Analysis of NO-suppressing activity of Strawberry Wine supplemented with ball-milled achenes

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Abstract Inflammation is generally thought to be involved in the development of several chronic diseases, therefore, phytochemicals to modulate immune responses has attracted great interests. The objective of the present study was to evaluate the potential anti-inflammatory effects of wine supplemented using ball-milled achene on modulating NO production and inducible nitric oxide synthase (iNOS) expression. Ball-milled achenes were added in strawberry must prior to fermentation, and the wine samples were then concentrated and extracted with water and/or ethanol prior to analysis. Bioactivities of wine extracts were evaluated using the cell viability assay, cell cycle measurements, NO production and iNOS expression in lipopolysaccharide (LPS)-stimulated RAW264.7 cells. Treatments of achenes supplemented strawberry wine extract up to 100 µg/mL inhibited the proliferation of LPS-stimulated RAW264.7 cell via affecting the progression of cell cycle. Moreover, no detectable cytotoxicity in RAW264.7 cells was observed. The supplemented wine extract suppressed the action of LPS and led to a decreased NO production in stimulated cells. The inhibitory effect of the wine extract on NO production was determined to be a 25–40% decrease in the level of 25–100 µg/mL, in contrast

to a 10% decrease for conventional wine samples. Additionally, an alcoholic wine extract (100 µg/mL) led to a 40.31% decrease in iNOS expression in LPS-stimulated cells, which was more effective than the same dose of tocopherol. The results show that strawberry wine supplemented with ball-milled achenes causes a substantial inhibition of NO production, and this biofunction is exerted via the down-regulation of iNOS expression.

Keywords Anti-inflammatory · Ball-milled achenes · Strawberry wine · RAW264.7 · NO production · iNOS

Introduction

Inflammation is a complicated reaction that protects living tissues against invasion of foreign substances and is also a major factor in the development of a variety of chronic diseases (Calixto et al. 2003, 2004). The immune process generally involves a sequential release of mediators, including pro-inflammatory cytokines, nitric oxide (NO) and prostaglandin E₂ (PGE₂). NO plays a key role in the development of inflammation, and its production is typically a response against viral and bacterial infections. This process contributes to the development of pathogenesis by promoting oxidative stress, tissue injury, and even certain types of cancer (Hanada and Yoshimura 2002; Lin and Tang 2008). NO is a metabolic by-product that is produced during the conversion of L-arginine to L-citrulline by the action of nitric oxide synthase (NOS). Three isoforms of NOS have been identified, including neuronal NOS (nNOS or NOS1), which is expressed constitutively by neurons in the brain and the enteric nervous system, whereas the expression of endothelial NOS (eNOS or NOS3) is confined to endothelial cells lining the vasculature. The third

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isoform of NOS is inducible NOS (iNOS or NOS2), which is expressed in response to certain inflammatory stimuli such as bacterial products, cytokines and lipid mediators (Laroux et al. 2001; Sacco et al. 2006). Both eNOS and nNOS generate relatively small amounts of NO, whereas iNOS catalyzes the synthesis of relatively high levels of NO and this production can occur over an extended duration. Therefore, the inactivation of iNOS and/or a reduction in NO production can be beneficial in terms of anti-inflammatory effects (Chang et al. 2010; Kwon et al. 2010).

Phenolic compounds have been shown to impart protective effects in the metabolic syndromes. Among those functional compounds, ellagic acid (EA) and ellagitannins naturally occur in certain fruits (such as berries), vegetables and nuts. A number of in vivo and in vitro studies have confirmed that EA and ellagitannins have important pharmacological properties including antioxidant, anti-inflammatory and anticarcinogenic activities (Wadsworth and Koop 1999; Seeram et al. 2006; Lin and Tang 2008; Corbett et al. 2010; Jeong et al. 2010; Landete 2011; Tangney and Rasmussen 2013; Joseph et al. 2014). EA has been demonstrated to have anti-inflammatory activities by down-regulating iNOS, COX-2, TNF- α , and IL-6, and was reported exerting a chemopreventive effect on colon carcinogenesis in rats (Umesalma and Sudhandiran 2010; Landete 2011). Berries, such as strawberries, black currants, and blueberries, typically contain relatively high levels of polyphenolic compounds. Among these berries, strawberry is rich in anthocyanins, quercetin, catechin, chlorogenic, ferulic, ellagitannins and ellagic acid (Wang and Millner 2009; Giampieri et al. 2012). It has been reported that the consumption of strawberries could affect pathways related to cardiovascular health by inhibiting inflammation and platelet aggregation, increasing LDL resistance to oxidation, suggesting that antioxidant activity is one possible and relevant mechanism (Henning et al. 2010; Larrosa et al. 2010). Additionally, phytochemicals in strawberries have also been reported to modulate cellular processes associated with cancer progression, including signaling pathways associated with cell proliferation and differentiation, cell-cycle arrest and inflammation (Wang et al. 2005). Products containing strawberries have been reported inhibitory effects on selected pro-inflammatory markers. A daily intake of 60 g of freeze-dried strawberry powder in a water-based drink for 6 months resulted in a lower protein expression of iNOS of up to 80% (Basu et al. 2010). In addition to pulp, strawberry achenes also contain high levels of ellagitannins and ellagic acid, but their hard pericarp limits their use (Aaby et al. 2005). The application of a milling process to pulverize achenes is beneficial to enhancing the release of bioactive compounds (Lee and Chen 2016), thus, could be used to avoid immune disorder diseases via modulating inflammatory expression.

In our previous study, the addition of ball-milled achenes in must before fermentation led to higher ellagic acid and total phenol contents in strawberry wine, then resulted in enhanced antioxidant activities (Lee and Chen 2016). Ellagic acid and polyphenols not only exert antioxidant activities, but also prevent inflammation. Therefore, it would be logical to assume that the use of milled achenes to increase ellagic acid and total phenolic compounds in wine would confer enhanced anti-inflammatory activities. The objective of this research was to evaluate the effect of adding ball-milled achenes in fruit must during the fermentation process on the anti-inflammatory activity of strawberry wine.

Materials and methods

Chemicals

Pectinase (3.2.1.15) was obtained from Trump Chemical Company (Taipei, Taiwan), and potassium metabisulfite ($K_2S_2O_5$), yeast and nutrients were supplied by the Whole Earth Trading Company (Taipei, Taiwan). Acetic acid, acetonitrile, dimethyl sulfoxide (DMSO), naphthylethylene-diamine dihydrochloride, sulfanilamide, lipopolysaccharide (LPS) 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT), propidium iodide (PI), lipopolysaccharides, $NaNO_3$ were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Fetal bovine serum (FBS), bovine serum albumin (BSA) and phosphate buffer saline (PBS) were purchased from Gibco BRL-Life Technologies (Thermo Fisher Scientific Inc., Taipei, Taiwan). Triton X-100 was purchased from BIONOVAS biotechnology Co. (Toronto, Ontario, Canada). The murine RAW264.7 macrophage cells were obtained from the Bioresource Collection and Research Center (BCRC) (Hsinchu, Taiwan).

Preparation of ball-milled achene supplemented strawberry wine

Freshly harvested strawberry fruit (*Fragaria x ananassa* Duch. cv. TY. No. 1) were obtained from the Dahu Wineland Resort (Miaoli County, Taiwan). The berries were selected and rinsed upon arrival, then stored in a $-20\text{ }^{\circ}\text{C}$ freezer prior to use.

Frozen berries were thawed, mixed with $K_2S_2O_5$ (150 mg/kg fruit), and processed to a puree in a Waring blender. The puree was then freeze-dried by a benchtop type freeze-dryer (FD24-3S-12P, Kingmech Co., New Taipei City, Taiwan) under a vacuum of $250\text{--}300 \times 10^{-3}$ Torr at $-20\text{ }^{\circ}\text{C}$ for 96 h. Strawberry achenes were collected using a sieve (Tyler 20 mesh, 850 μm), and then

pulverized using a planetary Ball Miller (Retsch PM100, Haan, Germany) with a ratio of achene samples and zircon (0.7 mm diameter) of 1:1 at 300 rpm for 0.5 h at 25 ± 2 °C. Strawberry wine was prepared using 800 mL of juice with a level of 60 ppm of pectinase. The sugar content and the titratable acidity were adjusted to 20°Brix and 0.6%, respectively, and the 1% (w/v juice) ball-milled achenes were then added to the must. The mixture was inoculated with a precultured yeast strain (*Saccharomyces cerevisiae* var. *bayanus*) with yeast nutrients, and transferred to an 18 °C room for a month fermentation period (Lee and Chen 2016).

Preparation of wine extract

An aliquot of 100 mL wine sample was de-alcoholated and concentrated by rotary evaporation under reduced pressure at 40 ± 2 °C. The concentrate was pre-frozen for 1 h and then dried using a freeze-dryer. Those freeze-dried substances were extracted with either 250 mL water and/or 400 mL ethanol in a sonicator for 30 min at room temperature, and successively freeze-dried in a vacuum condition. The dried materials were dissolved in d-H₂O to prepare a water and/or alcoholic extract solution. Those extracts were then filtered through 0.22 µm aseptic filter membranes prior to further analyses.

Analyses of cell viability and cell cycle

The cytotoxicity of the wine extract on cultured cells was analyzed using an MTT assay (Mosmann 1983). Murine RAW264.7 macrophages were preserved in 5% FBS/DMSO at 37 °C in a humidified incubator with an atmosphere of 5% CO₂, and the cell cultures were passaged every 2–3 days by means of a standard aseptic process. Cell suspensions (diluted to 1×10^4 RAW264.7 cells/mL) were seeded in a 96-well flat-bottom plate, and then the cells were incubated with LPS (100 ng/mL) and/or treated with either water or ethanol extracts in a 5% CO₂ atmosphere at 37 °C for 24 h. Afterwards, 100 µL of MTT was added to each well, and cells were then incubated in humidified atmosphere with 5% CO₂ at 37 °C for 1 h. The absorbance was measured at 550 nm using an ELISA microplate reader (Thermo Fisher Scientific Inc., USA), and cell viability was calculated as the ratio of viable cells exposed to the extract compared to untreated cells (controls).

The effect of chemicals on cell cycle profile was examined using PI staining (Liou et al. 2015). RAW264.7 cells were cultured, starved for 3 h then treated with LPS and/or wine extracts. After a 24 h incubation at 37 °C, the attached cells were harvested, washed twice with ice-cold PBS, and fixed in ice-cold 70% ethanol overnight at

– 20 °C. The fixed cells were washed with ice-cold PBS, and stained with a PI buffer prepared by treatment with 1 mL of 1X PBS, 2 µL of RNase (0.5 mg/mL), 1 µL of Triton X-100 and 5 µL of PI (5 µg/mL) for 30 min at 37 °C in dark condition. The cell sample was filtered, and the cell cycle distribution was then analyzed by Cytomics FC 500 Flow Cytometry (Beckman Coulter Inc., USA).

Morphological observations were also accomplished to confirm the viability of LPS-stimulated cells treated with wine extract. RAW 264.7 cells (1×10^4) were seeded in 96-well plates, and allowed to attach overnight. The cells were incubated with LPS and/or wine extracts in a 5% CO₂ atmosphere at 37 °C for 24 h, and analysis of alteration in cell morphology was conducted using an inverted microscope (Nikon Eclipse TS100, Tokyo, Japan).

NO inhibition assay

NO production is generally used to indicate inflammation based on the level of nitrite formed in the supernatant of LPS-stimulated RAW 264.7 cells. The activity of the wine extract for suppressing NO production in cell cultures was determined according to a previous method (Chang et al. 2010; Kwon et al. 2010). The murine macrophage RAW264.7 cells (1×10^4 cells) were seeded in a 96-well plate, and incubated at 37 °C for 24 h. The adherent cells were washed three times with PBS. NO production in the LPS (100 ng/mL)-stimulated cells was determined alone, or in association with vitamin E or wine extracts after incubation at 37 °C for 24 h. Subsequently, 100 µL of the supernatant media was mixed with 100 µL of Griess reagent (1 g/L *N*-(1-naphthyl) ethylene diamine dihydrochloride in water mixed with 10 g/L sulfanilamide in 50 mL/L H₂PO₄), and incubated at room temperature for 5 min. The absorbance at 550 nm was measured and recorded with an ELISA microplate reader (Thermo, Waltham, MA, USA). The concentration of nitrite was calculated from a standard curve prepared using NaNO₂.

Immunoblotting assay

The anti-inflammatory activity of ball-milled supplemented strawberry wine was also evaluated by analyzing the down-regulating expression of proteins that are associated with inflammation. Immunoblotting was used for the analysis of iNOS protein expression (Chang et al. 2010; Kwon et al. 2010). RAW264.7 cells were grown in a nutrient agar medium at 37 °C for 24 h, and the adherent cells were then washed with PBS. Cells were replaced with fresh medium containing LPS (100 ng/mL), alone or associated with vitamin E and/or wine extracts, and then incubated at 37 °C for 24 h. RAW264.7 cells were collected from the

medium plate, and rinsed with 2 mL of PBS buffer, then transferred to centrifuge tubes. Cells were separated from the nutrient plate by centrifugation at $12,000 \times g$ for 5 min at 4°C , and the supernatant was discarded. Cells were then added to RIPA lysis buffer (including 50 mmol/L Tris-HCl (pH 7.4), 10 g/L NP-40, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethanesulfonyl fluoride, 1 mmol/L Na_3VO_4 , 1 mmol/L NaF, and protease inhibitor cocktail tablet), and total protein in the cells was then extracted in a -20°C incubator overnight. The cell suspension was removed from the incubator and centrifuged at $12,000 \times g$ for 5 min at 4°C , and the supernatant was collected and used in determining the protein content of the sample. A 2 μL aliquot of the supernatant was added to BSA, then mixed with 1000 μL of Bradford protein-binding dye and the sample incubated for 5 min. A 200 μL sample was placed in a 96-well plate, and the absorbance at 595 nm measured. Total protein in the cell was determined based on the BSA-absorbance response curve. A protein extract containing 40 μg of protein (at a concentration of 2.5 mg/mL) was separated by running on a 5% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel for 1 h using 100 V, and the SDS-PAGE was then placed over a polyvinylidenedifluoride (PVDF) membrane after electrophoresis. The transfer process involved 100 V for 1 h, and the proteins that were transferred to the PVDF were removed by cutting. The blots were incubated overnight in blocking buffer (containing 3% BSA and 0.02% sodium) with mouse immunoglobulin G (IgG) anti iNOS antibody (1: 1000) and mouse IgG anti- β -actin (1: 5000) (Abcam Co., Cambridge, MA, USA) at room temperature for 1 h. The PVDF member was rinsed with phosphate buffer (containing 1% Tween 20, pH 7.2) for 5 min three times. Antibodies were diluted in 50 g/L non-fat milk and 1 g/L Tween 20 in PBS. Bands on the gel were incubated with a 1/2000 dilution of alkaline phosphatase (AP)-conjugated anti-mouse IgG (1: 25000) (Abcam Co.). The bound antibodies were enhanced by an ECL (electrochemical luminescence) substrate for 10 s, and protein expression was visualized using the BioSpectrum AC[®] Image System (Ultra-Violet Product, LLC. Upland, CA, USA), and quantified using a Gel-Pro Analyzer (V4.0, Cybermetric Inc.) software.

Statistical analysis

Variance analysis (ANOVA) was used to analyze the effect of wine extract on viability and LPS-induced NO production in the cell, and $p < 0.05$ was considered to be a threshold for statistical significance. Duncan's multiple range test was used to determine whether the differences among treatments were significant.

Results and discussion

Cytotoxicity of ball-milled achene supplemented strawberry wine extracts

The bioactivities of the strawberry wine were initially measured by the anti-inflammatory effect on the murine RAW264.7 macrophages. An MTT assay was used to analyze the potential cytotoxicity of a wine sample against cells. Since wine contains more than 99% liquid (water and ethanol), extracts of wine samples were used in the cell tests to avoid discrepancies associated with volume differences. After exposing wine extracts, both aqueous and ethanolic extracts of strawberry wine at the level of 10–100 $\mu\text{g}/\text{mL}$ for 24 h, no detectable difference was found compared to normal cells (Fig. 1). Jeong et al.

Fig. 1 Effect of a strawberry wine extract on the viability of RAW264.7 cells. **a** Water extracts, **b** ethanol extracts. LPS: cells were treated with 100 ng lipopolysaccharide/mL. Ww: water extract of strawberry wine. WBw: water extract of strawberry wine supplemented with ball-milled achenes. We: ethanol extract of strawberry wine. WBe: ethanol extract of strawberry wine supplemented with ball-milled achenes. The data are the mean $\pm$ SD of 3 replicates

(2010) reported that black raspberry wine also had measurable anti-inflammatory activities, however, cell viabilities (RAW264.7 cells) were reduced by 10–25% when treated with 1000–2000 µg/mL (freeze-dried extract/medium). This previous finding illustrates the importance of adequate intake. In this study, cell viabilities were not significantly different when treated with 10–100 µg of wine extract/mL, indicating that the extracts were not toxic against RAW264.7 cells. Therefore, the concentrations of extracts in 10–100 µg/mL range were used in the following studies.

Cell cycle profile

To investigate the effect of water and/or ethanol extracts of strawberry wine, untreated or supplemented with ball-milled achenes, on the down-regulation of the cell cycle in LPS-stimulated RAW264.7 cells was analyzed by flow cytometry with PI staining. Neither water extracts (100 µg/mL), from supplemented and conventional strawberry wines, showed any effects on the LPS-stimulated cells ($p > 0.05$) (Fig. 2). The alcoholic extracts of ball-milled achenes supplemented and conventional strawberry wines induced a significant ($p < 0.05$) increased LPS-stimulated cell population in the G0/G1 stage, along with a significant ($p < 0.05$) decrease in cells in the S and G2/M phase (Fig. 2). The results demonstrated that the proliferative signal by LPS (100 ng/mL) was arrested in the presence of wine extracts (100 µg/mL).

Proliferative or injurious stimulus cause G1 phase cell accumulation (thus, G0/G1 ratio decreases), and then an

Fig. 2 Effect of strawberry wine extracts on cell cycle profile. LPS: cells were treated with 100 ng lipopolysaccharide/mL. Ww: water extract of strawberry wine. WBw: water extract of strawberry wine supplemented with ball-milled achenes. We: ethanol extract of strawberry wine. WBe: ethanol extract of strawberry wine supplemented with ball-milled achenes. The data are the mean $\pm$ SD of 3 replicates, and values with the different lowercase letters indicate a significant difference in the same cell phase ($p < 0.05$)

explosive S phase progression. Cell cycle arrest is an important mechanism which enables cells to repair cell damage, e.g. to DNA, or to obtain deprived nutrients, before a continuation of the cell cycle that would otherwise lead to damaged cells or cell death. The phase distribution of the cell cycle was reported to be altered significantly by the active compound concentration and exposure time (Seeram et al. 2006; Zhang et al. 2011; Qiao et al. 2013). The ethanolic extracts showed greater inhibitory effectiveness on inhibiting the LPS action compared to aqueous substances, this may be due to the low water solubility of ellagic acid (Bala et al. 2006); however, the mechanism is uncertain. Profiles of bioactive compounds of supplemented wine and/or its extract are needed, and identification and quantification of bioactive compounds using HPLC–MS will be taken into account. The present study reported that supplemented wine extracts inhibit the proliferation of LPS-stimulated RAW264.7 cell via affecting the progression of cell cycle. Indeed, wine extracts induced a significant increase in the proportion of G0/G1 phase as well as a reduction in the proportion of S phase of LPS-stimulated cells, indicating a potential anti-inflammatory activity.

Cell morphology

Dendritic cells comprise a class of leukocytes that function as an antigen to naïve T cells in a primary immune response, therefore, an anti-inflammatory compound would be expected to suppress the induction of RAW264.7 cell to differentiate into a dendritic form (Saxena et al. 2003). To evaluate the effect of wine extracts on LPS-stimulated RAW264.7 cells, changes in cell morphology were monitored under an inverted microscope (Fig. 3). Untreated RAW264.7 cells (controls) appeared round, with a low degree of cell spreading (Fig. 3a). As expected, LPS stimulation significantly induced cell spreading and pseudopodia formation, leading to the development of a dendritic-like morphology (Fig. 3b). When cells were co-treated with LPS and wine extracts, they adopted a round shape (Fig. 3c–f). Supplemented strawberry wine inhibited the activation of RAW264.7 cells induced by LPS in the morphological analysis. Polyphenols were found to inhibit LPS-induced hepatic neutrophil infiltration, indicating less lesion from inflammation (Kao and others 2009). Those phenomena indicate that polyphenols have the ability to prevent inflammation.

Anti-inflammatory activities on NO production and iNOS expression

To address the potential anti-inflammatory effect of strawberry wine supplemented with ball-milled achenes,

Fig. 3 Changes in the cellular morphology of RAW264.7 cells under LPS and ball-milled achene supplemented strawberry wine extract (a) control (untreated cells) (b) stimulated by LPS (100 ng/mL) (c) LPS and a water extract of strawberry wine (d) LPS and an ethanol extract of strawberry wine (e) LPS and a water extract of strawberry

wine supplemented with ball-milled achenes (f) LPS and an ethanol extract of strawberry wine supplemented with ball-milled achenes. Supplemented wine extracts used in this study were at the level of 100 $\mu\text{g/mL}$. Samples were observed under a microscope and images were obtained with a digital camera at $\times 100$

the inhibitory effects on NO production and iNOS expression in LPS-stimulated RAW264.7 macrophage cells were evaluated in the presence of wine extracts.

The potential anti-inflammatory activities of the strawberry wine extracts in down-regulating LPS (100 ng/mL)-stimulated RAW 264.7 cells were observed. A significant increase in NO production, from 0.29–0.30 to 10.69–11.13 μM , was found for RAW264.7 cells exposed to LPS (Fig. 4), indicating that an immune response had been induced. A significant inhibition of NO production in the presence of a wine extract, both aqueous and alcoholic, was observed. Dose-dependent anti-inflammatory activities were determined for LPS-stimulated RAW264.7 cells that had been treated with achene-supplemented strawberry

wine aqueous extracts ranging from 10 to 100 $\mu\text{g/mL}$. Moreover, the effectiveness of a wine extract at a concentration of 100 $\mu\text{g/mL}$ was similar to that observed when the same amount of (100 $\mu\text{g/mL}$) tocopherol was used (Fig. 4a). Additionally, an alcoholic extract of supplemented wine also caused the LPS-stimulated inflammatory response to be down-regulated. The inhibitory effect of supplemented strawberry wine on NO production was determined to be 25–40% in the presence of a 25–100 μg extract/mL, where the effect of the typical wine product (control) was much less (10%, Fig. 4b). The inhibition of NO production by an alcoholic extract of supplemented strawberry wine (100 $\mu\text{g/mL}$) was 20% more compared to the control in LPS-stimulated RAW264.7 cells (Fig. 4b).

Fig. 4 Effect of strawberry wine extract on NO production in LPS-stimulated RAW264.7. **a** Water extract, **b** ethanol extract. NC: normal cell, control: extract from conventional process strawberry wine. Values with the different lowercase letters indicate a significant difference ($p < 0.05$) among treatments ($n = 3$)

Previous studies have demonstrated that various phenolic components such as anthocyanins and ellagic acid derivatives are found in strawberry achenes. Ellagic acid derivatives, comprised of ellagic acid, ellagic acid glycosides, and ellagitannins, represent an important group of

phenolic compounds in strawberries, together with raspberries and blackberries (Clifford and Scalbert 2000). Raspberry extracts with seeds crushed had an inhibitory effect on NO production (Jeong et al. 2010). This, therefore, suggests that the inclusion of achenes in the

Fig. 5 Effect of strawberry wine extract on iNOS expression in LPS-stimulated RAW264.7. **a** Aqueous extract, **b** ethanol extract. NC: normal cell. Values with different lowercase letters indicate a significant difference ($p < 0.05$) among treatments ($n = 3$)

fermentation process of strawberry wine may reduce inflammation in cells.

The iNOS levels were markedly increased in LPS-treated RAW264.7 cells, in which iNOS activities were increased by ca. 10 folds compared to normal cells (Fig. 5). In the presence of tocopherol, the extent of iNOS expression in the LPS-stimulated cells was decreased by more than 20%, indicating anti-inflammatory effectiveness (Fig. 5a, b). No effective inhibition on immune responses was found when LPS-stimulated RAW264.7 cells were treated with aqueous extracts of supplemented wine at levels of 10–100 µg/mL, where the relative protein expression was 83.81–129.33% (Fig. 5a). In contrast to water extracts, the ethanol extract caused a significant decrease in iNOS expression in LPS-stimulated cells at concentrations of 50 µg/mL and up, that relative iNOS levels were 59.69 ± 7.82 and $63.99 \pm 20.63\%$ in the presence of 50 and 100 µg/mL of alcoholic extract, respectively. Additionally, the alcohol extract had a more inhibitory effect than tocopherol at the same level (100 µg/mL) (Fig. 5b). However, an alcoholic extract less than 50 µg/mL showed no effect on iNOS expression in the presence of LPS, indicating the anti-inflammatory effect of the alcoholic extract was in dose-dependent.

NO is an important cellular second messenger and a regulator of inflammatory responses. iNOS-derived NO is a ubiquitous mediator of a wide range of inflammatory processes, and reflects the extent of inflammation (Laroux et al. 2001; Sacco et al. 2006). Above certain levels, NO affects the function of immune cells and/or causes inflammatory damage to tissue during an infection. Therefore, the suppression of iNOS-mediated NO production is the major contributor to the down-regulation of an inflammation response (Laroux et al. 2001; Chang et al. 2010; Kwon et al. 2010). In the present study, LPS was found to activate iNOS expression and, thus, stimulate NO production. Immune responses were down-regulated in the case of LPS-stimulated RAW264.7 cells that were treated with ball-milled achene supplemented strawberry wine extracts, indicating a potential anti-inflammatory effect on the NO/iNOS pathway. Since ellagic acid has been demonstrated to modulate inflammation processes (Wang et al. 2005; Umesalma and Sudhandiran 2010; Basu et al. 2010), the anti-inflammatory activities of ball-milled supplemented strawberry wine might be due to its higher ellagic acid contents (Lee and Chen 2016). Although NO production was effectively suppressed in the presence of an aqueous wine extract, no significant difference in iNOS expression was detected (Fig. 4a, 5a). The reason for these observations could be due to the fact that the aqueous extracts modulated NO production by inhibiting the expression of other forms of nitric oxide synthase, clearly, more studies will be needed to understand the mechanism

responsible for this observation. Additionally, information regarding the inhibitory effects of such extracts on the inducible inflammatory gene regulator (NF-κB) and/or pro-inflammatory cytokines (such as IL-1β, TNFα, IL-6, and IL-18) are also needed.

Conclusion

Ball-milled achene supplemented strawberry wine inhibits the activation of RAW264.7 cells induced by LPS. The inhibitory effect of supplemented strawberry wine on NO production was determined to be 25–40% in the presence of 25–100 µg of extract/mL, while the inhibition was less (ca. 10%) in a typical wine sample (control). Also, the inhibitory effect of ball-milled achene supplemented strawberry wine on LPS-stimulated NO production can be attributed to the suppression of the expression of iNOS enzyme. Many reports have addressed high flavonoid and phenolic contents in berries, thus, the consumption of berry products, including fruit wine, would be beneficial for reducing the risk of many metabolic syndromes. The hydrolysis of tannins in achenes to ellagic acid and phenols appears to be a key mechanism for increasing the levels of bioactive compounds in the final wine product. Since the ball-milling process is used to pulverize the pericarp of achenes and lead to the release of bioactive compounds, more ellagic acid and other phenol compounds are formed in the subsequent fermentation. In the future, the use of microbial tannases to produce non-alcoholic fruit beverages may be beneficial for improving the quality of soft drinks, and an immobilized enzyme technique should also be taken into account.

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