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Antinociceptive activities and the mechanisms of anti-inflammation of asiatic acid in mice

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Abstract

Asiatic acid (**AA**), a pentacyclic triterpene compound identified in the medicinal plant *Centella asiatica*, was evaluated for the antinociceptive and anti-inflammatory effects. Treatment of male ICR mice with **AA** (1, 5, and 10 mg/kg) significantly inhibited the numbers of acetic acid-induced writhing response in 10 minutes. Also, our result showed that **AA** (10 mg/kg) significantly inhibited the formalin-induced pain in the late phase ($p < 0.001$). In the anti-inflammatory test, **AA** (10 mg/kg) decreased the paw edema at the fourth and fifth h after λ-carrageenan (Carr) administration and increased the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) in the liver tissue. We also demonstrated that **AA** significantly attenuated the malondialdehyde (MDA) level in the edema paw at the fifth h after Carr injection. **AA** decreased the nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) levels on serum level at the fifth h after Carr injection. Western blotting revealed that **AA** (10 mg/kg) decreased Carr-induced inducible nitric oxide synthase (iNOS), cycloxyclase (COX-2) and nuclear factor-κB (NF-κB) expressions at the fifth h in the edema paw. An intraperitoneal (i.p.) injection treatment with **AA** also diminished neutrophil infiltration into sites of inflammation as did indomethacin (Indo). The anti-inflammatory mechanisms of **AA** might be related to the decrease in the level of MDA, iNOS, COX-2, and NF-κB in the edema paw *via* increasing the activities of CAT, SOD, and GPx in the liver through the suppression of NO, TNF-α, and IL-1β.

Key words: Chinese medicine- asiatic acid-anti-inflammation-NO-TNF-*α*.

Introduction

Triterpenes are biosynthesized in plants by the cyclization of squalene, and are widely distributed in the plant kingdom. Moreover, their biological activities have attracted much attention. Many triterpenoids have shown promising effects when applied as anti-inflammatory agents (1). In particular, **AA** is a member of the ursane-type triterpenoids and is derived from the medicinal plant *Centella asiatica*, which is used as a medicine in tropical regions (2). **AA** has been found to prevent UVA-mediated photoaging, to inhibit β-amyloid-induced and glutamate-induced neurotoxicity, and to possess anti-ulcer and anti-hepatofibric activities (3). In addition, it has been reported to exhibit a cytotoxic effect on liver, colon and breast cancer cells (4), and neuroprotective in a mouse model of focal cerebral ischemia (5).

Carr-induced paw edema is a useful model to assess vascular changes associated with inflammation. Subplantar injections of Carr in mice induce a biphasic edema. The first phase peaks at 3 h and the delayed phase peaks at 48 h after Carr injection. In the early phase, there is a diffuse cellular infiltrate with polymorphonuclear leukocytes (PMNs), whereas the infiltrate of the delayed phase is composed by macrophages, eosinophils and lymphocytes (6). The inflammatory effect induced by Carr could be associated with free radical on. Free radical, prostaglandin and NO will be released when administrating with Carr for 1~5 h. The edema effect was raised to maximum at the third h and its MDA production was due to free radical attack plasma membrane (6). Thus, inflammatory effect would result in the accumulation of MDA. Therefore, in this paper we examined the analgesic effects of **AA** on nociception induced by acetic acid and formalin. We also evaluated the anti-inflammatory effects of **AA** on paw edema induced by Carr in mice,

and we detected the levels of MDA, NO, TNF-α, iNOS and COX-2 in either paw edema or serum. Also, the activities of CAT, SOD and GPx in the liver at the fifth h after Carr injection were investigated to understand the relationship between the anti-inflammatory mechanism of the **AA** and antioxidant enzymes.

Methods

Chemicals

Asiatic acid, Carr and indomethacin (Indo) were obtained from Sigma (St. Louis, MO, USA). Acetic acid was purchased from Merck (Darmstadt, Germany). Formalin was purchased from Nihon Shiyaku Industries (Japan). TNF-α and IL-1β were purchased from Biosource International Inc. (Camarillo, CA, USA). Anti-iNOS, anti-COX-2, anti-NF-κB (p50), and anti-β-actin antibody (Santa Cruz, USA) and a protein assay kit (Bio-Rad Laboratories Ltd., Watford, Herts, U.K.) were obtained as indicated. Poly (vinylidene fluoride) membrane (Immobilon-P) was obtained from Millipore Corp. (Bedford, MA, USA).

Animals

6-8 weeks male ICR mice were obtained from the BioLASCO Taiwan Co., Ltd. The animals were kept in plexiglass cages at a constant temperature of 22 ±1°C, relative humidity 55 ± 5 % with 12 h dark-light cycle for at least 2 week before the experiment. They were given food and water *ad libitum*. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals. And all tests were conducted under the guidelines of the International Association for the Study of

Pain (7).

After a 2-week adaptation period, male ICR mice (18-25 g) were randomly assigned to five groups (n=6) of the animals in acetic acid-induced writhing (1%, 0.1 mL/10 g i.p.) and formalin-induced licking (5%, 20 μL/per mice i.p.) experiments. These include a pathological model group (received acetic acid or formalin), a positive control (acetic acid or formalin + Indo), and the **AA** administered groups (acetic acid or formalin+ **AA**: 1, 5, and 10 mg/kg). In the Carr-induced edema experiment, there were randomly assigned to six groups (n=6) of the animals in the study. The control group receives normal saline (i.p.). The other five groups include a Carr-treated, a positive control (Carr + Indo) and **AA** administered groups (Carr + **AA**: 1, 5, and 10 mg/kg).

Acetic acid-induced writhing response

The test was performed as described by Chang et al., (8). Writhing was induced by an intraperitoneal (i.p.) injection of 0.1 mL/10 g acetic acid solution (10 mL/kg). Positive control animals were pretreated with Indo (10 mg/kg, i.p.) 25 min before acetic acid. Each **AA** administered group was pretreated with 1 mg/kg, 5 mg/kg, or 10 mg/kg (dissolved in 0.5% carboxymethylcellulose) i.p. 25 min before acetic acid. Five minutes after the i.p. injection of acetic acid, the number of writhing and stretching was recorded.

Formalin test

The antinociceptive activity of the drugs was determined using the formalin test (8). Twenty microliters of 5% formalin was injected into the dorsal surface of the right hind paw of mice 30 min after i.p. administration of **AA** (1, 5, and 10 mg/kg), or Indo. The

mice were observed for 30 min after the injection of formalin, and the amount of time spent licking the injected hind paw was recorded. The first 5 min post formalin injection is referred to as the early phase and the period between 15 min and 40 min as the late phase. The total time spent licking or biting the injured paw (pain behavior) was measured with a stop watch. The activity was recorded in 5 min intervals.

λ-carrageenin-induced edema

Carr-induced hind paw edema model was used for determination of anti-inflammatory activity (8). Animals were i.p. treated with **AA** (1, 5, and 10 mg/kg), Indo or normal saline, 30 min prior to injection of 1% Carr (50 µL) in the plantar side of right hind paws of the mice. Paw volume was measured immediately after Carr injection and at 1, 2, 3, 4, and 5 h intervals after the administration of the edematogenic agent using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by the ratio a/b, where is the volume of the right hind paw after Carr treatment, and b is the volume of the right hind paw before Carr treatment. Indo was used as a positive control. After 5 hrs, the animals were sacrificed; the Carr-induced edema feet were dissected and stored at -80 ºC. Also, blood were withdrawn and kept at -80 ºC. The protein concentration of the sample was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

MDA assay

MDA from Carr-induced edema foot was evaluated by the thiobarbituric acid reacting substances (TRARS) method (8). Briefly, MDA reacted with thiobarbituric acid in the

acidic high temperature and formed a red-complex TBARS. The absorbance of TBARS was determined at 532 nm.

Measurement of Nitric oxide/Nitrite

NO production was indirectly assessed by measuring the nitrite levels in serum determined by a colorimetric method based on the Griess reaction (8). Serum samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g/L) to a final concentration of 15 g/L. After centrifugation at 10,000×*g* for 5 min at room temperature, 100 μL supernatant was applied to a microliter plate well, followed by 100 μL of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid). After 10 min of color development at room temperature, the absorbance was measured at 540 nm with a Micro-Reader (Molecular Devices, Orleans Drive, Sunnyvale, CA). By using sodium nitrite to generate a standard curve, the concentration of nitrite was measured by absorbance at 540 nm.

Measurement of serum TNF-α and IL-1β by ELISA

Serum levels of TNF-α and IL-1β were determined using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Biosource International Inc., Camarillo, CA) according to the manufacturer's instruction. TNF-α and IL-1β were determined from a standard curve. The concentrations were expressed as pg/mL.

Antioxidant enzyme activity measurements

The following biochemical parameters were analyzed to check the hepatoprotective activity of **AA** by the methods given below.

Total SOD activity was determined by the inhibition of cytochrome c reduction (9). The reduction of cytochrome c was mediated by superoxide anions generated by xanthine/xanthine oxidase system and monitored at 550 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome c reduction by 50%. Total CAT activity was based on that of Aebi (10). In brief, the reduction of 10 mM H_2O_2 in 20 mM of phosphate buffer (pH 7.0) was monitored by measuring the absorbance at 240 nm. The activity was calculated using a molar absorption coefficient, and the enzyme activity was defined as nmoles of dissipating hydrogen peroxide per mg protein per min. Total GPx activity in cytosol was determined according to Paglia and Valentine's method (11). The enzyme solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2) and the absorbance at 340 nm was measured. Activity was evaluated from a calibration curve, and the enzyme activity was defined as nmoles of NADPH oxidized per mg protein per min.

Western blot analysis of iNOS, COX-2, and NF-κB

Soft tissues were removed from individual mice paws and homogenized in a solution containing 10 mM CHAPS, 1mM phenylmethylsulphonyl fluoride (PMSF), 5 μg/mL, aprotinin, 1 μM pepstatin and 10 μM leupeptin. The homogenates were centrifuged at 12,000*g* for 20 min, and 30 μg of protein from the supernatants was then separated on 10% sodium dodecylsulphate–polyacrylamide gel and transferred to polyvinylidene

188 difluoride membranes. Following transfer, the membrane was blocked for 2 h at room
189 temperature with 5% skim milk in Tris-buffered saline-Tween (TBST; 20 mM Tris, 500
190 mM NaCl, pH 7.5, 0.1% Tween 20). The membranes were then incubated with mouse
191 monoclonal anti-iNOS, anti-COX-2 or anti-NF-κB (p50) antibody in 5% skim milk in
192 TBST for 2 h at room temperature. The membranes were washed three times with TBST
193 at room temperature and then incubated with a 1 : 2000 dilution of anti-mouse IgG
194 secondary antibody conjugated to horseradish peroxidase (Sigma, St Louis, MO, U.S.A.)
195 in 2.5% skim milk in TBST for 1 h at room temperature. The membranes were washed
196 three times and the immunoreactive proteins were detected by enhanced
197 chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International
198 plc., Buckinghamshire, U.K.). The results of Western blot analysis were quantified by
199 measuring the relative intensity compared to the control using Kodak Molecular Imaging
200 Software (Version 4.0.5, Eastman Kodak Company, Rochester, NY) and represented in
201 the relative intensities.
202
203 **Histological examination**
204 For histological examination, biopsies of paws were taken 5 h following the
205 interplanetary injection of Carr. The tissue slices were fixed in (1.85% formaldehyde, 1%
206 acetic acid) for 1 week at room temperature, dehydrated by graded ethanol and embedded
207 in Paraffin (Sherwood Medical). Sections (thickness 5 μm) were deparaffinized with
208 xylene and stained with H & E stain. All samples were observed and photographed with
209 Nikon microscopy. Every 3~5 tissue slices were randomly chosen from Carr, Indo and
210 **AA**-treated (10 mg/kg) groups. Histological examination of these tissue slices revealed an

excessive inflammatory response with massive infiltration of PMNs by microscope. The numbers of neutrophils were counted in each scope (400 x) and thereafter obtain their average count from 5 scopes of every tissue slice.

Statistical analysis

Data are expressed as mean ± S.E.M. Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range test). Statistical significance is expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Results

Effects of AA on acetic-induced writhing response

The cumulative amount of abdominal stretching correlated with the level of acetic acid induced pain (Fig. 2). **AA** treatment (1 mg/kg) significantly inhibited the number of writhing in comparison with the normal controls (p <0.05). **AA** (5 or 10 mg/kg) further reduced the number of writhing ($p < 0.01$ or $p < 0.001$) and **AA** (10 mg/kg) demonstrates more inhibition than Indo (10 mg/kg).

Formalin test

AA (1 mg/kg) significantly ($p < 0.05$) inhibited formalin-induced pain in the late phase (Fig. 3); however, it did not show any inhibition in the early phase. The positive control Indo (5 or 10 mg/kg) also significantly ($p < 0.01$ or $p < 0.001$) inhibited the formalin induced pain in the late phase.

Effects of AA on λ-Carrageenan-induced mice paw edema

As shown in Fig. 4, Carr induced paw edema. **AA** (5 or 10 mg/kg) inhibited ($p < 0.01$ or $p < 0.001$) the development of paw edema induced by Carr after 4 and 5 h of treatment, significantly. Indo (10 mg/kg) significantly decreased the Carr induced paw edema after 4 and 5 h of treatment ($p < 0.001$).

Effects of AA on MDA level

MDA level increased significantly in the edema paw at the 5 h after Carr injection ($p < 0.001$). However, MDA level was decreased significantly by treatment with **AA** (5 mg/kg) ($p < 0.001$), as well as 10 mg/kg Indo (Fig. 5).

Effects of AA on NO level

In Fig. 6A, the NO level increased significantly in the edema serum at the 5 h after Carr injection ($p < 0.001$). **AA** (5 or 10 mg/kg) significantly decreased the serum NO level ($p < 0.01$ or $p < 0.001$). The inhibitory potency was similar to that of Indo (10 mg/kg) at 5th h after induction.

Effects of AA on TNF-α and IL-1β levels.

TNF-α and IL-1β levels increased significantly in serum at the 5th h after Carr injection ($p < 0.001$). However, **AA** (5 or 10 mg/kg) decreased the TNF-α and IL-1β levels in serum at the 5th h after Carr injection ($p < 0.01$ or $p < 0.001$), as well as 10 mg/kg Indo (Fig. 6B and 6C).

Effects of AA on activities of antioxidant enzymes

The acute inflammatory response is associated with the production of reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and peroxynitrite. In a number of pathophysiological conditions associated with inflammation or oxidant stress, these ROS have been proposed to mediate cell damage in the liver (1). At the 5th h following the intrapaw injection of Carr, liver tissues were analyzed for the biochemical parameters such as CAT, SOD and GPx activities (Table 1). CAT, SOD and GPx activities in liver tissue were significantly decreased by Carr administration. CAT, SOD, and GPx activity were increased significantly after treated with 10 mg/kg **AA** and 10 mg/kg Indo (P<0.01) (Table 1).

Effects of AA on λ-Carrageenan-induced iNOS, COX-2, and NF-κB protein expressions in mice paw edema

Transcription of pro-inflammatory mediators such as iNOS, COX-2, TNF-α, and IL-1β is regulated by activation of transcription factor NF-κB (Kubes and McCafferty, 2000). The effect of **AA** on iNOS, COX-2, and NF-κB protein expression was studied by western blot. Equal amounts of protein (30 μg/lane) were resolved by SDS-PAGE and then transferred to a nitrocellulose membrane and iNOS, COX-2, and NF-κB were detected using a specific antibody. The results showed that injection **AA** (10 mg/kg) on Carr-induced for 5 h inhibited iNOS, COX-2 and NF-κB proteins expression in mouse paw edema (Fig. 7A). The detection of β-actin was also performed in the same blot as an internal control. The intensity of protein bands was analyzed using Kodak Quantity software (Molecular Imaging Software System, Kodak) in three independent experiments

and showed an average of 77.6%, 72.4%, and 62.8% down-regulation of iNOS, COX-2, and NF-κB protein, respectively, after the treatment with **AA** at 10 mg/kg compared with the Carr-induced alone (Fig. 7B). And the protein expression showed an average of 43.6%, 41.1%, and 36.4% down-regulation of iNOS, COX-2, and NF-κB protein after treatment with Indo at 10 mg/kg compared with the Carr-induced alone (Fig. 7B). The down-regulation of iNOS, COX-2, and NF-κB activity of **AA** (10 mg/kg) was better than Indo (10 mg/kg).

Histological examination

Paw biopsies of Carr model animals showed marked cellular infiltration in the connective tissue. The infiltrates accumulated between collagen fibers and into intercellular spaces. Paw biopsies of animals treated with **AA** (10 mg/kg) showed a reduction in inflammatory response Carr-induced. Inflammatory cells were actually reduced in number and confined to near the vascular areas. Intercellular spaces did not show any cellular infiltrations. Collagen fibers were regular in shape and showed a reduction of intercellular spaces. Moreover, the hypoderm connective tissue was not damaged (Fig. 8). Neutrophils were notified increased with Carr treatment ($P < 0.001$). As Indo and **AA** (10 mg/kg) could significantly decrease the neutrophils numbers as compared to the Carr-treated group ($P < 0.001$) (Fig. 8E).

Discussion

We have evaluated the putative analgesic and anti-inflammatory activities of **AA** to clarify the pain and inflammation relieving effects. Two different analgesic testing

303 methods were employed with the objective of identifying possible peripheral and central
304 effects of the test substances. The acetic writhing test is normally used to study the
305 peripheral analgesic effects of drugs. Although this test is nonspecific (e.g.,
306 anticholinergic, antihistaminic and other agents also show activity in the test), it is widely
307 used for analgesic screening (12). In our study, we found that **AA** (1, 5, and 10 mg/kg)
308 exhibited antinociceptive effect in acetic acid-induced writhing response (Fig. 2). This
309 effect may be due to inhibition of the synthesis of the arachidonic acid metabolites (13).
310 The *in vivo* model of pain, formalin-induced paw pain has been well established as a
311 valid model for analgesic study. It is well known that the formalin test produces a distinct
312 biphasic nociception, a first phase (lasting the first 5 min) corresponding to acute
313 neurogenic pain, and a second phase (lasting from 15 to 30 min after injection of formalin)
314 corresponding to inflammatory pain responses (14). Therefore, the test can be used to
315 clarify the possible mechanism of an antinociceptive effect of a proposed analgesic.
316 Centrally acting drugs such as opioids inhibit both phases equally, but peripherally acting
317 drugs such as aspirin, Indo and dexamethasone only inhibit the late phase (15). The
318 inhibitory effect of **AA** on the nociceptive response in the late phase of the formalin test
319 suggested that the anti-nociceptive effect of **AA** could be due to its peripheral action (Fig.
320 3).
321 The injection of Carr in mice produces a typical biphasic edema associated with the
322 production of several inflammatory mediators, such as bradykinin, prostaglandins, nitric
323 oxide, and cytokines. The Carr test is highly sensitive to nonsteroidal antiinflammatory
324 drugs, and has long been accepted as a useful phlogistic tool for investigating new drug
325 therapies (16). The degree of swelling of the Carr-injected paws was maximal at 3 th after

injection. Statistical analysis revealed that **AA** (10 mg/kg) and Indo significantly inhibited the development of edema at 4 th after treatment (p<0.001) (Fig. 4). They both showed anti-inflammatory effects in Carr-induced mice edema paw. It is well known that the third phase of the edema-induced by Carr, in which the edema reaches its highest volume, is characterized by the presence of prostaglandins and other compounds of slow reaction (17) found that the injection of Carr into the rat paw induces the liberation of bradykinin, which later induces the biosynthesis of prostaglandin and other autacoids, which are responsible for the formation of the inflammatory exudates. In addition, the classification of antinociceptive drugs is usually based on their mechanism of action either on the central nervous system or on the peripheral nervous system (18).

NO plays an important role in Carr induced paw edema. iNOS is expressed in this model within 4 h after injection of Carr. The subsequent production of NO maintains the edema. In the studies of mechanism on the inflammation, L-arginine–NO pathway has been proposed to play an important role in the Carr-induced inflammatory response (19). Our present results also confirm that Carr-induced paw edema model results in the production of NO. The expression of the inducible isoform of NO synthase has been proposed as an important mediator of inflammation (20). In our study, the level of NO was decreased significantly by treatment with 1, 5 and 10 mg/kg **AA**. We suggest the mechanism of anti-inflammatory of **AA** may be through the L-arginine–NO pathway since **AA** significantly inhibits the NO production (Fig. 6A).

TNF-α is a major mediator in inflammatory responses, inducing innate immune responses by activating T cells and macrophages, and stimulating secretion of other inflammatory cytokines (21). Also, TNF-α is a mediator of Carr-induced inflammatory

incapacitation, and is able to induce the further release of kinins and leukotrienes, which is suggested to have an important role in the maintenance of long-lasting nociceptive response. IL-1β is also important in the regulation of the inflammatory response. Moreover, IL-1β increases the expression of adhesion factors on endothelial cells to enable transmigration of leukocytes, and is associated with hyperalgesia and fever (22). In this study, we found AA decreased the TNF-α and IL-1β levels in serum after Carr injection by treatment with 1, 5, and 10 mg/kg **AA**, significantly (Fig. 6B and 6C).

AA is one of the most common triterpenes and has a variety of pharmacological activities (23). Nonetheless, little information is available with respect to the molecular mechanisms underlying the anti-inflammatory effect of **AA**. The inhibitory effects of **AA** and asiaticoside on the LPS-induced pro-inflammatory molecules, including NO and prostaglandin E2, and found that **AA** is a more potent inhibitor than asiaticoside. These results suggest that the anti-inflammatory properties of **AA** might be the results from the inhibition of iNOS, COX-2, interleukin-6, IL-1β and TNF-α expression through the down-regulation of Nuclear factor-kappa B activation via suppression of IκB kinase and mitogen-activated protein kinase (p38, ERK1/2 and JNK) phosphorylation in RAW264.7 cells (24).

The Carr-induced inflammatory response has been linked to neutrophils infiltration and the production of neutrophils-derived free radicals as well as the release of other neutrophils-derived mediators (8). Some researches demonstrate that inflammatory effect induced by Carr is associated with free radicals. Free radicals, prostaglandin and NO will be released when administrating with Carr for 1-6 h. The edema effect was raised to the maximum at the third h. MDA production is due to free radical attack plasma membrane.

372 Thus, inflammatory effect would result in the accumulation of MDA. GSH is a known
373 oxyradical scavenger. Enhancing the level of GSH conducive toward favor reduces MDA
374 the production. Endogenous GSH plays an important role against Carr-induced local
375 inflammation. In a number of pathophysiological conditions associated with
376 inflammation or oxidant stress, these ROS have been proposed to mediate cell damage
377 via a number of independent mechanisms including the initiation of lipid peroxidation,
378 the inactivation of a variety of antioxidant enzymes and depletion of glutathione. Giving
379 the importance of the oxidative status in the formation of edema, the anti-inflammatory
380 effect exhibited by drug in this model might be related to its antioxidant properties (8). In
381 this study, there are significantly increases in CAT, SOD and GPx activities with **AA**
382 treatment (Table 1). Furthermore, there are significant decreases in MDA level with **AA**
383 treatment (Fig. 5). We assume the suppression of MDA production is probably due to the
384 increases of CAT, SOD and GPx activities.

385 During inflammatory processes, large amounts of the proinflammatory mediators, NO
386 and PGE_2, are generated by inducible iNOS and COX-2, respectively (25). INOS, is
387 generally not present in resting cells, but is induced by various stimuli, which include
388 bacterial LPS, TNF-α, IL-1β and interferon-γ (26). However, COX-2 is induced by
389 pro-inflammatory stimuli, including LPS and cytokines in cells *in vitro* and in inflamed
390 sites *in vivo*. Furthermore, COX-2 is believed to be the isoform responsible for the
391 production of pro-inflammatory prostaglandins (PGs) in various models of inflammation
392 (27). In this study, there are significantly decreased in iNOS and COX-2 activities with
393 **AA** treatment (Fig. 7A). We assume the suppression of NO production is probably due to
394 the decreases of iNOS and COX-2 activities. An inflammatory response implicates

395 macrophages and neutrophils, which secrete a number of mediators (eicosinoids, oxidants,
396 cytokine and lytic enzymes) responsible for initiation, progression and persistence of
397 acute or chronic state of inflammation (28). NO is the most important among these
398 mediators and is produced in macrophages by COX-2 and iNOS, respectively (29). COXs
399 are pro-inflammatory enzymes that are involved in arachidonic acid metabolism and
400 influence biological reactions such as tissue repair and immune responses, all of which
401 are associated with inflammation. COX-1 and COX-2 are the rate-limiting enzymes in the
402 synthesis of PGE_2. COX-1 is constitutively expressed and involved in the acute
403 inflammatory response, whereas COX-2 is expressed in specific cells (i.e., macrophages,
404 monocytes, and neutrophils) after stimulation COX-2-dependent PGE_2 is produced by
405 inflammatory cells and increased in disease (30).

406 NF-κB is known to be a major transcription factor to regulate the expressions of
407 pro-inflammatory enzymes and cytokines, such as iNOS, COX-2, and TNF-α (31).
408 NF-κB subunits (p65 and/or p50) are normally sequestered in the cytosol as an inactive
409 complex by binding to inhibitory factor IκB-α in un-stimulated cells. Upon stimulation of
410 pro-inflammatory signals including LPS, IκB-α is phosphorylated by IκB kinase (IKK)
411 and inactivated through ubiquitin-mediated degradation. The resulting free NF-κB is
412 translocated into the nucleus and acts as a transcription factor. As shown in Fig. 7A, the
413 treatment with **AA** blocks the degradation of NF-κB in Carr-induced paw edema.
414 Therefore, these results suggest that **AA** inhibits the expression of iNOS and COX-2, and
415 thus NO production through inactivation of NF-κB activation.

416 NO also is responsible for vasodilatation, increase in vascular permeability and
417 edema formation at the site of inflammation (32). NO along with superoxide ($O_2^{\cdot -}$) and

418 the products of their interaction, also initiates a wide range of toxic oxidative reactions
419 causing tissue injury (33). Likewise, the neutrophils produce oxidants and release
420 granular constituents comprising of lytic enzymes performing important role in
421 inflammatory injury (34). In this study, **AA** inhibition in the release of these mediators is
422 a potential strategy to control inflammation and is implicated in mechanism of action as
423 shown in Fig. 9.

424 In conclusion, these results suggested that **AA** possessed analgesic and
425 anti-inflammatory effects. The anti-inflammatory mechanism of **AA** may be related to
426 iNOS and associated with the increase in the activities of antioxidant enzymes (CAT,
427 SOD and GPx). **AA** may be used as a pharmacological agent in the prevention or
428 treatment of disease in which free radical formation is a pathogenic factor.

429

430 **Acknowledgements**

431 The authors want to thank the financial supports from the National Science Council (NSC
432 97-2313-B-039-001-MY3) and China Medical University (CMU) (CMU95-PH-11,
433 CMU96-113, CMU97-232, and CMU99-S-29). The authors would like to thank Dr
434 Jeffrey Conrad for critically reading the manuscript.

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436 **References**

437 1. Huang GJ, Huang SS, Lin SS, Shao YY, Chen CC, Hou WC, et al. Analgesic effects and
438 the mechanisms of anti-inflammation of ergostatrien-3β-ol from *Antrodia camphorata*
439 submerged whole broth in mice. *J Agric Food Chem* 2010; 58:7445–7452.

2. Coldren CD, Hashim P, Ali JM, Oh SK, Sinskey AJ, Rha C. Gene expression changes in the human fibroblast induced by *Centella asiatica* triterpenoids. *Planta Med* 2003; 69:725–732.

3. Dong MS, Jung SH, Kim HJ, Kim JR, Zhao LX, Lee ES, et al. Structure-related cytotoxicity and anti-hepatofibric effect of asiatic acid derivatives in rat hepatic stellate cell-line, HSC-T6. *Arch Pharm Res* 2004;27:512–517.

4. Lee YS, Jin DQ, Kwon EJ, Park SH, Lee ES, Jeong TC, et al. Asiatic acid, a triterpene, induces apoptosis through intracellular Ca^{2+} release and enhanced expression of p53 in $HepG_2$ human hepatoma cells. *Cancer Lett.* 2002;186:83–91.

5. Krishnamurthy RG, Senut MC, Zemke D, Min J, Frenkel MB, Greenberg EJ, Yu SW, Ahn N, Goudreau J, Kassab M, Panickar KS, Majid A. Asiatic acid, a pentacyclic triterpene from *Centella asiatica*, is neuroprotective in a mouse model of focal cerebral ischemia. *J Neurosci Res* 2009;87:2541-50.

6. Janero DR. Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radic. Biol. Med.* 1990;9: 515–540.

7. Zimmermann M. Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 1983;16:109-110.

8. Chang HY, Sheu MJ, Yang CH, Leu ZC, Chang Y, Peng WH, et al**.** Analgesic effects and the mechanisms of anti-inflammation of hispolon in mice. *Evidence-Based Compl. Altern. Med.* 2009; doi:10.1093/ecam/nep027.

461 9. Flohe L, Otting F. Superoxide dismutase assays. *Methods in Enzymology* 1984**;**105:
462 93–104.

463 10. Aebi H. Catalase *in vitro*. *Methods in Enzymology.* 1984;105:121–126.

464 11. Paglia ED, Valentine WN. Studies on the quantitative and qualitative characterization
465 of erythrocytes glutathione peroxidase. *J Lab Clin Med* 1967; 70:158–169.
466 12. Shibata M, Ohkubo T, Takahashi H, Inoki R. Modified formalin test: characteristic
467 biphasic pain response. *Pain* 1989;38:347–352.

468 13. Franzotti EM, Santos CV, Rodrigues HM, Mourao RH, Andrade MR, Antoniolli AR.
469 Anti-inflammatory, analgesic activity and acute toxicity of *Sida cordifolia* L.
470 (Malva-branca). *J Ethnopharmacol.* 2000;72:273–727.

471 14. Viana AF, Maciel IS, Motta EM, Leal PC, Pianowski L, Campos MM, Calixto JB.
472 Antinociceptive Activity of *Trichilia catigua* Hydroalcoholic Extract: New Evidence
473 on its Dopaminergic Effects. *Evidence-Based Compl. Altern. Med.*
474 2009;doi:10.1093/ecam/nep144.
475 15. Tjolsen A, Berge OG, Hunskaar S, Rosland JH, Hole K. The formalin test: an
476 evaluation of the method. *Pain* 1992; 51:5-17.

477 16. Spector WG, Willoughb DA. The inflammatory response. *Bacteriol Rev.* 1963;27:
478 117–154.

479 17. Tohda C, Nakayama N, Hatanaka F, Komatsu K. Comparison of anti-inflammatory
480 activities of six *Curcuma* rhizomes: a possible curcuminoid-independent pathway

481 mediated by *Curcuma phaeocaulis* extract. *Evidence-Based Compl. Altern. Med.* 2006;
482 3:255–260.

483 18. Salvemini D, Wang Z, Bourdon DM, Stern MK, Curne MG, Manning PT. Evidence
484 of peroxynitrite involvement in the carrageenan induced rat paw edema. *Eur. J. Clin.*
485 *Pharmacol.* 1996; 303:217–220.

486 19. Cuzzocrea S, Zingarelli B, Calapai G, Nava F, Caputi AP. Zymosanactivated plasma
487 induces paw oedema by nitric oxide and prostaglandin production. *Life Sci.* 1997;60:
488 215–220.

489 20. Liao H, Banbury LK, Leach DN. Elucidation of Danzhixiaoyao Wan and Its
490 Constituent Herbs on Antioxidant Activity and Inhibition of Nitric Oxide Production.
491 *Evidence-Based Compl. Altern. Med.* 2007; 4:425–430.

492 21. Saad B, Abouatta BS, Basha W, Hmade A, Kmail A, Khasib S, et al. *Hypericum*
493 *triquetrifolium*—Derived Factors Downregulate the Production Levels of LPS-Induced
494 Nitric Oxide and Tumor Necrosis Factor-α in THP-1 Cells. *Evidence-Based Compl.*
495 *Altern. Med.* 2007; 4:425–430.

496 22. Dawson J, Sedgwick AD, Edwards JC, Lees PA. comparative study of the cellular,
497 exudative and histological responses to carrageenan, dextran and zymosan in the
498 mouse. *Int. J. Tissue React.* 1991;13:171–185.

499 23. Lee YS, Jin DQ, Beak SM, Lee ES, Kim JA. Inhibition of ultraviolet-A-modulated
500 signaling pathways by asiatic acid and ursolic acid in HaCaT human keratinocytes.

501 *Eur J Pharmacol*. 2003; 476:173–178.

502 24. Yun KJ, Kim JY, Kim JB, Lee KW, Jeong SY, Park HJ, Jung HJ, et al. Inhibition of
503 LPS-induced NO and PGE_2 production by asiatic acid via NF-κB inactivation in
504 RAW 264.7 macrophages: Possible involvement of the IKK and MAPK pathways.
505 *Int Immunopharmacol* 2008; 8:431–441.

506 25. Lee SH, Soyoola E, Chanmugam P, Hart S, Sun W, Zhong H, et al. Selective
507 expression of mitogen-inducible cyclooxygenase in macrophages stimulated with
508 lipopolysaccharide. *J Biol Chem* 1992; 267:25934–25938.

509 26. Salvemini D, Ischiropoulos H, Cuzzocrea S. Roles of nitric oxide and superoxide in
510 inflammation. *Methods Mol Biol*. 2003; 225: 291–303.

511 27. Subbaramaiah K, Dannenberg AJ. Cyclooxygenase 2: a molecular target for cancer
512 prevention and treatment. *Trends Pharmacol Sci.* 2003;24:96–102.

513 28. Lefkowitz DL, Gelderman MP, Fuhrmann SR, Graham S, Starnes JD, Lefkowitz SS,
514 et al. Neutrophilic lysozyme-macrophage interactions perpetuate chronic
515 inflammation associated with experimental arthritis. *Clin Immunol* 1999;
516 91:145–155.

517 29. Harris SG, Padilla J, Koumas L, Ray D, Phipps RP. Prostaglandins as modulators of
518 immunity. *Trends Immunol* 2002;23:144–150.

519 30. Min SW, Ryu SN, Kim DH. Anti-inflammatory effects of black rice,
520 cyanidin-3-O-β-D-glycoside, and its metabolites, cyanidin and protocatechuic acid.
521 *Int Immunopharmacol* 2010;10:959-966.

522 31. Karin M, Ben-Neriah Y. Phosphorylation meets ubiquitination: the control of NF-κB
523 activity. *Annu Rev Immunol* 2000;18:621–663.

524 32. Moncada S, Palmer RMJ, Higgs EA. Nitric oxide physiology, pathophysiology and

pharmacology. *Pharmacol Rev* 1991;43:109–142.

33. Hogg N. Free radicals in disease. *Semin Reprod Endocrinol* 1998;16:241–248.

34. Yoshikawa T, Naito Y. The role of neutrophils and inflammation in gastric mucosal injury. *Free Radical Research* 2000;33:785–794.

Figure Legends

Figure 1. Chemical structure of asiatic acid (**AA**).

Figure 2. Analagesic effects of **AA** and indomethacin (Indo) on acetic acid-induced writhing response in mice. Each value represents as mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ as compared with the pathological model group (Con) (one-way ANOVA followed by Scheffe's multiple range test).

Figure 3. Effects of **AA** and Indo on the early phase and late phase in formalin test in mice. Each value represents as mean ± S.E.M. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the pathological model group (Con) (one-way ANOVA followed by Scheffe's multiple range test).

Figure 4. Effects of **AA** and Indo on hind paw edema induced by Carr in mice. Each value represents as mean ± S.E.M. $^{***}p < 0.001$ as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple range test).

Figure 5. Effects of **AA** and Indo on the tissue MDA concentration of paw in mice. Normal control received 0.9% normal saline. Animals treated with **AA** (1, 5, and 10 mg/kg) and Indo were assayed for their ability inhibiting MDA production in the right hind paws. The right hind paw tissues were dissected at the 5 h. Then the homogenate was centrifuged and the supernatant was obtained for the MDA assays. Each value represents as mean ± S.E.M. $^{###}p <$

571 0.001 as compared with the control group. $^{**}p < 0.01$ and $^{***}p < 0.001$ as
572 compared with the Carr group (one-way ANOVA followed by Scheffe's
573 multiple range test).
574
575 **Figure 6.** Effects of **AA** and Indo on Carr-induced (A) NO, (B) TNF-α, and (C)
576 interlukin-1β concentrations of serum at 5 h in mice. Normal control received
577 0.9% normal saline. Animals treated with **AA** (1, 5, and 10 mg/kg) and Indo
578 were assayed in the right hind paws. After 5 h, the animals were sacrificed and
579 blood was withdrawn. Then fresh blood was centrifuged and the supernatant
580 was obtained for measuring NO, TNF-α, and interlukin-1β levels. Each value
581 represents as mean ± S.E.M. $^{###}p < 0.001$ as compared with the control group.
582 $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared with the Carr group
583 (one-way ANOVA followed by Scheffe's multiple range test).
584
585 **Figure 7.** Inhibition of iNOS, COX-2, and NF-κB protein expression by **AA** induced by
586 Carr in mice paw edema for 5 h. Normal control received 0.9% normal saline.
587 Animals treated with **AA** (1, 5, and 10 mg/kg) and Indo to injection of Carr
588 right hind paws. The right hind paw tissues were taken at the 5 h. Then the
589 homogenate was centrifuged and tissue suspended were then prepared and
590 subjected to western blotting using an antibody specific for iNOS, COX-2 and
591 NF-κB. β-actin was used as an internal control. (A) Representative western
592 blot from two separate experiments is shown. (B) Relative iNOS, COX-2 and
593 NF-κB protein levels were calculated with reference to Carr-injected mouse.

###compared with sample of control group. The data were presented as mean ± S.D. for three different experiments performed in triplicate. **$p < 0.01$ and ***$p < 0.001$ were compared with Carr-alone group.

Figure 8. Histological appearance of the mouse hind footpad after a subcutaneous injection with Carr stained with H&E stain at the 5 h to reveal hemorrhage, edema and inflammatory cell infiltration in (A) control mice, (B) Carr-treated mice demonstrating hemorrhage with moderately extravascular red blood cells and a large amount of inflammatory leukocyte mainly neutrophils infiltration in the subdermis interstitial tissue of mice, and **(**C**)** mice given Indo (10 mg/kg) before Carr**. AA** significantly shows (D) morphological alterations (100×) and (E) the numbers of neutrophils in each scope (400x) compared to subcutaneous injection of Carr only. ###$p < 0.001$ as compared with the control group. **$P < 0.01$, and ***$p < 0.001$ compared with Carr group. Scale bar = 100 μm.

Figure 9. Propose the mechanism of **AA** in λ-carrageenan (Carr) -injected mouse. **AA** inhibit the production of TNF-α, free radicals and lipid peroxidation, which in turn decrease MDA level, iNOS, COX-2, and NF-κB activation in the paw edema and increase the CAT, SOD and GPx activities in the liver. MDA: malondialdehyde; TNF-α: tumor necrosis factor-α; IL-1β: interleukin-1β; NO: nitric oxide; CAT: catalase; SOD: superoxide dismutase; GPx: glutathione peroxidase; iNOS: inducible nitric oxide synthase; COX-2: cycloxyclase-2;

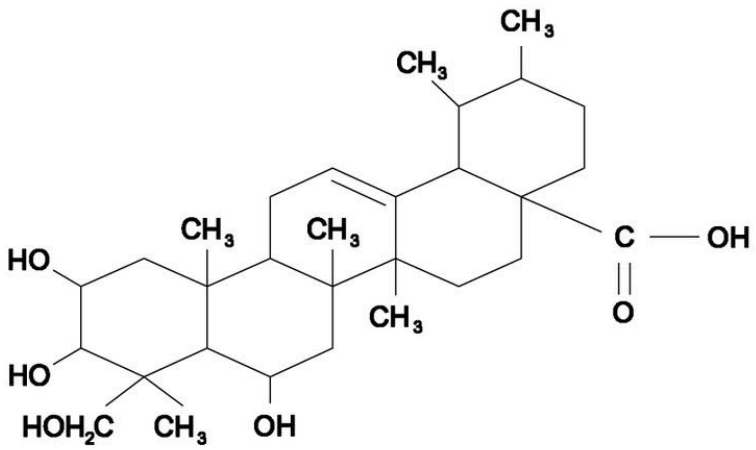
NF-κB: Nuclear factor- κB.

Table 1: Effects of **AA** and Indo on the liver CAT, SOD, and GPx activities in mice.

Groups	Catalase(U/mg protein)	SOD (U/mg protein)	GPx (U/mg protein)
Control	5.12 ± 0.21	24.39 ± 0.18	3.23 ± 0.18
Carr	3.46 ± 0.32$^{###}$	17.56 ± 0.31$^{###}$	1.96 ± 0.14$^{###}$
Carr+ Indo	4.53 ± 0.25**	22.13± 0.26**	2.76 ± 0.29**
Carr + **AA** (1 mg/Kg)	3.84 ± 0.17	19.47 ± 0.15	2.14 ± 0.19
Carr + **AA** (5 mg/Kg)	4.36 ± 0.25*	21.32 ± 0.19*	2.49 ± 0.27*
Carr + **AA** (10 mg/Kg)	4.67 ± 0.36**	23.06 ± 0.33**	2.93 ± 0.14**

Each value represents as mean ± S.E.M. $^{###}p < 0.001$ as compared with the control. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared with the Carr group (one-way ANOVA followed by Scheffe's multiple range test).

Figure 1.



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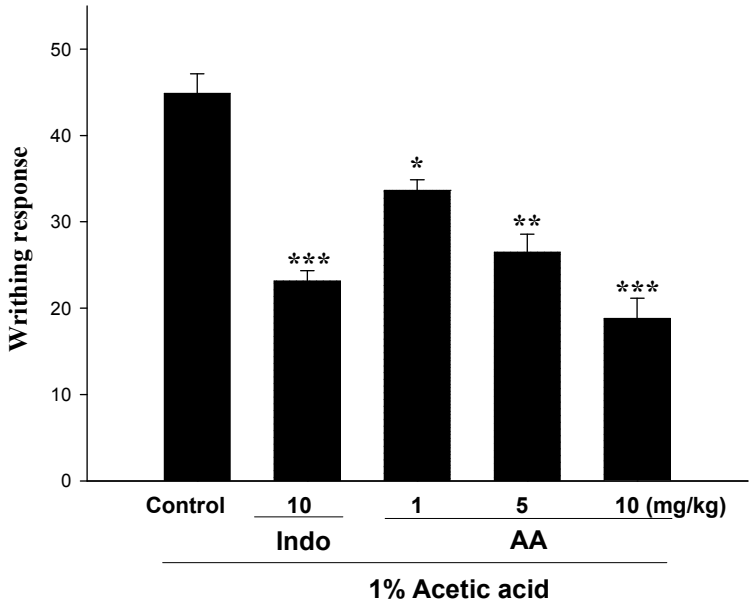
669 **Figure 2.**



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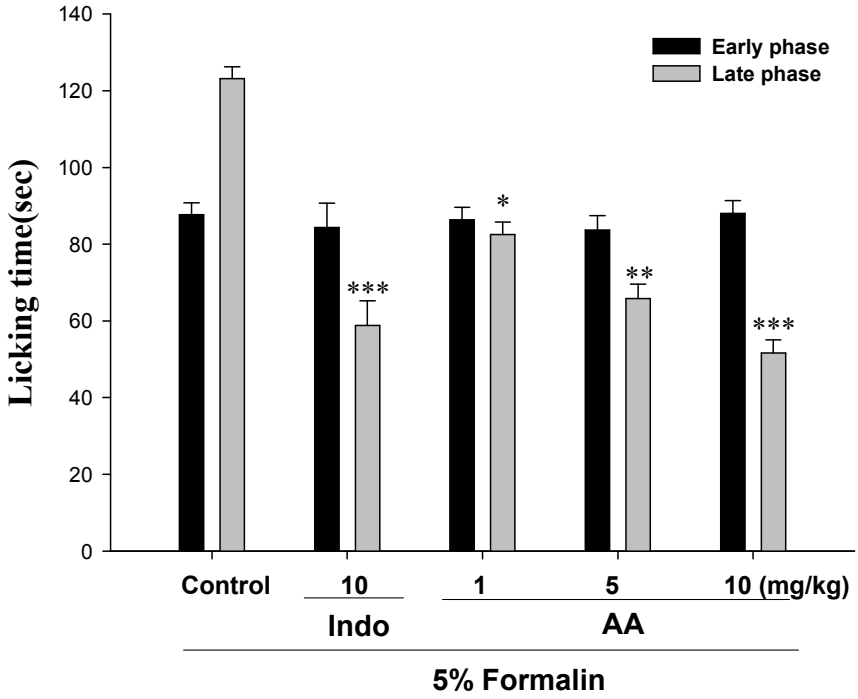
681 **Figure 3.**



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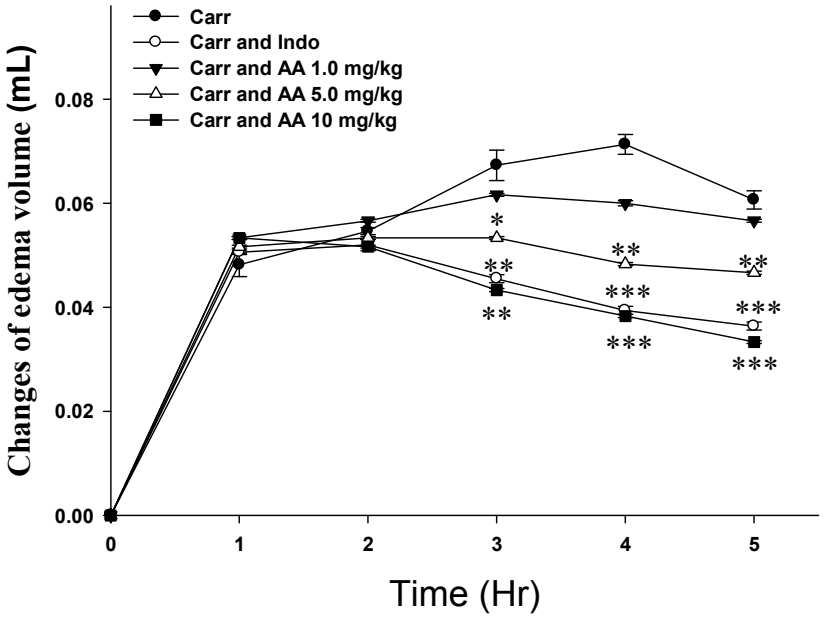
690 **Figure 4.**



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701 **Figure 5.**

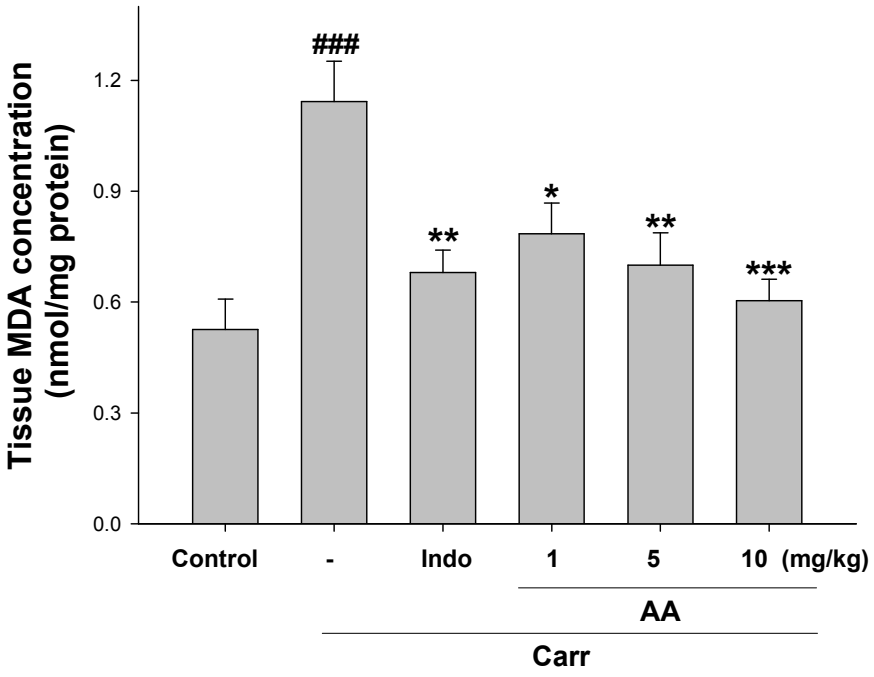

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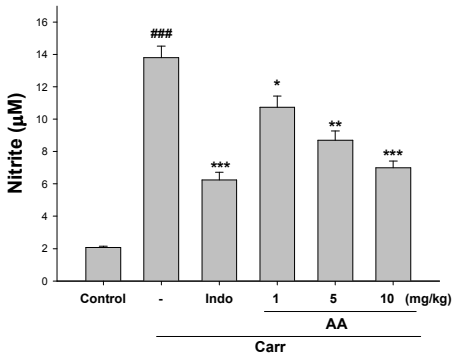
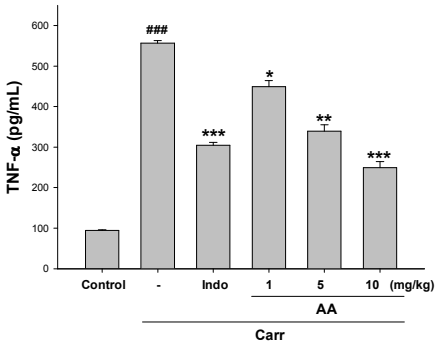
Figure 6.

A.


Nitrite (µM)
16
14
12
10
8
6
4
2
0
###
*
**
***
***
Control
-
Indo
1
5
10
(mg/kg)
AA
Carr

B.


TNF-α (pg/mL)
600
500
400
300
200
100
0
###
*
**
***
***
Control
-
Indo
1
5
10
(mg/kg)
AA
Carr

C.

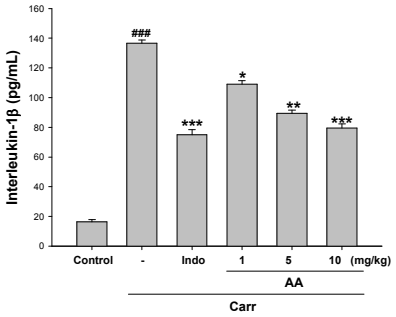
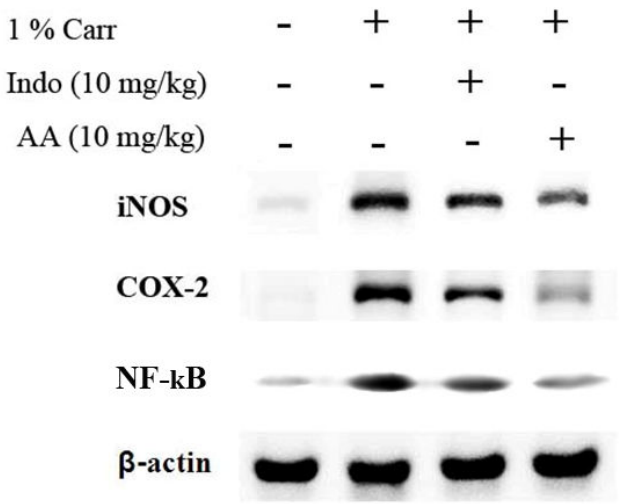

Interleukin-1β (pg/mL)
160
140
120
100
80
60
40
20
0
###
*
**
***
***
Control
-
Indo
1
5
10
(mg/kg)
AA
Carr

Figure 7.

A.


1 % Carr
Indo (10 mg/kg)
AA (10 mg/kg)
iNOS
COX-2
NF-kB
β-actin

B.

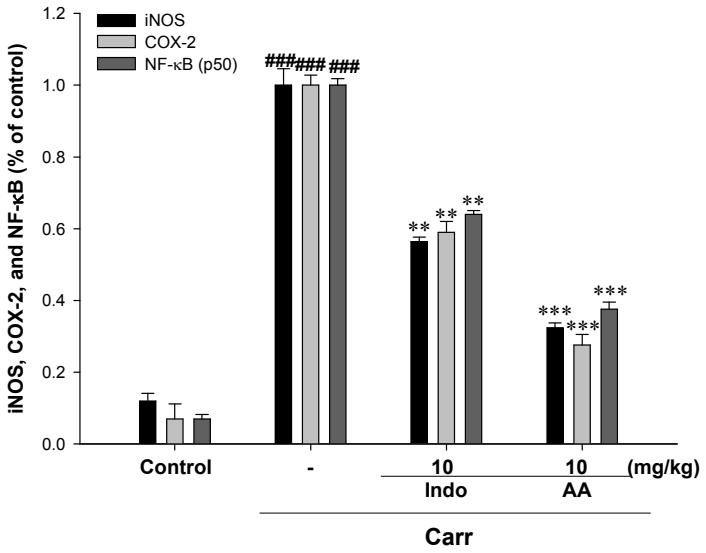
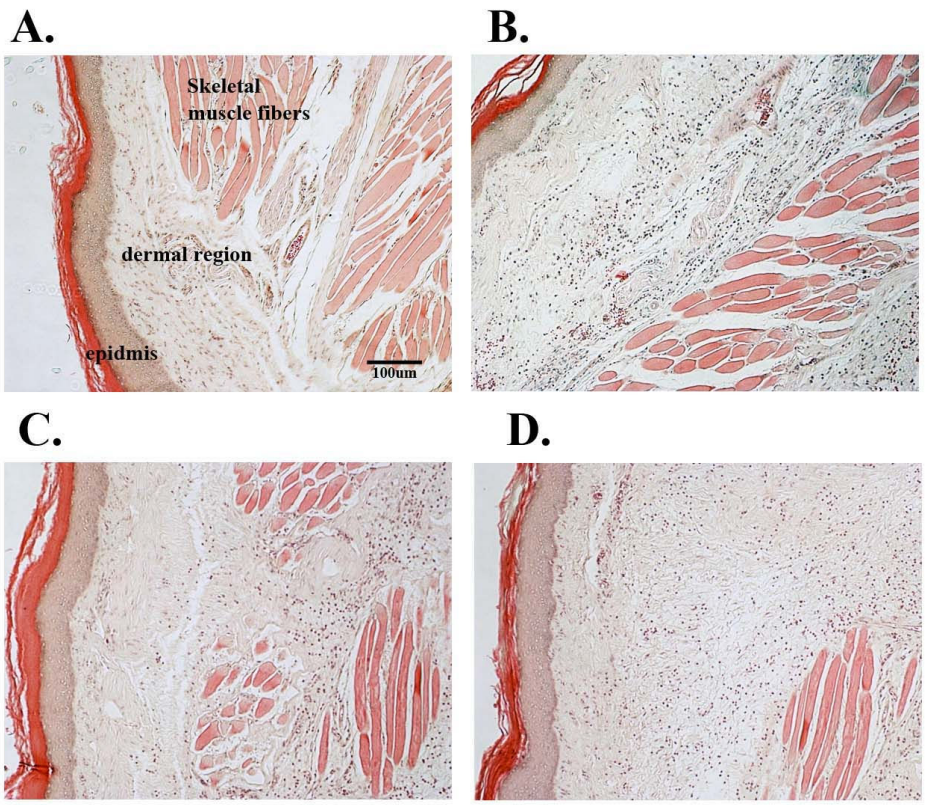
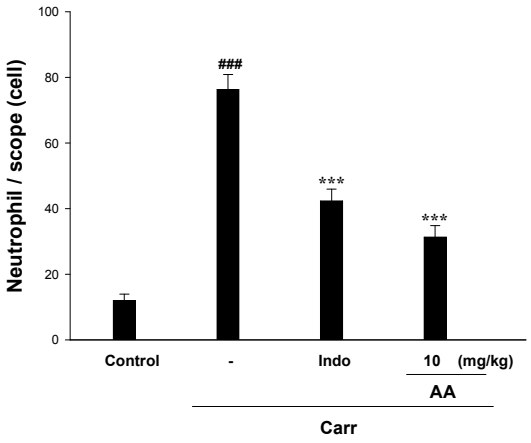

iNOS
COX-2
NF-κB (p50)
iNOS, COX-2, and NF-κB (% of control)
Control
Indo
AA
(mg/kg)
Carr

Figure 8.

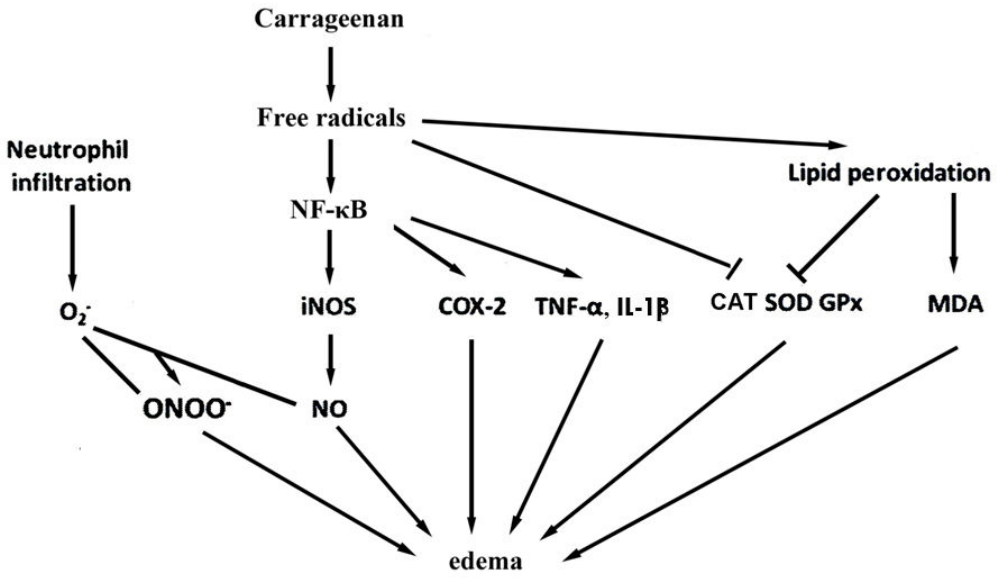

A.
B.
Skeletal
muscle fibers
dermal region
epidmis
100um
C.
D.

E.


Neutrophil / scope (cell)
100
80
60
40
20
0
###
***
***
Control
-
Indo
10 (mg/kg)
AA
Carr

728

729 **Figure 9.**



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