Original Paper

***Monascus Adlay* and Monacolin K Attenuates Arterial Thrombosis in Rats through the Inhibition of ICAM-1 and Oxidative Stress**

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Key Word

Arterial thrombosis • ICAM-1 • Monacolin K • *Monascus Adlay* • Reactive oxygen species • Platelet

ABSTRACT

Background/Aims: *Monascus Adlay* (MA) prepared from fungal fermentation of *Monascus purpureus* inoculating with cooked adlay contains high content of monacolin K (MK) and phenolic compounds. We explored whether MA and MK improve FeCl₃-induced arterial thrombosis in rats. **Methods:** The rats were divided into control, FeCl₃-treated rat carotid artery occlusion (TTO), TTO determined with one-week MA, and TTO determined with one-week MK. We compared MA or MK effects on oxidative stress by chemiluminescence amplification and immunohistochemistry, TTO by a transonic system, NFκB, ICAM-1, endoplasmic reticulum stress CHOP and Nrf2 signaling by western blotting. **Results:** MA or MK efficiently depressed O₂⁻, H₂O₂ and HOCl levels, platelet activation and aggregation and H₂O₂-enhanced ICAM-1 and VCAM-1 expression in the endothelial cells. FeCl₃ significantly increased NFκB p65, 3-nitrotyrosine, CHOP and ICAM-1 expression, and decreased nuclear Nrf2 translocation and induces arterial thrombus formation. MA or MK pretreatment significantly elongated the level of FeCl₃-induced TTO compared to TTO group, significantly decreased proinflammatory NF-κB/ICAM-1 signaling, endoplasmic reticulum stress CHOP expression and decreased thrombotic area. MA or MK significantly preserved nuclear Nrf2 translocation. MA and MK exerted a similar protective effect in attenuating thrombus formation. **Conclusions:** We suggest MA is better than MK to improve FeCl₃-induced arterial thrombosis.

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Introduction

Traditional Chinese herbal medicine has been widely used in modern medicine for health promotion, disease prevention and treatment. These include products made from fungi, such as *Monascus* spp., and grass crops, such as adlay (*Cois lachrymal-jobi* L. var. *ma-yuen* Stapf; also known as Chinese pearl barley and soft-shelled Job's tears). Products of fermented *Monascus* spp. (e.g., anka and red koji) were first mentioned in the ancient Chinese pharmacopoeia, Pen-Chow-Kang-Mu (Systematic Pharmacopoeia) by Li, S. C. in 1596, and are widely used as a Chinese cuisine [1-5] for its metabolic products, including monacolin K, γ -aminobutyric acid, dimeric acid, and polyketide pigments and so on. The major metabolic product from fermented *Monascus* species is monacolin K (MK), which is known to have hypocholesterolemic, anti-fibrosis, anti-inflammatory, antioxidant, and anti-apoptosis properties [6]. Adlay is widely and popularly planted in Taiwan, China, and Japan. It has a high nutritional value and is effective in the treatment of rheumatism and neuralgia as well as anti-inflammatory, antioxidant, anti-tumorigenic, and anti-helminthic properties [7, 8].

Excess production of reactive oxygen species (ROS) may contribute to abnormal signal transduction or cellular dysfunction, initiating an oxidative stress cascade that leads to ICAM-1 mediated endothelial dysfunction and thrombosis formation [9, 10]. Thrombus formation is a highly dynamic process that is mediated by proteins of the coagulation cascade, the subendothelial matrix as well as surface adhesion proteins on platelets, leukocytes and endothelial cells. In the pathological condition of thrombosis, oxidative stress or increased ROS is associated with impaired nitric oxide (NO) bioavailability and endothelial dysfunction, and contributes to the pathogenesis of acute vascular syndromes by predisposing to plaque rupture and intravascular thrombosis [9, 10].

A dominant mechanism of impaired vascular NO bioavailability relates to its oxidative inactivation by O_2^- . A central feature of impaired endothelial function is the presence of ROS manifested by oxidized low-density lipoprotein in hypercholesterolemia, glycoxidation products in hyperglycemia, redox-active compounds in tobacco smoke, and lipid peroxides in hyperhomocysteinemia [9]. Oxidized lipids can also be generated by metal-dependent Fenton oxidation; enzyme-catalyzed oxidation by lipoxygenase or reaction with HOCl generated by myeloperoxidase; cell-dependent oxidation via a diversity of O_2^- and H_2O_2 -generating oxidases; and oxidation by NO-derived reactive nitrogen species such as NO_2 , nitryl chloride (NO_2Cl), and peroxyxynitrite [9, 10]. The improvement of endothelial function by decreasing oxidative stress and inflammation would attenuate endothelial progenitor cell apoptosis and thrombosis formation [6].

We have demonstrated that inoculating cooked adlay with *Monascus* spp. produces monascus adlay (MA) containing both functional and active components [11]. MA extracts including rich MK and total phenol contents display higher antioxidant activity, reducing power, scavenging and chelating abilities than uninoculated adlay products [5, 11]. MA and MK can depress oxidative stress evoked endoplasmic reticulum stress, apoptosis, autophagy and pyroptosis [11]. MK or lovastatin can inhibit urotensin enhanced expression of VCAM-1 and ICAM-1 by modulating the Rho activation, and NF- κ B inhibitors [12].

In addition, phenolic components from adlay inhibit the release and secretion of inflammatory mediators/cytokines [13, 14] and decrease O_2^- production/generation [15]. However, the anti-thrombotic effect of MA has never been explored. We therefore explored whether dietary MA through its active component, MK, enhances antioxidant, anti-inflammatory, anti-endoplasmic reticulum stress and anti-adhesive mechanisms to counteracting oxidative stress-induced cardiovascular diseases, like thrombosis, in our $FeCl_3$ -induced thrombosis.

Methods and Materials

Preparation of Monascus adlay (MA)

Monascus fermented products were prepared on an adlay substrate, using a solid-state culture method as described previously [4, 11]. In brief, adlay (obtained from the Erhlin Farmers' Association, Changhua County, Taiwan) was immersed in deionized water for 2 h, dried and autoclaved at 121°C for 20 min. We inoculated *M. purpureus* Went (CCRC 31498; obtained from the Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu City, Taiwan) onto malt extract agar (Difco agar, Voigt Global Distribution Inc, Kansas City, Missouri, USA) at 25°C for 72 h. We inoculated the mycelium into potato dextrose broth (Difco) and incubated it at 25°C for 7 d. The culture was homogenized in a blender and inoculated into autoclaved adlay with 10% *M. purpureus* Went at an inoculation rate of 5%. MA products were harvested after the fungal mycelia had colonized for 7 d at 25°C. MA was autoclaved at 121°C for 20 min and air-dried in an oven at 40°C. The dried MA was ground into a coarse powder (20 mesh) using a Restsch Ultra Centrifugal Mill and Sieving Machine (Haan, Germany). Our previous report had indicated the MK content in MA extract by a high-performance liquid chromatography (HPLC) [11]. Briefly, 1 g of powdered MA was extracted with 5 mL ethyl acetate at 70°C for 1.5 h. After 5 min of centrifugation at 1800 rpm and filtration through a 0.45-μm membrane, the filtrate was dried under a vacuum. One milliliter of acetonitrile was added to the resulting mixture, which was then filtered with a 0.45-μm pore size filter and analyzed by an HPLC system (Model L-6200, Hitachi, Japan). MK content in our prepared MA extract was 1.57 mg/g (0.157%).

Animals and MA Treatments

The onset of hemolysis and subsequent thrombosis and tissue infarction is faster in female than in male rats [16]. Therefore, we explored the medical efficacy of an MA diet and MK on 24 female Wistar rats (200–220 g; mean body weight, 206 ± 10 g) housed at the Experimental Animal Center of National Taiwan Normal University. The standard rat chow diet contained 58% carbohydrates, 28.5% proteins, and 13.5% fat (Laboratory Rodent diet 5001; Young Li Trading Company Ltd., Sihhih City, New Taipei City, Taiwan). We mixed powdered rat chow diet and powdered MA at a ratio of 99:1 by combining them through a 20-mesh sieve (aperture = 0.94 mm) [11]. We used 2% corn starch and 3% soybean oil to re-form the product into the MA lump diet. The same methods were used to crush and re-form the control, standard rat chow diet into food lumps. Animals were provided with food and tap water *ad libitum*.

FeCl₃-induced carotid arterial time to occlusion (TTO)

All the rats were anesthetized by subcutaneous injection of 1.2 g/kg urethane (Sigma-Aldrich Inc., St. Louis, MO, USA). After arterial isolation, transonic flow probes (Probe# 0.5VBB517, Transonic Systems Inc., Ithaca, NY, USA) for carotid arterial blood flow measurement were applied and displayed on a small animal blood flow meter (Model 206, Transonic Systems Inc., Ithaca, NY, USA). All the blood flow signals were continuously recorded with an ADI System (PowerLab/16S, ADI Instruments, Pty Ltd, Castle Hill, Australia). The carotid arteries were injured as previously described¹¹ with a slight modification. In brief, a filter paper (1 mm × 2 mm), soaked with 30% FeCl₃ solution (Ferric chloride, Sigma, St. Louis, MO, USA), was applied to the artery for 3 minutes and the cavity was filled with saline immediately. The flow rate was continuously recorded, and the time to occlusion (TTO, arterial blood flow decreases to zero) was determined. For some animals, the arterial rings were fixed in 10% formalin after completing the thrombosis protocol, as described previously [11]. The injured arterial segments were excised, embedded in paraffin, sectioned, and subjected to hematoxylin and eosin staining.

Grouping

Rats were randomly divided into the following groups: control diet without TTO (n=6), control diet with TTO (n=6), MA diet with TTO (n=6), and MK pretreatment with TTO (n=6). Both MA and MK treatments lasted for 2 weeks. MK (lovastatin) (A.G. Scientific, CA, USA) was dissolved in deionized water at a concentration of 1.0 mg/100 mL. We found that rats in the MA group ingested 30±3 g apiece, or approximately 0.47 mg of MK per rat [11]. Animals in the MK group drank approximately 40–50 mL of treated water apiece, for a mean dosage of MK around 0.47 mg/rat. These doses were based on Boyd's formula for body surface area,

such that daily doses of MA and MK were equivalent to the daily recommended supplemental doses for adult humans (~2 g of *Monascus*-fermented products and ~20 mg of MK).

All surgical and experimental procedures were approved by the Institutional Animal Care and Use Committee of National Taiwan Normal University and are in accordance with the guidelines of the National Science Council of the Republic of China (NSC 1997). All possible efforts were made to reduce the numbers of animals used and to minimize animal suffering during the experiment.

Lucigenin-Enhanced Chemiluminescence (CL) Counts

We considered that MA or MK may affect the degree and process of oxidative stress in the carotid arteries. We selected 900 sec of FeCl₃ lesion to the carotid artery to determine the degree of ROS amount before its complete occlusion. We used lucigenin- and luminol-amplified chemiluminescence (CL) methods detect O₂⁻, H₂O₂ and HOCl amounts in MA (65 mg/mL containing 0.1 mg MK), and MK (0.1 mg/mL). These data were compared to the control values measured in distilled water [17]. The lucigenin-enhanced CL method has been confirmed to be a reliable assay for oxidative stress in damaged tissue [17]. We compared the differences in CL counts from carotid arteries in the rat subjected FeCl₃ injury as well as the MA or MK treatment. The carotid artery was removed after each treatment and homogenized with saline in a 0.1 mL volume. ROS levels in the homogenized arteries were determined by a CL analyzer (CLD-110; Tohoku Electronic Industrial, Japan) after administration of 1.0 ml of 0.1 mM lucigenin in phosphate-buffered saline (pH 7.4) into the tested samples. The assay was performed in duplicate for each sample, and total CL counts in 600 s were calculated by integrating the area under the curve.

Soluble form of Vascular ICAM-1 and ROS assay

To obtain the quantified data of oxidative stress, in some rats after FeCl₃ lesion for 900 s, the homogenates of arterial rings were used for measurement of soluble form of ICAM-1 (sICAM-1) by an ELISA kit (rat ICAM-1/CD54 Quantikine ELISA Kit) and H₂O₂-ROS amount by a luminol-amplified CL assay as described above. sICAM-1 is an important biomarker and the main cause for neutrophil adhesion to endothelium then to release ROS and to trigger thrombosis in the vessel wall [11, 18].

In situ demonstration of ROS production and amount in the carotid artery

High levels of ROS might promote the expression of 3-nitrotyrosine (3-NT) and ICAM-1 in the endothelium to trigger thrombotic cascades. We considered that MA or MK may affect the degree and process of oxidative stress in the carotid arteries. We selected 900 s of FeCl₃ lesion to the carotid artery to determine the degree of oxidative stress 3-NT and ICAM-1 by immunocytochemical stains. For immunocytochemical stains, the rats (n=3 in each group) were sacrificed at the end of experiment. 3-NT and the ICAM-1 [18] expression in the paraffin-embedded sections of the vascular rings were immunostained. The 5-μm cross-sections were stained with anti-3-NT antibody (Alpha Diagnostic International; San Antonio, TX) and with ICAM-1 antibody (R&D Systems, Minneapolis, MN). The 3-NT and ICAM-1 stains were photographed on a Leica microscope (Leica Microsystems Wetzlar, Wetzlar, Germany).

The percentage of staining in the vascular rings was calculated by the formula:

% staining = stained curved length/total curved length.

The thrombus size was determined by Adobe Photoshop 7.0.1 imaging software using the following formula:

% thrombus size = thrombus area of intravascular area/total intravascular area × 100.

Immunoblot analysis for NF-κB, 3-NT, ICAM-1, CHOP, and Nrf2

To explore proteins expression, we selected 4 h of FeCl₃ lesion to the carotid artery to determine the degree of proinflammation transcription factor, NF-κB, oxidative stress biomarkers, 3-NT and ICAM-1, endoplasmic reticulum stress biomarker, CCAAT/-enhancer-binding protein homologous protein (CHOP), by western blotting. The immunoblotting method for western blotting was performed as described previously [18]. We determined the expression of 3-NT, ICAM-1, CHOP and β-actin in the total homogenates of carotid arterial tissues subjected to 30% FeCl₃ lesion. We evaluated NF-κB p65 and Nrf2 in the nuclear

proteins. The isolated arteries were placed in ice-cold isolation buffer containing 0.5 M saccharose, 10 mM Tris-HCl, 1.5 mM MgCl₂, 10 mM KCl, 10% glycerol, 1 mM EDTA, 1 mM DTT, 2 mg/mL aprotinin, 4 mg/mL leupeptin, 2 mg/mL chymostatin, 2 mg/mL pepstatin, and 100 mg/mL 4-(2-aminoethyl)-benzenesulfonyl fluoride at pH 7.4 and were homogenized by using a tissue grinder. Then, the supernatant was resuspended in isolation buffer and the aliquots (nuclear fractions) were stored at -70°C. Antibodies raised against NFκB (R&D Systems, Minneapolis, MN), LaminA/C, Nrf2, CHOP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 3-NT (Alpha Diagnostic International, San Antonio, TX), ICAM-1 (R&D Systems, Minneapolis, MN, USA) and β-actin (catalog no. A5316, clone AC-74, Sigma) were used. The immunoreactive bands were detected by incubation with each respective antibody; the secondary antibody alkaline phosphatase; and, finally, nitroblue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate, and a toluidine salt (Roche Diagnostic, Mannheim, Germany) stock solution for 30 minutes at room temperature.

Preparation of Platelet Suspensions for Platelet activation and aggregation

Rat platelet suspensions were prepared as described previously [18]. In brief, blood (5 mL) was drawn from the carotid artery into plastic tubes containing 1 mL of 3.8% sodium citrate buffer (blood : sodium citrate = 9 : 1). Platelet-rich plasma was obtained by low-speed centrifugation (1500 rpm for 10 min) and further centrifuged at 15,000 rpm for 10 min to obtain a platelet pellet. The platelets were suspended in 190 μL aliquots of Ca²⁺-free Tyrode's solution (pH 7.35) and were incubated with the indicated concentrations of MA extract, MK or vehicle (DMSO) for 30 min at 37 °C. MA or MK was dissolved in DMSO as a stock solution and stored at -20 °C. The platelet number was adjusted to 3 × 10⁸/mL before use. A turbidimetric method was adapted to measure platelet aggregation with a lumi-aggregometer (Payton Scientific, Scarborough, ON, Canada). Specifically, 10 μL of 2 mM ADP was administered to the platelet suspension (0.4 mL) for 5 min and the response of aggregation was expressed in light-transmission units.

For platelet activation, the washed platelets, pretreated with MA or MK were stimulated with collagen (2.5 μg/mL) and incubated for 5 min at 37°C. A detailed procedure was described below. The reaction was terminated, and the platelets were centrifuged followed by resuspension in ice-cold phosphate buffered saline (PBS) containing 10% fetal calf serum (FCS) and 1% sodium azide. The platelets were incubated with CD62P primary antibody (P-selectin, Thermo Fisher Scientific Taiwan, Co., Ltd., Taipei, Taiwan) in 3% bovine serum albumin (BSA)/PBS for 30 min at 4 °C in the dark, then fixed and washed three times by centrifugation at 400×g for 5 min. The platelets were resuspended in ice-cold PBS followed by FITC-conjugated secondary antibody (Santa Cruz Biotechnology) incubation in 3% BSA/PBS for 30 min at 4 °C in the dark. Platelets were again washed three times by centrifugation at 400×g for 5 min and resuspended in ice-cold PBS, 3% BSA and 1% sodium azide. The samples were analyzed with a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA, USA).

Expression of ICAM-1 and VCAM-1 on ROS-treated endothelial cells

To investigate the effect of MA or MK on ROS-treated cells, human umbilical vein endothelial cell (HUVEC), HUVEC were exposed to H₂O₂ (100 nmol/L) in the presence of MA extract (255 μg/mL), MK (400 ng/mL) or isovolumetric solvent control (0.5% DMSO) for 24 h. HUVECs (ATCC CRL-2873) were cultured in EGM2 medium supplemented with 2% (v/v) fetal calf serum as described [18]. Cells were used at passages 3–4, and all cells exhibited the specific characteristics of endothelial cells. HUVECs were seeded at 1 × 10⁵ cells/mL on 0.8% gelatin-coated 24-well plates. After 24 h, the medium was replaced with serum-free medium, and cells were incubated overnight. After 1 h, the medium was replaced with normal growth medium or medium containing MA, MK or vehicle (1–50 μg/mL). Cell plates were examined by fluorescence microscopy (Nikon TS100, Tokyo, Japan). The ICAM-1 and VCAM-1 expression level in HUVEC were determined by immunofluorescence staining [19, 20]. For ICAM-1 and VCAM-1 expression studies, cells were stained by a rat anti-human/mouse ICAM-1 or VCAM-1 antibody (Santa Cruz Biotechnology) at 4 °C overnight. The secondary antibody, FITC-labeled goat anti-rat (Santa Cruz Biotechnology), was applied at 37 °C for 30 min followed by staining of the nuclei with 4,6-diamidino-2-phenylindole (DAPI; Sigma).

Statistical analyses

All data were expressed as mean ± standard error mean. Differences within groups were evaluated by a paired *t* test. Two-way ANOVA was used to establish differences among groups. Intergroup comparisons

were made by Duncan's multiple-range test. Differences were regarded as significant if $P < 0.05$ was attained.

Results

MA or MK pretreatment delays FeCl_3 -induced TTO

As shown in Figure 1A, the definition of TTO was indicated in one control, MA- and MK-treated rat subjected to FeCl_3 lesion, respectively. Topical application of 30% FeCl_3 efficiently induced carotid arterial blood flow cessation, which was designated to TTO within 10–20 min. The use of MA or MK pretreatment for two weeks significantly ($P < 0.05$) elongated FeCl_3 -induced TTO levels (Figure 1B) when compared to TTO group. The value of TTO between MA+TTO and MK+TTO groups was not significantly different.

MA or MK reduces FeCl_3 -induced arterial thrombosis and endothelial 3-NT and ICAM-1

As shown in Figures 2B–2D, we found that FeCl_3 -induced carotid arterial thrombosis by the appearance of thrombus in the carotid artery when compared to non-treated control rat (Figure 2A). A less expression

of endothelial 3-NT (Figure 2E) and ICAM-1 (Figure 2I) was found in the control carotid arteries. FeCl_3 stimulation markedly enhanced endothelial 3-NT (Figures 2F–2H) and ICAM-1 (Figures 2J–2L) expression in the carotid arteries. MA or MK pretreatment effectively reduced carotid arterial thrombotic area by the inhibition of endothelial 3-NT and ICAM-1 accumulation indicated by black arrows in Figure 2.

MK or MK inhibited platelet activation and aggregation and endothelial ICAM-1 and VCAM-1 expression

MA or MK significantly decreased collagen-enhanced platelet activation by the downregulation of P selectin expression in the washed platelets (Figure 3A). In addition, MA or MK significantly inhibited platelet aggregation stimulated by ADP (Figure 3B). The statistic data showed that MA seems to have a high tendency but not significant in inhibition of platelet activation and aggregation compared with MK. H_2O_2 significantly increased fluorescent ICAM-1 expression (Figure 3C) and fluorescent VCAM-1 expression (Figure 3D) in the cultured HUVECs. MA or MK significantly depressed these ICAM-1 and VCAM-1 expressions with a similar effect between MA and MK.

Scavenging O_2^- , H_2O_2 and HOCl ability by MA and MK

As shown in Figure 4, MA or MK significantly ($P < 0.05$) reduced O_2^- , H_2O_2 and HOCl

Fig. 1. Effects of MA and MK treatment on FeCl_3 -reduced carotid arterial blood flow (A) and the statistical data of TTO levels (B) in three groups of control, MA or MK treated rats ($n=6$ each). MA or MA treatment significantly delayed FeCl_3 -induced carotid arterial blood flow occlusion to zero. *, $P < 0.05$ versus the Control group.

Fig. 2. Effects of MA or MK treatment on FeCl_3 -induced carotid arterial thrombosis (A-D) by H&E stain and oxidative stress (E-H) analyzed by 3-NT stain and I-CAM-1 (I-L) in the carotid arteries from Control, TTO, TTO+MA and TTO+MK groups. FeCl_3 markedly induced thrombosis and increased 3-NT and ICAM-1 stains in the endothelium indicated by black arrows. MA or MK treatment efficiently reduced thrombosis and 3-NT and ICAM-1 stains in the endothelial area.

Fig. 3. Effect of MA or MK on platelet activation and aggregation in washed platelets and H_2O_2 -induced endothelial ICAM-1 and VCAM-1 expression in endothelial cells. A: Collagen activated the platelets by the increase of P selectin expression. MA or MK significantly inhibited platelet activation by downregulating P selectin expression. B: ADP enhanced platelets aggregation, whereas MA or MK significantly inhibited ADP-enhanced platelet aggregation. H_2O_2 significantly increased fluorescent endothelial ICAM-1 (C) and VCAM-1 expression (D) in endothelial cells. MA or MK treatment significantly depressed H_2O_2 -enhanced endothelial ICAM-1 and VCAM-1 expression in endothelial cells. * $P < 0.05$ versus DMSO-control (Con) group; # $P < 0.05$ versus H_2O_2 group.

Fig. 4. $O_2^{\bullet-}$, H_2O_2 - and $HOCl$ scavenging activity of the control (distilled water; Con), MA, and monacolin K (MK). MA is better than MK in scavenging $O_2^{\bullet-}$, H_2O_2 - and $HOCl$ activity. *, $P < 0.05$ in comparison with Con; #, $P < 0.05$ in comparisons with MA.

Fig. 5. Effect of MA or MK treatment on $FeCl_3$ -enhanced ROS (A) and sICAM-1 (B) levels in the $FeCl_3$ -treated carotid arteries in Control, TTO, TTO+MA or TTO+MK group. A: TTO significantly increased ROS CL counts by luminol-amplified CL methods when compared to Control arteries. MA or MK treatment significantly attenuated TTO-enhanced ROS CL counts compared with TTO group. B: TTO significantly elevated sICAM-1 levels by ELISA assay compared to Control group. MA or MK treatment significantly decreased $FeCl_3$ -enhanced arterial sICAM-1 levels compared with TTO group. * $P < 0.05$ when compared to Control group. # $P < 0.05$ when compared to TTO group.

counts implicating that their wide-range antioxidant ability in MA or MK. This data also informed that MA via its active component, MK, can inhibit $O_2^{\bullet-}$, H_2O_2 and $HOCl$ activity. Our data also evidence that MA is more effective than MK in reducing $O_2^{\bullet-}$, H_2O_2 and $HOCl$ counts, implicating the phenolic compounds from adlay work synergistically with MK to increase the antioxidant ability of MA.

MA or MK treatment on $FeCl_3$ -enhanced arterial ROS and sICAM-1 levels

To further confirm $FeCl_3$ -induced oxidative stress in the carotid arteries, we determined the level of ROS and sICAM-1 by biochemical assay in the carotid arterial homogenates in the control, MA and MK groups. Figure 5A demonstrated that $FeCl_3$ significantly ($P < 0.05$) increased ROS CL counts in the control, MA and MK group by luminol-amplified CL methods when compared to non-treated control arteries. MA or MK treatment significantly ($P < 0.05$)

Fig. 6. Effect of MA or MK treatment on FeCl_3 -enhanced nuclear NF- κ B translocation (A), 3-NT (B), ICAM-1 (C), CHOP (D) and nuclear Nrf2 (n-Nrf2) (E) expression in the FeCl_3 -treated carotid arteries in Control, TTO (T), TTO+MA (T+MA) or TTO+MK (T+MK) group. FeCl_3 significantly enhanced nuclear NF- κ B translocation, increased arterial 3-NT, ICAM-1 and CHOP expression and significantly decreased n-Nrf2 expression when compared to Control arteries. MA or MK treatment significantly attenuated FeCl_3 -enhanced nuclear NF- κ B translocation, 3-NT, ICAM-1 and CHOP expression and partly preserved n-Nrf2 expression compared with TTO group. * $P < 0.05$ when compared to Con group. # $P < 0.05$ when compared to T group.

decreased FeCl_3 -enhanced ROS CL counts compared to TTO group. Figure 5B also showed that FeCl_3 significantly ($P < 0.05$) elevated sICAM-1 levels in the control, MA or MK group by an ELISA assay compared to non-treated control group. Similarly, MA or MK treatment significantly ($P < 0.05$) depressed FeCl_3 -enhanced arterial sICAM-1 levels compared with TTO group. There is no significance between MA and MK groups.

MA or MK on 3-NT, ICAM-1, CHOP and n-Nrf2 expression in FeCl_3 -treated arteries

With western blot analysis, FeCl_3 significantly enhanced 3-NT (Figure 6A), ICAM-1 (Figure 6B), and CHOP expression (Figure 6D) and decreased n-Nrf2 expression (Figure 6C) in the damaged carotid arteries of the TTO group when compared to Control group. MA or

MK treatment significantly attenuated FeCl₃-enhanced 3-NT, ICAM-1 and CHOP expression and partly preserved n-Nrf2 expression compared with TTO group.

Discussion

Our prepared MA has been characterized by a high content of MK and total phenolic compounds and stronger anti-O₂^{•-} and anti-H₂O₂ (e.g., antioxidant) activities than either of its source materials (*M. purpureus* Went and adlay) alone and prevents smoke-induced lung injury [11]. The present study further indicates that daily intake of MA or MK can significantly scavenge ROS, suppress P selectin-mediated platelet activation, ADP-stimulated platelet aggregation and decrease H₂O₂-enhanced endothelial ICAM-1 and VCAM-1 expression *in vitro*. In *in vivo* study, MA or MK treatment FeCl₃-induced oxidative stress, NF-κB p65 mediated ICAM-1 expression and adhesion molecules expression, ER stress CHOP expression and thrombosis in the rat carotid artery. MA or MK also preserved nuclear Nrf2 translocation to increase cytoprotective ability in response to FeCl₃ injury. We found that the MA through its active component MK protects the vessels against FeCl₃-induced thrombosis formation.

The MA products contain high nutritional potential for the higher levels of crude ash, fat, fiber, and protein than are found in uninoculated adlay [4]. In addition, MA has a bitter taste probably due to its high MK and total phenolic compound content [4, 11]. Methanolic extracts from MA is more effective than adlay in scavenging 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals and chelating ferrous ions [5]. Our previous study [11] and the present study consistently show that MA displays a strong and effective tendency than MK in reducing O₂^{•-}, H₂O₂ and HOCl amounts. Trans-coniferylaldehyde, a phenolic compound in adlay, was recently found to efficiently scavenge DPPH radicals and inhibit O₂^{•-} production [15]. Generally, all these data implicate that naturally antioxidant components, such as phenolic compounds, work synergistically with MK to increase the antioxidant activity of MA. According to our data MA and MK presenting the same effect, we further indicated that MA contains high MK, total phenolic compounds, and other nutritional factors, confers higher antioxidant activity than MK, and can be used as functional food to prevent or as therapeutic drugs to treat the cardiovascular diseases like thrombus formation in the future.

FeCl₃ treatment would induce a H₂O₂-dependent Fenton reaction to oxidize macromolecules like lipid and protein in the vessels. According to our data of FeCl₃-treated carotid artery *in vivo*, the thrombus formation occurred rapidly within 10–20 min and was characterized by endothelial disruption, extensive platelet, white and red blood cell clumps, and interspersed fibrin. These data were consistent with previous findings [7, 11]. The ease of thrombus formation and similarity to human thrombus make this model appropriate for studying the influence of different agents that can inhibit platelet and blood cell aggregation. Our evidence found that FeCl₃-increased vascular ROS generation, 3-NT expression and ICAM-1 activity and expression locally in the endothelial area of the vessel wall. All the enhanced oxidative parameters observed in the artery of the FeCl₃-treated rats was significantly eliminated by the dietary MA or MK pretreatment, implicating that FeCl₃-increased vessel wall production of H₂O₂ could be scavenged by MA or MK.

In organs subjected to several kinds of oxidative injury like septic shock, smoke, hemorrhage, and ischemia/reperfusion, the excess ROS production oxidized several macromolecules, triggered pro-inflammatory signaling pathways responsible for the activation of NF-κB and AP-1 and promoted atherosclerosis and thrombosis [21–23]. Locally vascular ROS formation inhibited the bioavailability of nitric oxide (NO), impaired vascular relaxation, and increased leukocytes, platelets and fibrin adhesion to and aggregation in injured vessels [23, 24]. ROS can increase fractalkine (CX3CL1) and ICAM-1 expressions on injured endothelium, attract fractalkine receptor (CX3CR1)-expression inflammatory cells to the inflamed area, and provoke atherosclerosis and vascular inflammation [25]. These activated cascades resulted in up-regulation of the ICAM-1 gene in the vascular endothelium

and subsequent accumulation of activated neutrophils and other leukocytes in the tissue [11]. Likewise, we demonstrated that FeCl_3 stimulation increased vascular ROS formation that may lead to the early activation of nuclear translocation of the p65 subunit of NF- κ B and AP-1, which, in turn, promoted the expression of ICAM-1 protein and other inflammatory cytokines like CX3CL1. One previous study stated that after arterial-venous fistula surgery, increased monocyte/macrophage infiltration and pro-inflammatory cytokine-mediated adhesion molecules (i.e., ICAM-1) were highly expressed in the damaged venous wall [26]. Our recent data also demonstrated that increases in intracellular ROS, ICAM-1, and apoptosis occur in the damaged vessels easily contributing to thrombosis formation [18].

The induction of many cytoprotective enzymes in response to reactive chemical stress is regulated primarily at the transcriptional level. Activation of gene transcription is mediated primarily by Nrf2 (nuclear factor E2-related factor 2). Nrf2 controls basal expression of its genes clearly indicates that it is a constitutively and functionally active transcription factor and, notably, implies its presence in the nucleus under homeostatic conditions. Our data found that nuclear Nrf2 decreased after FeCl_3 lesion, but was preserved by MA or MK treatment. We have evidenced that FeCl_3 lesion enhanced nuclear p65 subunit of NF- κ B expression in the carotid arteries (Figure 6). We also noted that FeCl_3 lesion increased arterial 3-NT oxidative stress associated ICAM-1 and CHOP expression. We suggest that FeCl_3 induced thrombus formation through the upregulation of NF- κ B p65-mediated ICAM-1 and VCAM-1 expression associated oxidative stress and endoplasmic reticulum stress and downregulation of Nrf2 translocation to nucleus.

Platelet activation and aggregation is considered a crucial step in the initiation and aggravation of arterial thrombosis. ADP from activated platelets is recognized as major factor in thrombus formation and is a potent stimulator of ROS release from neutrophils [27]. MK affects platelet-neutrophil interactions by altering Rho-GTPase-dependent adenosine nucleotide function and consecutively inhibiting thrombin-activated platelets primed neutrophils for enhanced ROS release [27]. MK also known as simvastatin treatment of hypercholesterolemic mice and monkeys reduced oxLDL, monocyte procoagulant protein tissue factor (TF) expression, microparticle TF activity, activation of coagulation, and inflammation, without affecting total cholesterol levels [28]. MK decreased the rise in neutrophil adhesion and ROS generation following stimulation of saphenous vein endothelial cell culture with advanced glycation end products *in vitro* and *in vivo* data from diabetic patients administered with MK showed a similar significant reduction in neutrophil adhesion and ROS generation [29]. As far we know, there is no report about MA effect on leukocytes. MK content in our MA extract was 1.57 mg/g (0.157%). All these data implied that MA may through MK inhibit platelet-neutrophil interaction and monocyte activity and reduce ROS release and inflammation.

Conclusion

We have prepared MA from inoculating cooked adlay with *Monascus* spp. and obtained rich content of MK. MA or MK can scavenge O_2^- , H_2O_2 and HOCl amount, inhibit P selectin expression in collagen-activated platelets, depress platelet aggregation and inhibit H_2O_2 -enhanced endothelial ICAM-1 and VCAM-1 expression *in vitro*. Pretreatment of MA or MK for two weeks in the rats *in vivo*, MA or MK significantly extended the time to FeCl_3 -induced arterial occlusion, reduced vascular ROS production, decreased vascular NF- κ B, ICAM-1, 3-NT, and CHOP expression and ICAM-1 expression and preserved nuclear Nrf2 translocation. We conclude that MA is better than MK in the status containing total phenolic compounds and other nutritional factors and confers higher antioxidant activity than MK. We further suggest that MA is better than MK in state why we can use MA instead of MK (because MK is a drug) and MA can be used as functional food and natural product to treat the cardiovascular diseases like thrombus formation in the future.

Disclosure Statement

The authors of this manuscript state that they do not have any conflict of interests and nothing to disclose.

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