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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.5b04861 • Publication Date (Web): 09 Dec 2015

Downloaded from <http://pubs.acs.org> on December 11, 2015

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**Protective effects of diallyl sulfide on ovalbumin-induced pulmonary
inflammation of allergic asthma mice by microRNA-144, -34a and
-34b/c-modulated Nrf2 activation**

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Running title: Diallyl sulfide alleviates allergic asthma *in vivo*

Keywords: Diallyl sulfide; Ovalbumin; Asthma; Inflammation; Nrf2

Abbreviations

ARE, antioxidant response element; BALF, bronchoalveolar lavage fluid; DAS.

26 diallyl sulfide; DEX, dexamethasone; HO-1, heme oxygenase-1; Keap1, Kelch-like
27 ECH-associated protein 1; 8-iso-PGF2 α , 8-iso-prostaglandin F2 α ; NF- κ B, nuclear
28 factor kappa B; Nrf2, nuclear factor-erythroid 2 related factor 2; 8-OHdG,
29 8-hydroxy-2'-deoxyguanosine; OVA, ovalbumin; TNF α , tumor necrosis factor alpha
30

31 **Abstract**

32

33 Allergic airway disorder is characterized by an increase in the level of reactive oxygen
34 species (ROS). The induction of inflammation and hyperresponsiveness by an
35 allergen was ameliorated by antioxidants *in vivo*. This study investigated the
36 protective effects and underlying mechanism of diallyl sulfide (DAS) on ovalbumin
37 (OVA)-induced allergic asthma of BALB/c mice. The animals were intraperitoneally
38 sensitized by inhaling OVA to induce chronic airway inflammation. By administrating
39 DAS, a decrease of the infiltrated inflammatory cell counts and the levels of IL-4 and
40 IL-10 in bronchoalveolar lavage fluid as well as the OVA-specific immunoglobulin E
41 levels in sera were observed. DAS also effectively inhibited OVA-induced
42 inflammatory cell infiltration and mucus hypersecretion in lung tissue. Several
43 OVA-induced inflammatory factors (ROS, 8-hydroxy-2'-deoxyguanosine,
44 8-iso-prostaglandin F2 α , and NF- κ B) were inhibited by DAS. In addition, DAS
45 increased OVA inhalation-reduced levels of Nrf2 activation by regulating
46 microRNA-144, -34a and -34b/c. Together, the pathogenesis of OVA-induced asthma
47 is highly associated with oxidative stress, and DAS may be an effective supplement to
48 alleviate this disease.

49

50

51 INTRODUCTION

52 Asthma is a chronic inflammatory disease that is underpinned by the recruitment
53 and activation of inflammatory cells into the airways. The inflammation in allergic
54 asthma is characterized by increasing numbers of inflammatory cells in lung tissue
55 and the blood, and the impacts subsequently enhance production of Th2 cytokines
56 (IL-4, IL-5, and IL-10).¹ Antioxidant defenses can be overwhelmed due to the
57 generation of reactive oxygen species (ROS), which cause oxidative stress in the
58 lung.² High oxidative stress has been commonly observed in the airways of asthmatic
59 subjects,^{3, 4} and airway function is therefore gradually damaged.²

60 Nuclear factor-erythroid 2 related factor 2 (Nrf2) is a transcription factor that
61 heterodimerizes at *cis*-acting antioxidant response elements (AREs). AREs are located
62 at proximal promoters of genes, which encode proteins for managing adaptive
63 antioxidant defense, xenobiotic detoxification, proteasome maintenance, cell
64 proliferation, and cell cycle regulation.⁵ Moreover, Nrf2 has direct or indirect
65 modulatory effects on various immune and inflammatory pathways.⁶ Disrupting Nrf2
66 expression can augment airway inflammation and hyperresponsiveness in a mouse
67 model of allergic asthma, suggesting that Nrf2 pathway plays a key role *in vivo* for
68 protecting allergic and asthmatic responses.⁷

69 Dietary antioxidants have the capacity to boost the antioxidant defenses of the host,
70 thereby suppressing oxidative stress.^{3, 4} Diallyl sulfide (DAS), an enriched component
71 found in garlic, is one of several natural organosulfur compounds that are beneficial to
72 human health.^{8, 9} It has been reported that DAS possesses an anti-cancer effect to
73 induce colon cancer cell apoptosis.¹⁰ Moreover, DAS shows biological activities in
74 the modulation of phase II drug metabolizing enzymes, including glutathione
75 *S*-transferase (GST) and NAD(P)H:quinone oxidoreductase 1 (NQO1). The

76 detoxifying capability of DAS may potentially reduce the oxidative stress in the liver
77 and protect against oxidative injury in tissues.¹¹ Recently, DAS was demonstrated to
78 enhance pulmonary antioxidant enzyme activity through the activation of Nrf2 in SD
79 rats and in human embryonic MRC-5 lung cells by extracellular signal-regulated
80 kinase (ERK)/p38 signaling.¹² The reduction of proinflammatory responses which
81 were induced by tumor necrosis factor alpha (TNF- α) and IL-1 β through the Nrf2
82 pathway has also been observed in rat aortic smooth muscle A7r5 cells.¹³ In addition,
83 diallyl disulfide, which is another sulfur compound present in garlic oil, has been
84 shown to stimulate Nrf2/HO-1 signaling and to modulate NF- κ B pathway in airway
85 inflammation *in vivo*.¹⁴ In this study, we investigated the potential protective effect of
86 DAS against oxidative stress and lung damage in a murine model of ovalbumin
87 (OVA)-induced allergic airway inflammation compared to treatment with a steroid
88 drug, dexamethasone (DEX).

89

90

91 MATERIALS AND METHODS

92 **Chemicals.** Diallyl sulfide (DAS) was obtained from Alfa Aesar (Ward Hill, MA,
93 USA). Ovalbumin, Alhydrogel and DEX were obtained from Sigma-Aldrich (St.
94 Louis, MO, USA). Anti-phospho-I κ B α , anti-phospho-p65, anti-lamin B1, anti-Keap1,
95 anti-DJ-1, and anti- β -actin antibodies were obtained from Cell Signaling Technology
96 (Beverly, MA, USA). Antibodies against Nrf2 and HO-1 were obtained from Santa
97 Cruz Biotechnology (Santa Cruz, CA, USA). All fine chemicals were obtained from
98 Showa Chemical (Tokyo, Japan) or Sigma-Aldrich (St. Louis, MO, USA).

99

100 **Animals.** Male BALB/c mice (age 6-8 weeks) were purchased from BioLASCO

101 Taiwan Co. Ltd. (Taipei, Taiwan). The mice were divided into seven groups (eight
102 mice per each group) and administered food and water *ad libitum*. All experimental
103 animal procedures were conducted according to the guidelines of the National
104 Institutes of Health. The experimental protocols were approved by the Institutional
105 Animal Care and Use Committee of National Chung Hsing University, Taichung,
106 Taiwan (IACUC Approval No: 100-77).

107

108 **Induction of Allergic Airway Inflammation.** Five groups of mice were sensitized
109 by intraperitoneal injection with 0.2 mL sensitizing solution containing 100 µg OVA
110 emulsified in 1 mg aluminum hydroxide on day 7 and 14. The animals were then
111 challenged with 1% aerosolized OVA for 30 min daily on day 22-24. DAS (50, 100
112 and 250 mg/kg bw) or DEX positive control (0.2 mg/kg bw) was orally administered
113 for ten consecutive days beginning on day 15. The control group and the DAS-treated
114 (250 mg/kg bw) group received the same sensitization schedule.

115

116 **Collection and Analysis of Inflammatory Cells in the Blood and**
117 **Bronchoalveolar Lavage Fluid (BALF).** Blood from mice was collected to obtain
118 serum, and the serum was stored at -80°C until analysis. BALF was obtained by
119 cannulating the trachea and washing the airway lumina with 1 mL Hank's buffered
120 salt solution (HBSS). The instilled fluid was recovered after each wash. The fluid was
121 then pooled and centrifuged (150×g, 4°C, 5 min) to collect the cell pellet. The cell
122 pellet was treated with erythrocyte lysis buffer and resuspended in HBSS (100 µL).
123 Cell number was detected utilizing a standard hemocytometer. Cells were
124 cytocentrifuged and stained with Diff-Quik staining kit (International Reagents Co.,
125 Kobe, Japan), and cell types were enumerated according to morphological criteria

126 with a minimum of 200 cells counted per slide. Slides were examined in a blinded
127 fashion.

128

129 **Histological Examination.** The left lung was staining with either
130 hematoxylin-eosin (HE) or periodic acid-Schiff (PAS) after being immersed overnight
131 in 10% neutral buffered formalin, embedded in paraffin, and sectioned (5 μ m
132 thickness). The samples were examined and conducted by a light microscope attached
133 to an image-analysis system (Image-Pro Plus, Media Cybernetics Inc., Warrendale,
134 PA, USA). The number of PAS-negative and PAS-positive cells (goblet cells) in an
135 individual bronchus was determined as previously described^{15, 16} following by
136 quantifying the level of mucus expression in the airway. The percentage of
137 PAS-positive cells per bronchus was calculated in total epithelial cells.¹⁷

138

139 **Determination of Cytokine Production and Biomarkers of Oxidative Stress.**

140 Supernatant obtained from the BALF was stored at -80°C until measurement, and
141 ELISA experiments were performed according to the manufacturer's instructions. The
142 levels of cytokines, including IL-4, IL-10, IL-12 and IFN- γ , and biomarkers,
143 including 8-hydroxy-2'-deoxyguanosine (8-OHdG) and 8-iso-prostaglandin F 2α
144 (8-iso-PGF 2α), in supernatants of BALF were measured with ELISA kits from
145 Abcam (Cambridge, MA, USA) and Cayman Chemicals (Ann Arbor, MI, USA),
146 respectively.

147

148 **Assay of Intracellular ROS Production.** The intracellular ROS was detected
149 using a fluorescent probe, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA,
150 Molecular Probes, Eugene, OR, USA). An aliquot of 95 μ L BALF was loaded into a

151 96-well plate, and 5 μ L DCFH-DA (final concentration: 20 μ M) was added. The
152 intensity of DCF fluorescence was monitored using a FLUOstar galaxy fluorescence
153 plate reader (BMG LABTECH, Ortenberg, Germany).

154

155 **Measurement of Total IgE and OVA-specific IgE in Serum.** ELISA analyses
156 were performed according to the manufacturer's instructions. The levels of total and
157 OVA-specific IgE in serum were determined by ELISA kit (Mouse IgE ELISA Set,
158 BD OptEIA, San Diego, CA, USA)

159

160 **RNA Extraction and RT-PCR.** Lung tissues were washed with PBS, and TRIzol
161 Reagent (Invitrogen, Carlsbad, CA, USA) was used to extract the intracellular RNA.
162 Next, the RevertAid First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD,
163 USA) was used to reverse-translate RNA into cDNA, and Taq DNA polymerase
164 (Fermentas) was used to amplify the desired cDNA sequence. The primers used to
165 amplify target genes were as follows: TNF- α , forward:
166 AGCACAGAAAGCATGATCCG, reverse: CTGATGAGAGGGAGGCCATT; IL-1 β ,
167 forward: GCGAGACTTCTCACCAAACA, reverse:
168 GTTACCCCCATGACTCCAGA; catalase, forward:
169 AGAGCGGATTCCTGAGAGAGTG, reverse: CAAACCCAC
170 GAGGGTCACGAAC; superoxide dismutase (SOD), forward:
171 GTTGGAGACCTGGGCAATGTGG, reverse: CCACAAGCCAAGCGGCTTCCAG;
172 glutathione peroxidase (GPx), forward: GTGCGAGGTGAATGGTGAGAAG,
173 reverse: TGCTGTATCTGCGCACTGGAAC; glutathione reductase (GRd), forward:
174 ACATCCCTACCGTGGTCTTCAG, reverse: GCCAACCACCTTCTCCTCTTTG;
175 heme oxygenase-1 (HO-1), forward: CCGTGGCAGTGGAATTTATGC, reverse:

176 TGCCAGGCATCTCCTTCCATTC; NQO1, forward:
177 AGCGGCTCCATGTACTCTCTGC, reverse: TCCTCCCAGACAGTCTCCAGAC;
178 GAPDH, forward: TTGGAGGGCAAGTCTGGTG, reverse:
179 CCGCTCCCAAGATCCAACTA. The PCR products were electrophoresed on a 1.0%
180 agarose gel. The gel was photographed using a BioDoc-It system (UVP Inc., Upland,
181 CA, USA) after the DNA was stained with SYBR Safe DNA gel stain (Invitrogen).
182 The results are expressed as a ratio of the DNA signal relative to the corresponding
183 GAPDH signal from each sample.

184

185 **Preparation of Nuclear/cytosol Extracts.** The lung tissues were centrifuged at
186 13,000×g for 5 min at 4°C and lysed using the Nuclear/Cytosol Fractionation Kit
187 (BioVision, Milpitas, CA, USA). The lysate was centrifuged at 13,000×g for 5 min,
188 and Nuclear Extraction Reagents were added, followed by vortexing for 15 sec. This
189 mixture was then centrifuged at 13000×g for 10 min. Protein concentration of cellular
190 extracts was detected by a bicinchoninic acid assay, using a commercial protein
191 reagent kit (Bio-Rad, Hercules, CA, USA).

192

193 **Western Blotting.** Lung tissues were lysed in RIPA buffer (Millipore, Billerica,
194 MA, USA) and boiled at 100°C for 10 min with 4× protein loading dye as previously
195 described.¹² Samples were then subjected to SDS polyacrylamide gel electrophoresis.
196 Proteins were transferred onto cellulose nitrate membranes (Sartorius Stedim Biotech,
197 Goettingen, Germany), and the membranes were incubated with the indicated primary
198 antibodies (phospho-IκBα, phospho-p65, Keap1, DJ-1, Nrf2, HO-1, lamin B1, or
199 β-actin) overnight (1:1000 dilutions). The horseradish peroxide-conjugated secondary
200 antibody (1:5000 dilutions) was then incubated and further analyzed using the

Chemiluminescent ECL detection system (Millipore). Protein levels were normalized by comparison with the corresponding lamin B1 or β -actin. Signal intensity was quantified using VisionWorks LS 6.3.3 Software (UVP Inc.) as previously described.¹⁸

Expression Profiling of miRNA by Real-time PCR. Lung tissues were washed three times with PBS, and TRIzol Reagent (Invitrogen) was used to extract the intracellular RNA. cDNA was prepared from 2 μ g total RNA with universal reverse transcription primer for cDNA synthesis by the Universal cDNA Synthesis Kit II (Exiqon, Woburn, MA, USA). Quantitative RT-PCR was performed using the ABI 7900 Sequence Detection System (Thermo Fisher Scientific, Waltham, MA USA). The expression of microRNA under various conditions was quantified using a fluorescein quantitative real-time PCR detection system (ExiLENT SYBR Green master mix, Exiqon). The primer pairs were obtained from microRNA LNA PCR primer sets (Exiqon). Amplification was performed according to the manufacturer's cycling protocol and performed in triplicate. U6 snRNA (hsa, mmu) was used as an internal control. U6 snRNA PCR primer pairs were obtained from commercialized PCR primer set (Exiqon). The real-time PCR data were quantified using relative quantification (the $2^{-\Delta\Delta CT}$ method).

Statistical Analysis. All data are expressed as the mean $\pm$ standard deviation (SD). ANOVA was used to evaluate the differences of variances between multiple groups. The significant differences in the means between two specific groups were subjected to Duncan's test. A *P* value <0.05 was defined to be significant.

226

227 **RESULTS**

228 **Effect of DAS on the Tissue Weights of BALB/c Mice.** The weights of lungs,
229 hearts, and livers excised from mice after administration with DAS (50, 100 and 250
230 mg/kg bw) or 0.2 mg/kg bw DEX for 14 consecutive days showed no significant
231 differences compared to the control group (data not shown). The good health status of
232 all mice was maintained throughout the experimental period.

233

234 **DAS Decreases Th2 Responses in BALF.** OVA sensitization and challenge
235 significantly ($p<0.05$) induced the production of IL-4 and IL-10 in BALF. These
236 increased Th2 cytokines were strongly depressed by the treatment of DAS (100 and
237 250 mg/kg bw) (**Figures 1A and 1B**). In contrast, IFN- γ and IL-12 levels in the
238 OVA-sensitized group were lower than those in the control group, whereas DAS (100
239 and 250 mg/kg bw) administration significantly ($p<0.05$) raised the levels of IFN- γ
240 and IL-12 compared to OVA-treated mice (**Figures 1C and 1D**).

241

242 **DAS Inhibits Total IgE and OVA-specific IgE Productions in Serum.** Because
243 the level of IgE in BALF is dependent on the secretion of IL-4, IL-5, and IL-13, IgE is
244 therefore considered an additional index of Th2 cytokine secretion.¹ We found that
245 OVA significantly ($p<0.05$) increased the serum levels of total and OVA-specific-IgE
246 compared to the normal group. Furthermore, the increased levels of total and
247 OVA-specific-IgE were significantly ($p<0.05$) attenuated by the treatment with DAS
248 and DEX (**Figures 2A and 2B**). These results suggest that DAS and DEX suppressed
249 the response of Th2-type immunization and the activity of mast cells in this animal
250 model of asthma.

251

DAS Suppresses OVA-induced Pulmonary Inflammatory Cell Infiltration.

BALF was collected at 24 h after the last OVA or saline aerosol challenge. The total inflammatory cells, including eosinophils, macrophages, neutrophils, and lymphocytes, were markedly increased by the inhalation of OVA compared to the saline aerosol control. DAS (100 and 250 mg/kg bw) significantly ($p<0.05$) suppressed OVA-induced total inflammatory cells (**Figures 3A-3D**). Based on the results, DAS was found to have an anti-inflammatory activity on pulmonary cell infiltration, the impact of which might be similar to DEX. Additionally, DAS did not affect the profile or number of cells in BALF of mice (**Figure 3**).

261

DAS Attenuates Airway Inflammation and Antigen-induced Mucus Production.

We then studied the *in vivo* effect of DAS on OVA-induced airway inflammation. A histopathological analysis revealed that the inflammatory cells infiltrated into the airways of OVA-challenged mice, but DAS (100 and 250 mg/kg bw) administration significantly ($p<0.05$) reduced this infiltration. The infiltration of inflammatory cells in OVA-challenged mice was also significantly ($p<0.05$) reduced by DEX treatment (**Figures 4A and 4B**).

Mucus hypersecretion is a feature of airway inflammation.⁷ We stained lung sections with PAS to assess the capability of DAS to suppress mucus overproduction caused by goblet cell hyperplasia in OVA-challenged mice. A bright violet stain used to indicate mucus overproduction was applied to detect the bronchial airways of OVA-challenged mice. The results revealed that the mucus overproduction induced by OVA was markedly diminished by either DAS or DEX treatment (**Figures 5A and 5B**).

276

277 DAS Inhibits OVA-induced Oxidative Damage and Activation of NF- κ B p65.

278 8-OHdG is a marker of oxidative DNA damage, and 8-iso-PGF2 α also serves as an
279 indicator of lipid peroxidation in BALF. The levels of these two molecules reflect the
280 inflammatory processes and redox imbalance in asthmatic airways.^{2, 19} The exposure
281 under OVA aerosol enhanced ($p<0.05$) 8-OHdG levels up to threefold in BALF
282 compared to the saline aerosol control (**Figure 6A**). Both DAS and DEX have a
283 similar potential to significantly ($p<0.05$) block oxidative DNA damage by
284 suppressing 8-OHdG. A substantial increase in 8-iso-PGF2 α was also observed in
285 BALF upon OVA challenge (**Figure 6B**). Both DAS and DEX were able to
286 significantly ($p<0.05$) reduce oxidative damage of lipids by abating 8-iso-PGF2 α
287 formation.

288 NF- κ B is a well-known transcription factor that is involved in the transduction of
289 ROS stimulation.²⁰ We therefore evaluated the status of phosphorylated p65 in mice
290 as sensitized and challenged with OVA with or without DAS and DEX treatments.
291 The immunoblots demonstrated that the levels of cytosolic phosphorylated I κ B α and
292 nuclear phosphorylated p65 as well as the NF- κ B transcriptionally active subunit were
293 increased in OVA-challenged mice. The accumulation of nuclear phosphorylated p65
294 was inhibited *via* the administration of DAS and DEX (**Figure 6C**). Additionally, the
295 expression of the NF- κ B-downstream inflammatory factors (TNF- α and IL-1 β) was
296 also suppressed by DAS and DEX (**Figure 6D**). These results suggest that the
297 inhibitory mechanism of DAS on inflammatory mediators is similar to that of DEX,
298 which is at least partly through the suppression of NF- κ B activation.

299

300 DAS Negatively Modulates ROS Production through the Promotion of

301 **Nrf2-mediated Antioxidants Levels in OVA-challenged Mice.** DAS modulated the
302 activity of antioxidant enzymes to protect lung tissue against oxidative damage. Acute
303 OVA challenge significantly ($p<0.05$) decreased the mRNA expression of several
304 antioxidant enzymes, including SOD, catalase, GPx, GRd, HO-1, and NQO1, in
305 mouse tissue. Both DAS (100 and 250 mg/kg bw) and DEX restored the mRNA
306 expression ($p<0.05$) of antioxidant enzymes in lungs from OVA-challenged mice
307 **(Figure 7A).**

308 The transcription factor Nrf2 is important in redox homeostasis by regulating the
309 activation of glutathione synthesis and antioxidant defense genes, such as HO-1.²¹ We
310 further determined the influence of Nrf2 by DAS administration in acute
311 OVA-challenged mice. Levels of Nrf2 in nuclear protein extracts from lung tissues in
312 mice were dramatically decreased after OVA inhalation, and DAS significantly
313 increased nuclear Nrf2 expression in OVA-induced mice **(Figure 7B)**. The protein
314 expression of DJ-1 was also decreased under OVA-challenge conditions. Nevertheless,
315 the levels of nuclear Nrf2 and cytosolic DJ-1 were increased by the administration of
316 DAS or DEX, indicating that DAS and DEX exhibited an up-regulatory effect on the
317 downstream protein HO-1 **(Figure 7C)**.

318 To verify that whether OVA-induced ROS can be inhibited by activating Nrf2 and
319 Nrf2-related antioxidative enzymes, DCFH-DA staining was employed to determine
320 the intracellular accumulation of ROS in BALF. OVA-induced ROS were
321 significantly ($p<0.05$) decreased through the administration of DAS and DEX
322 **(Figure 7D)**. According to these data, DAS down-regulated ROS production through
323 the promotion of Nrf2-mediated antioxidants in OVA-challenged mice.

324

325 **The Dynamic Change in miRNA Expression in Lung of OVA-challenged Mice.**

To elucidate the role of the miRNAs (-574, -223, -196, -155, -146a, -146b, -144, -34a and -34b/c) in Th2 inflammation of asthma, we compared the expression of these miRNAs in lungs from OVA-challenged group and the control group (**Figure 8**). The expression levels of miRNA-155, -146a, -146b, -144 and -34a were significantly ($p<0.05$) higher, but the expression of miRNA-34b/c was significantly ($p<0.05$) lower in the OVA-challenged group than that in control group (**Figures 8D-8I**). Three miRNAs, miRNA-574, -223 and -196, showed no significant difference between the treated and untreated groups (**Figures 8A-8C**). After DAS administration in the OVA-challenged group, the levels of miR-144 and -34a were reduced (**Figures 8G-8H**), and miR-34b/c levels were significantly ($p<0.05$) increased (**Figure 8I**). Interestingly, a lower expression of miR-146a occurred in the DEX group than that in the OVA group (**Figure 8E**).

338

339

DISCUSSION

Airway inflammation, mucus secretion, airway hyperresponsiveness, and even insensitivity to steroids in asthma are associated with oxidative stress.²² Normally, low oxidative stress is well tolerated due to the protection of antioxidants, such as SOD, catalase, and GPx, present in lung.^{19, 23} In the case of asthma, the production of ROS and RNS, including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hydroxyl radical ($OH^\bullet$), and nitric oxide ($NO^\bullet$), may be upregulated during allergen exposure.³ High levels of oxidative stress finally overwhelm the antioxidant defense in the lung and result in the induction of proinflammatory cytokines, chemokines, and adhesion molecules. If oxidative stress was elevated sequentially, direct toxic effects will lead to cellular apoptosis, necrosis, and oxidative damage in the lung tissue. Oxidative

351 stress is a major contributor to steroid therapy resistance in patients with severe
352 asthma.²⁴ Hence, an effective drug, compound or phytochemical which possess both
353 anti-inflammatory and antioxidant properties for the treatment of chronic severe
354 asthma is in high demand. DAS is rich in garlic which is a well-known oriental
355 medicine used to treat inflammation and allergic diseases.^{8, 9} However, the
356 anti-inflammatory effects of DAS administration on OVA-induced immune system
357 imbalance and hyperresponsiveness in a murine model of asthma have not yet been
358 fully investigated *in vivo*.

359 In the present study, we evaluated the effect of DAS on OVA-induced airway
360 inflammation in an experimental model of acute asthma. Our results revealed that
361 DAS attenuated airway inflammation, including inflammatory cell infiltration,
362 inflammatory cytokine expression, and mucus secretion, as well as oxidative damage
363 in allergic mice.

364 Nrf2 is the primary transcription factor for regulating the expression of phase II
365 antioxidant and detoxifying enzymes. Nrf2 is indirectly involved in the modulation of
366 various immune and inflammatory pathways.⁶ Therefore, the depression of Nrf2
367 expression may exacerbate lung function in response to multiple oxidative stimuli (i.e.,
368 allergens, hyperoxia, mechanical ventilation, viruses, bacterial endotoxins,
369 environmental pollution, and other inflammatory agents).²⁵ Pulmonary Nrf2
370 expression is found mainly in the epithelium and alveolar macrophages.²⁶ It has been
371 confirmed that Nrf2 plays a central role in the expression of survival and
372 inflammatory genes in the lung and acts as a critical mediator of cellular adaptation in
373 response to proinflammatory and other noxious stimuli.⁵ The disruption of the *Nrf2*
374 gene may lead to severe allergen-driven airway inflammation and
375 hyperresponsiveness in mice. Notably, Nrf2 disruption causes an increase in the

amounts of Th2 cytokines IL-4 and IL-13 in BALF and splenocytes after allergen challenge,⁷ indicating the important protective role of Nrf2 pathway in allergic and asthmatic responses *in vivo*. Indeed, Nrf2 protection against allergic airway inflammation *in vivo* has been demonstrated in the mouse dendritic cells after exposure to environmental particulate matter and ragweed allergens.^{27, 28} Importantly, Shin *et al.*¹⁴ also indicated that Nrf2 activation contributed to enhancement of the antioxidant status to suppress airway inflammation in OVA-induced asthmatic mice after administration with diallyl disulfide from garlic, which agrees with our study regarding Nrf2 function. To the best of our knowledge, this is the first study to demonstrate that DAS can upregulate the activation of Nrf2 in the lung tissues of an OVA-induced airway inflammation model *in vivo*.

Our results showed that DAS elevated the Nrf2 translocation from the cytosol to the nucleus in the lungs of OVA-challenged mice, indicating that the inflammation in allergen-driven airway was reduced by DAS. A pronounced mucus cell hyperplasia and infiltration of eosinophils into lungs can be found in OVA-challenged mice. Increasing evidence indicates that eosinophils play an essential role in the pathogenesis of allergic airway inflammation, and mucus over-production and goblet cell over-differentiation are the characteristics of allergic airway inflammation.²⁹ Our data revealed that DAS could significantly reduce the numbers of eosinophils in BALF and prevent inflammatory cells infiltration and the generation of Th2 cytokines (IL-4 and IL-10) in BALF *via* an Nrf2-dependent mechanism. Thus, DAS is a potential compound for the alleviation of allergic airway inflammation.

Aerosolized OVA not only promotes inflammatory cell infiltration but also increases oxidative damage in the lung.⁷ Our finding demonstrated that the oxidative damage was increased in the levels of 8-OHdG and 8-isoprostane in the lung. These

401 types of oxidative damage in the lung have been observed in asthmatic patients.^{30, 31}
402 In this study, the biomarkers of oxidative damage in inflamed lung can be suppressed
403 by DAS, suggesting that DAS could markedly reduce ROS and thereby prevent
404 oxidant-induced damage in lung.

405 The relationship between miRNA and Th2 lymphocytes of asthma has attracted
406 significant attention due to the rapid and efficient intervention of miRNAs on target
407 genes. Until now, few studies have been conducted regarding mainly miRNAs from
408 airway biopsies or lung tissue.^{32, 33} In this study, six miRNAs (miRNA-155, -146a,
409 -146b, -144, -34a and -34b/c) were found to play proinflammatory roles in asthma,
410 and the inhibitory effect of DAS on inflammation may be explained partially through
411 the regulation of miRNA-144, -34a and -34b/c. It has been reported that the increased
412 expression of miR-144 in erythroid cells leads to a reduction in Nrf2 protein levels
413 and causes a decrease in the levels of the major intracellular antioxidant glutathione
414 (GSH); the total antioxidant defense capacity is consequently altered.³⁴ In addition to
415 targeting Nrf2 mRNA, miR-34a has been reported to directly regulate the microsomal
416 glutathione *S*-transferase 1 gene in HEK293 cells, and the level of miR-34a is
417 elevated in the livers of aged rats,³⁵ suggesting that miR-34a can elicit a dual
418 dampening effect on ARE-regulated antioxidant responses. Moreover, it is of
419 particular interest that a similar study suggests that miR-34a additionally targets
420 sirtuin 1 (Sirt1), a nicotinamide adenine dinucleotide-dependent class III HDAC
421 involved in maintaining antioxidant defense during aging.³⁶ The additional key
422 members of the Nrf2 regulome, DJ-1 and small Maf proteins, which act through
423 binding to Nrf2,^{37, 38} are thought to be regulated by microRNAs. The association
424 between low levels of DJ-1 and miR-34b/c in Parkinson disease brains has been
425 reported as the depletion of miR-34b/c in SH-SY5Y neuronal cells results in the

reduction of DJ-1 levels and leads to mitochondrial dysfunction, oxidative stress, and compromised cell viability,³⁹ which are consistent with this study that miR-34b/c and DJ-1 play a vital role in the activation of the Nrf2/ARE pathway. Moreover, corticosteroids are the most potent anti-inflammatory drug used to treat asthma. Corticosteroids act not only to suppress the accumulation and activity of inflammatory cells, such as eosinophils, lymphocytes, and macrophages, but also to inhibit the synthesis of inflammatory mediators.⁴⁰ Our study revealed that the levels of miR-144, -34a, and -34b/c were unchanged but that miR-146a was down-regulated in the DEX-treated group. These results imply that the DEX-regulated miR-146a may be partly involved in the anti-inflammatory action of DEX.

Together, these results suggest a relationship between airway inflammation and Nrf2 signaling that is becoming gradually clearer. Nrf2 is activated in response to allergen challenge in the lungs of DAS-treated mice, which activates the transcription of many antioxidant genes. Consequently, the development of airway inflammation, immune cell infiltration, and Th2 cytokine release were blocked. Moreover, the maintained balance between ROS and Nrf2-regulated antioxidants is essential for the appropriate inflammatory response to allergen challenge. We hereby conclude that DAS should be a potential compound used to prevent the development of airway inflammation *via* the modulation of the oxidative stress pathway.

ACKNOWLEDGEMENTS

This research work was supported in part by the Minister of Science and Technology, MOST101-2313-B-005-046-MY3, Taiwan and by the Ministry of Education, Taiwan under the ATU plan. The authors thank Dr. J. W. Liao of the Graduate Institute of Veterinary Pathobiology for his technical assistance on histological examination.

451

452 Conflict of interest statement

453 The authors have declared no conflict of interest.

454

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581

582

583

584 **Figure legends**

585 **Figure 1.** Levels of cytokine (A) IL-4, (B) IL-10, (C) IFN- γ , and (D) IL-12 in the
586 BALF of BALB/c mice. The animals were sensitized by ovalbumin (OVA) and
587 treated with DAS (50, 100 and 250 mg/kg bw) or DEX positive control (0.2 mg/kg
588 bw) as described in Materials and Methods. The vertical axis shows the level of
589 cytokine concentration in BALB/c mice. Values are recorded as the means $\pm$ SD
590 (n=8). Different letters (a-c) show statistically significant differences in each group
591 ($p<0.05$).

592

593 **Figure 2.** Levels of cytokine (A) total IgE and (B) OVA-specific IgE in the serum of
594 BALB/c mice. The animals were sensitized by ovalbumin (OVA) and treated with
595 DAS (50, 100 and 250 mg/kg bw) or DEX positive control (0.2 mg/kg bw) as
596 described in MATERIALS AND METHODS. The vertical axis shows the level of
597 cytokine concentration in BALB/c mice. Values are recorded as the means $\pm$ SD
598 (n=8). Different letters (a-d) show statistically significant differences in each group
599 ($p<0.05$).

600

601 **Figure 3.** Numbers of inflammatory cells (A) eosinophils, (B) macrophages, (C)
602 neutrophils, and (D) lymphocytes in the BALF of BALB/c mice. The animals were
603 sensitized by ovalbumin (OVA) and treated with DAS (50, 100 and 250 mg/kg bw) or
604 DEX positive control (0.2 mg/kg bw) as described in Materials and Methods. The
605 vertical axis shows the numbers of inflammatory cells in BALB/c mice. Values are
606 recorded as the means $\pm$ SD (n=8). Different letters (a-c) show statistically significant
607 differences in each group ($p<0.05$).

608

609 **Figure 4.** Effect of DAS treatment on infiltration of inflammatory cells in the lung
610 tissues of OVA-challenged mice. The animals were sensitized by ovalbumin (OVA)
611 and treated with DAS (50, 100 and 250 mg/kg bw) or DEX positive control (0.2
612 mg/kg bw) as described in Materials and Methods. (A) The lung tissues from mice
613 were sectioned and stained with hematoxylin-eosin (HE). The physiology slides were
614 examined by light microscopy (400×), and the arrows (↑) indicate the infiltration
615 inflammatory cells. (B) The infiltration score of inflammatory cells was graded as
616 follows: 1, minimal (< 1%); 2, slight (1-25%); 3, moderate (26-50%); 4,
617 moderate/severe (51-75%); 5, severe/high (76-100%). Values are recorded as the
618 means ± SD (n=8). Different letters (a-d) show statistically significant differences in
619 each group ($p<0.05$).

620

621 **Figure 5.** Effect of DAS treatment on hyperplasia of goblet cells in the lung tissues of
622 OVA-challenged mice. The animals were sensitized by ovalbumin (OVA) and treated
623 with DAS (50, 100 and 250 mg/kg bw) or DEX positive control (0.2 mg/kg bw) as
624 described in Materials and Methods. (A) The lung tissues from mice were sectioned
625 and stained with periodic acid-Schiff (PAS). The physiology slides were examined by
626 light microscopy (400×), and the arrows (↑) indicate the hyperplasia of goblet cells.
627 (B) Quantitative analysis for the number of PAS-positive cells per bronchiole. Values
628 are recorded as the means ± SD (n=8). Different letters (a-d) show statistically
629 significant differences in each group ($p<0.05$).

630

631 **Figure 6.** Effect of DAS administration on level of inflammatory mediators in
632 OVA-induced BALB/c mice. The animals were sensitized by ovalbumin (OVA) and
633 treated with DAS (50, 100 and 250 mg/kg bw) or DEX positive control (0.2 mg/kg

634 bw) as described in Materials and Methods. Levels of (A)
635 8-hydroxy-2'-deoxyguanosine (8-OHdG) and (B) 8-iso-prostaglandin F2 α
636 (8-iso-PGF2 α) (both biomarkers of oxidative stress) in the BALF were determined by
637 ELISA. (C) The p-IkBa and p-p65 in cytosolic or nuclear extracts were detected by
638 Western blot with specific antibodies. (D) The gene expression of TNF- α and IL-1 β
639 was analyzed by RT-PCR. The intensity of each gene-specific band was normalized to
640 the corresponding internal control (GAPDH). Values are recorded as the means $\pm$ SD
641 (n=8). Different letters (a-c and A-C) show statistically significant differences in each
642 group ($p < 0.05$).

643

644 **Figure 7.** Effect of DAS administration on (A) mRNA expression of Nrf2-related
645 antioxidant genes [catalase, superoxide dismutase (SOD), glutathione peroxidase
646 (GPx), glutathione reductase (GRd), heme oxygenase-1 (HO-1), NAD(P)H:quinone
647 oxidoreductase 1 (NQO1)], (B) protein level of Keap1, DJ-1 and Nrf2 activation, and
648 (C) HO-1 protein expression in the lung tissues of OVA-induced BALB/c mice as
649 described in Materials and Methods. (D) The production of ROS in BALF, which was
650 determined using a fluorescent probe DCFH-DA (20 μ M). Values are recorded as the
651 means $\pm$ SD (n=8). Different letters (a-d) show statistically significant differences in
652 each group ($p < 0.05$).

653

654 **Figure 8.** Effect of DAS administration on the expression of miRNAs in the lung
655 tissues of OVA-challenged BALB/c mice. The animals were sensitized by ovalbumin
656 (OVA) and treated with DAS (50, 100 and 250 mg/kg bw) or DEX positive control
657 (0.2 mg/kg bw) as described in Materials and Methods. These miRNAs include (A)
658 miRNA-574, (B) miRNA-223, (C) miRNA-196, (D) miRNA-155, (E) miRNA-146a,

659 (F) miRNA-146b, (G) miRNA-144, (H) miRNA-34a, and (I) miRNA-34b/c. Each
660 data was normalized to the value of the U6 snRNA. Values are recorded as the means
661 $\pm$ SD (n=8). Different letters (a-c) show statistically significant differences in each
662 group ($p < 0.05$).

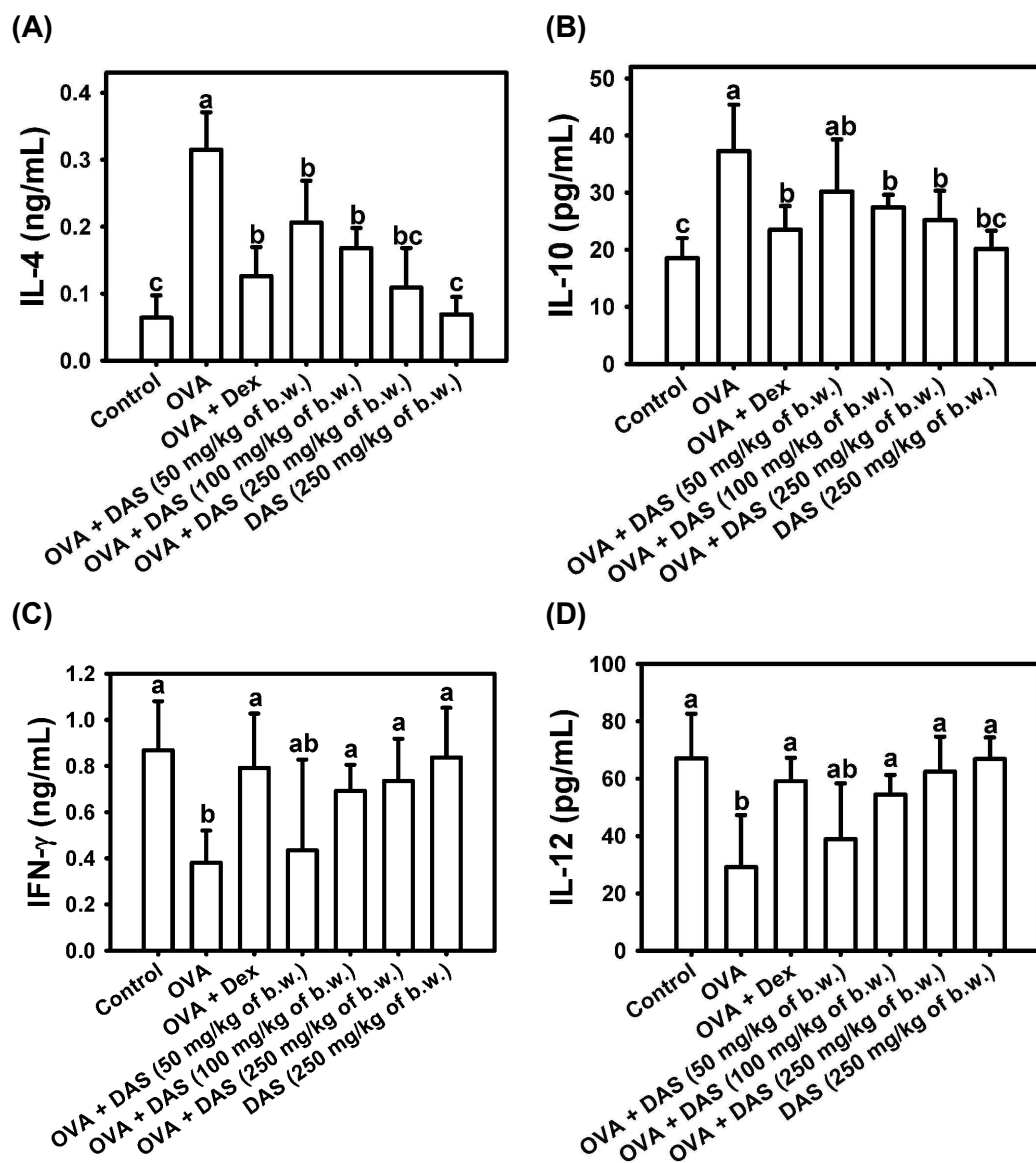


Figure 1.

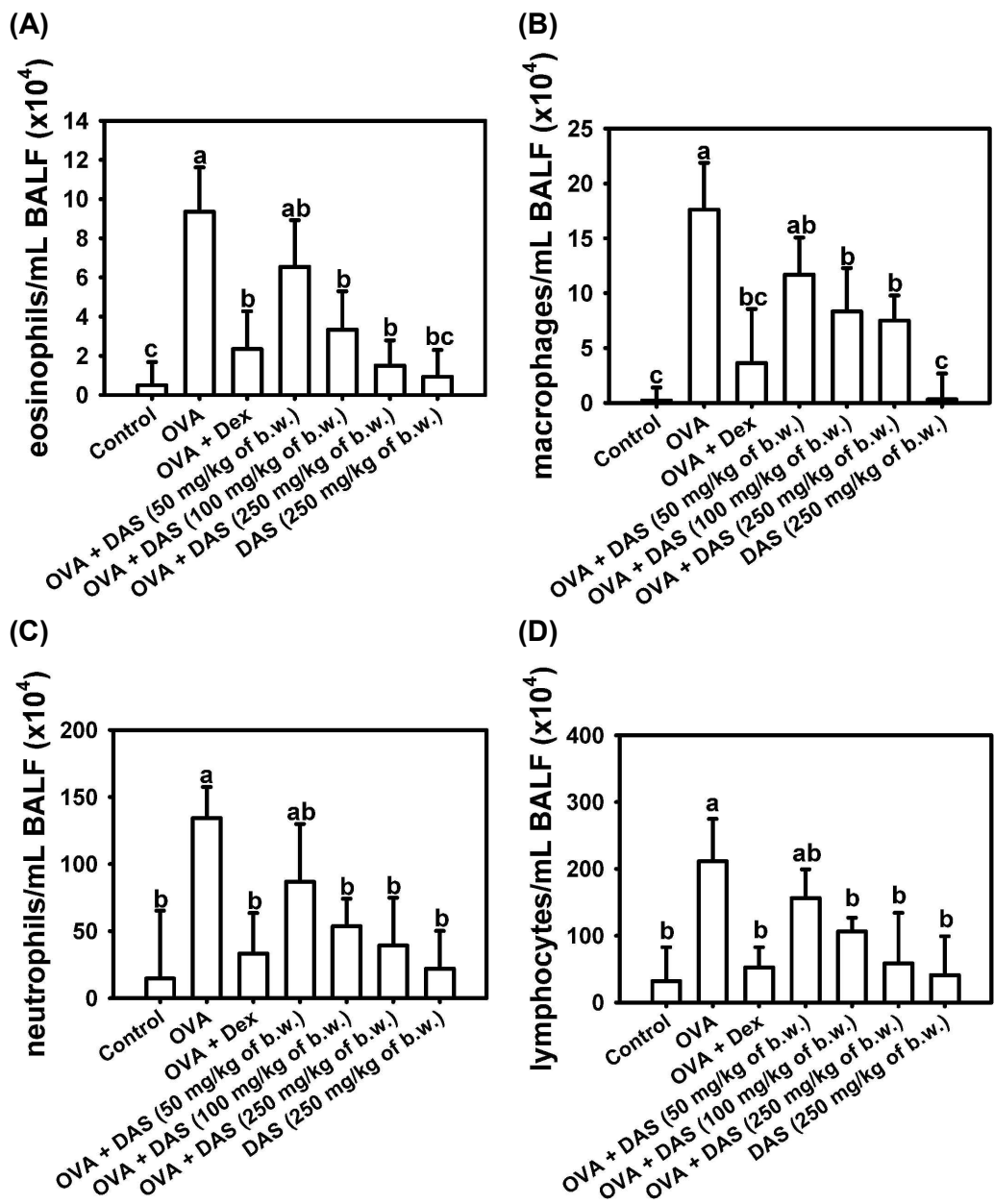
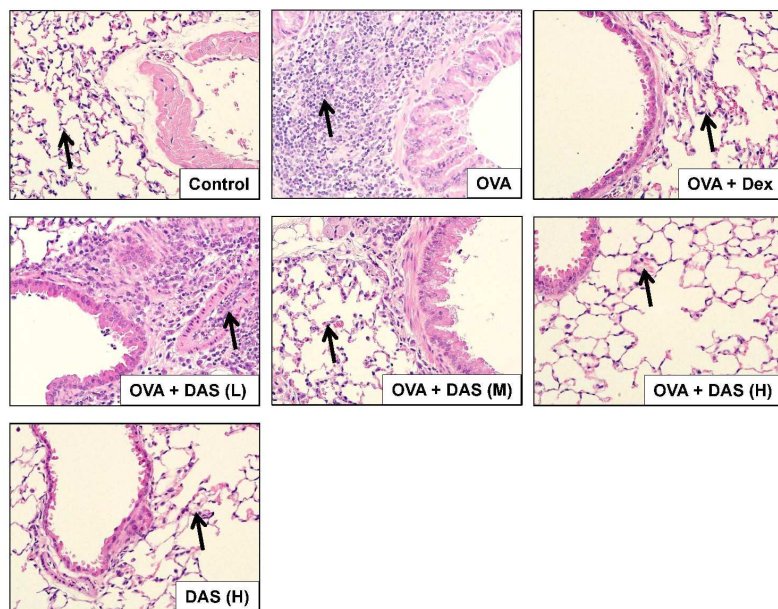


Figure 3.

(A)



(B)

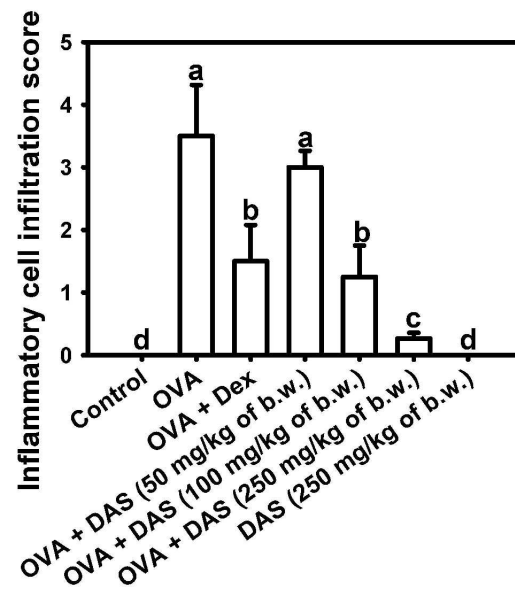


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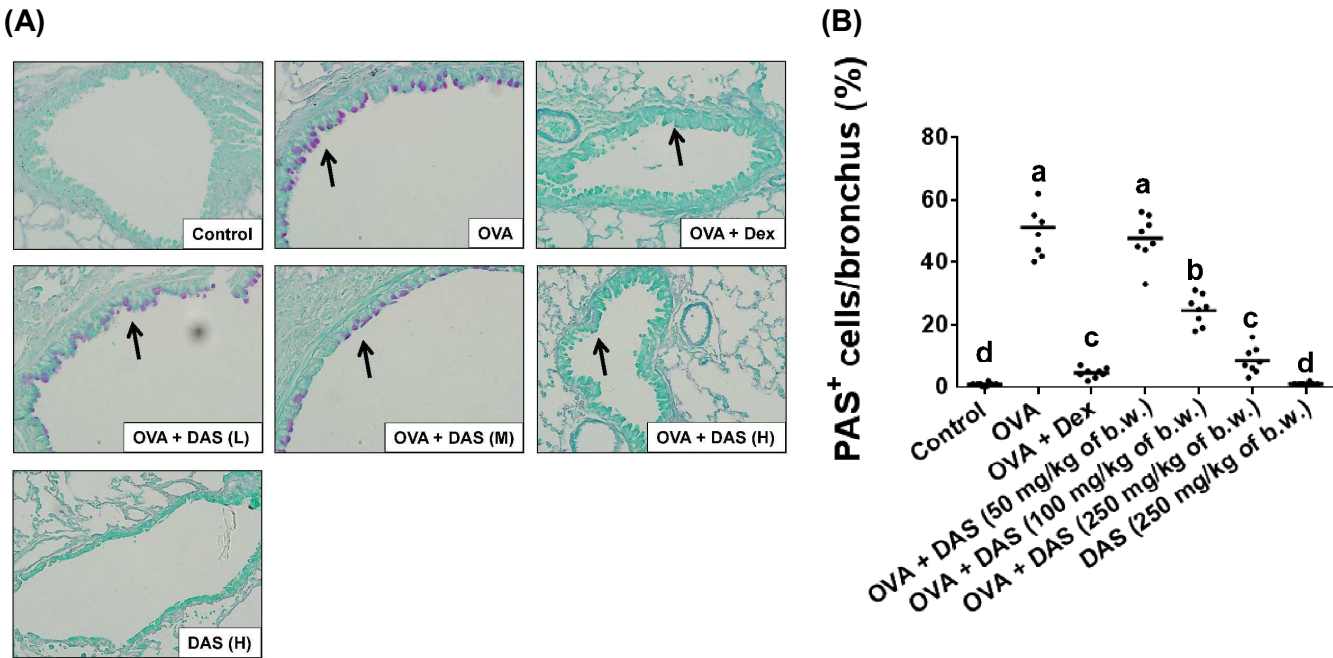


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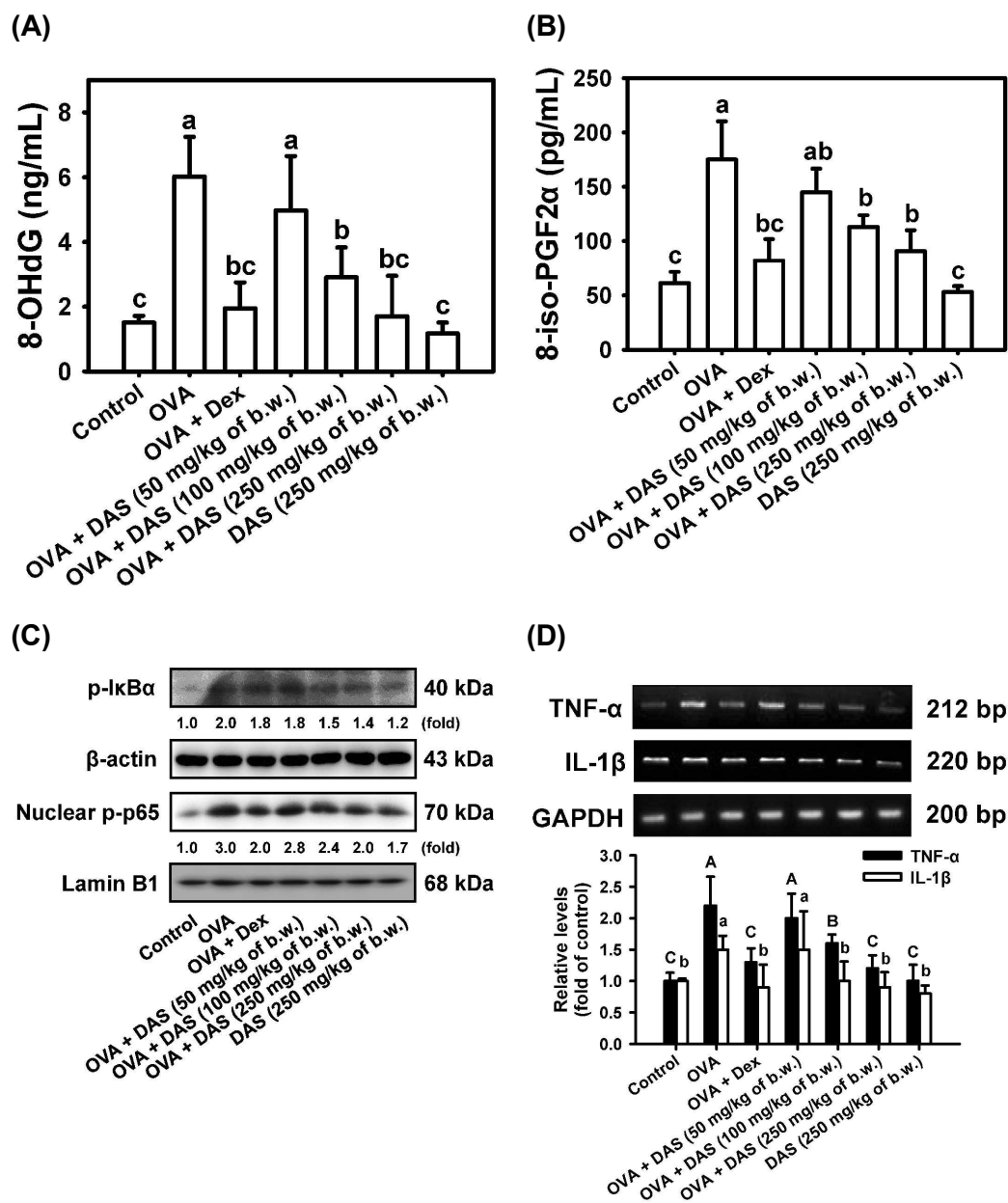


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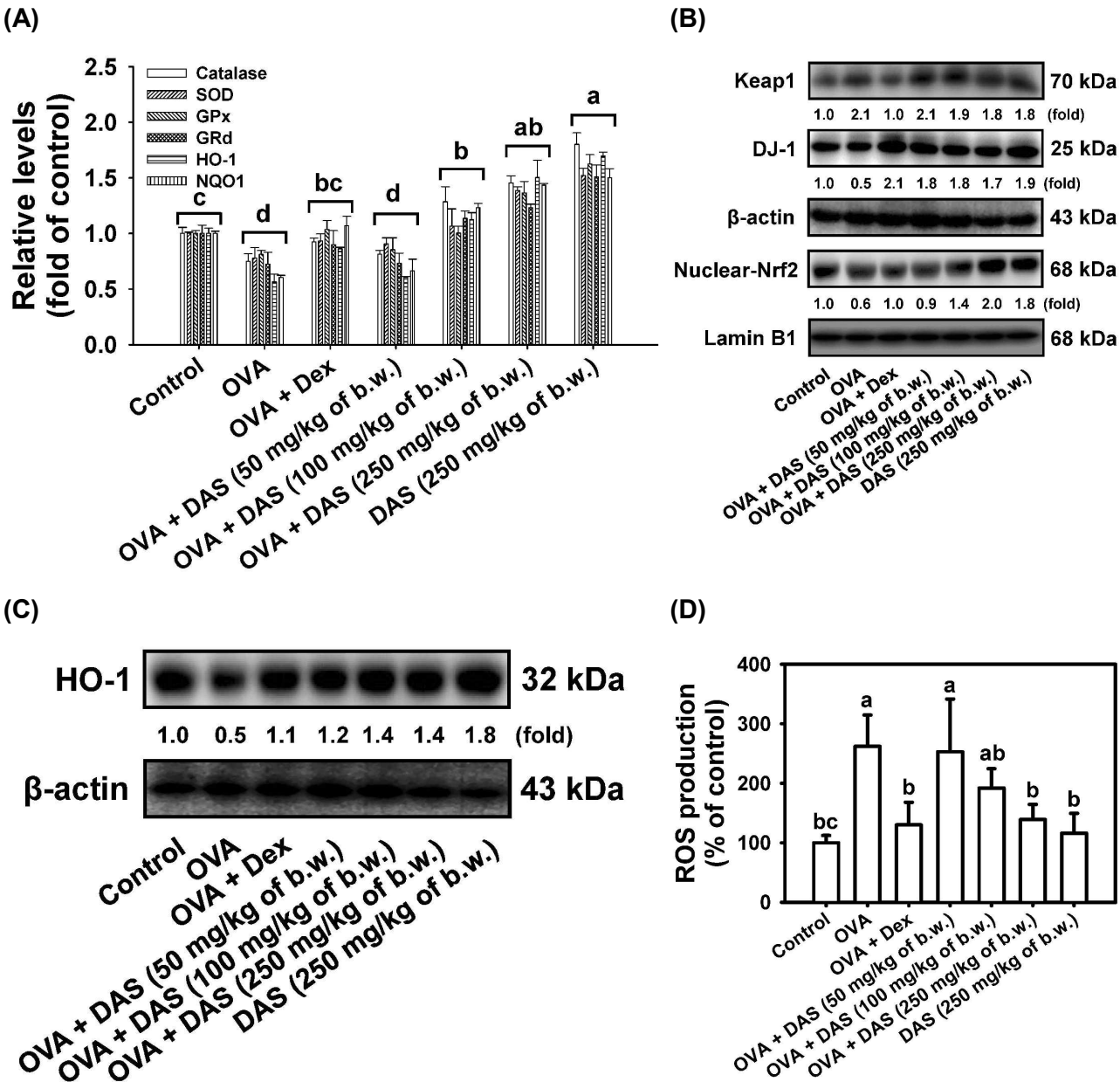


Figure 7.

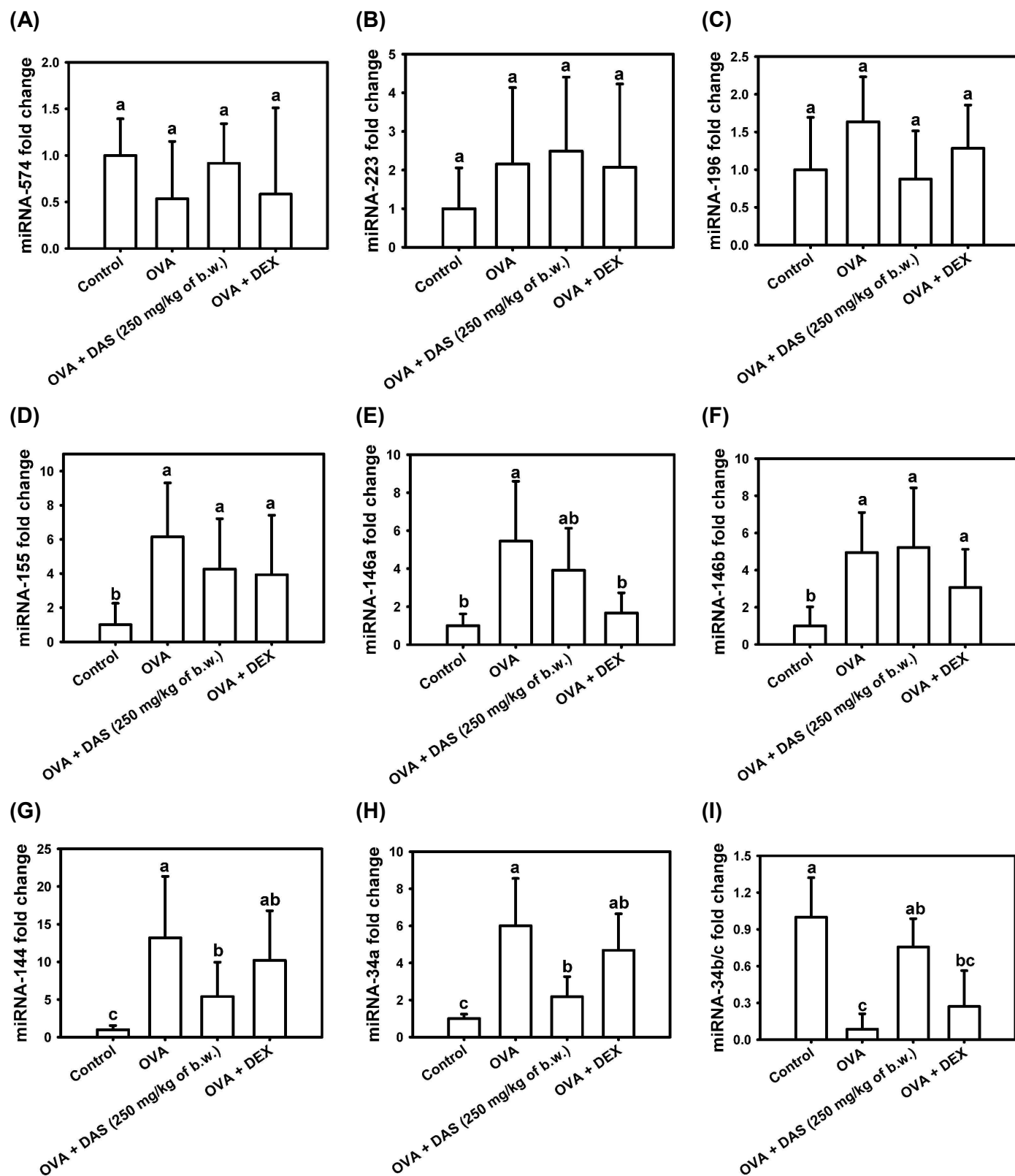


Figure 8.

Table of Contents Graphic

