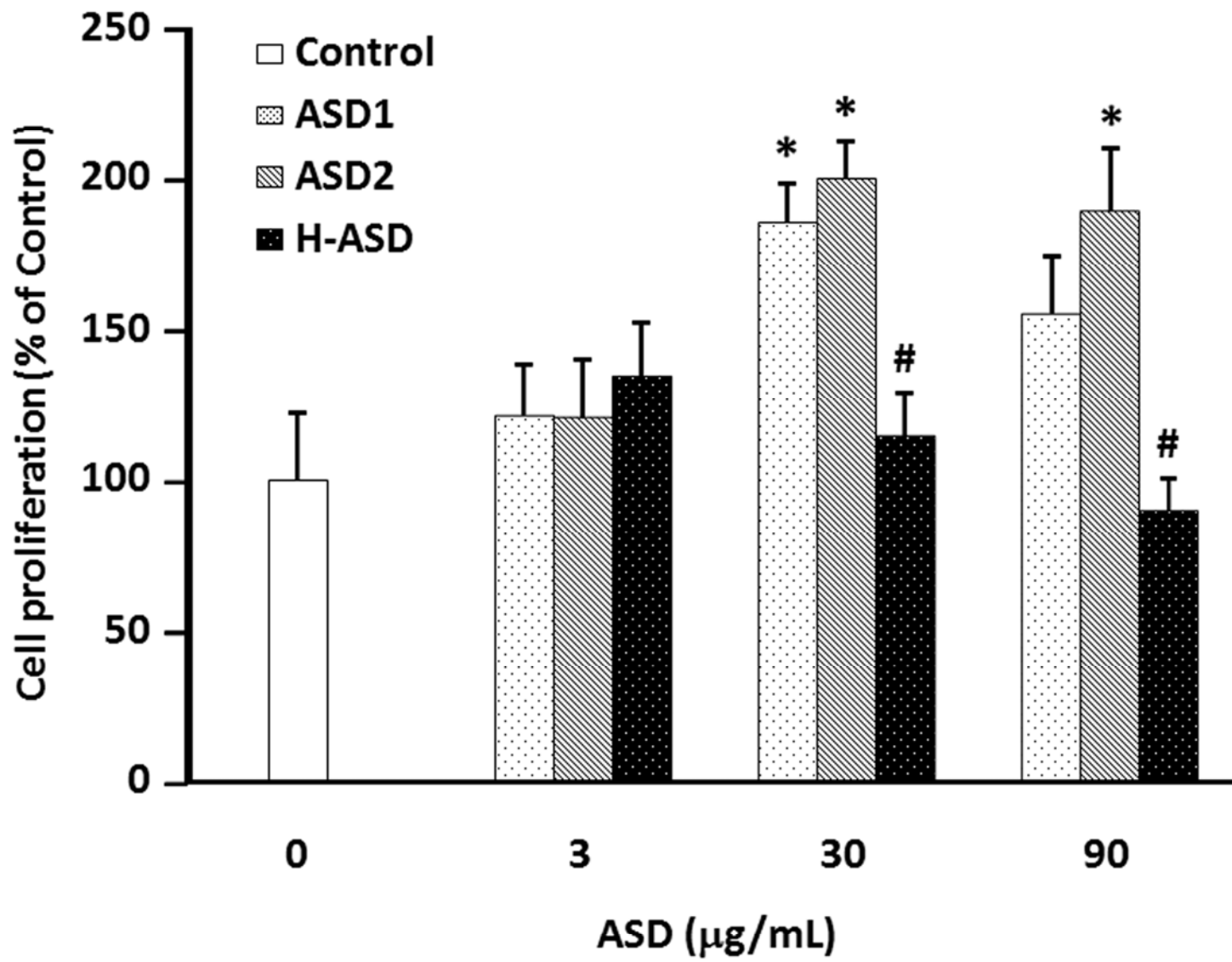


Figure 5