Apple Polyphenols Regulate Mitochondrial Superoxide Generation and Extend Survival in a Mouse Model of Dilated Cardiomyopathy

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***Abstract*- Apple polyphenols (AP), which contain procyanidins as major components, have been reported to display potent antioxidant activity and several beneficial health effects. To investigate the protective effect of AP intake against murine cardiomyopathy caused by endogenous oxidative stress, we orally administered AP to heart/muscle-specific manganese-superoxide dismutase (Mn-SOD)-deficient (H/M-*Sod2*$^{-/-}$) mice [Nojiri *et al*., 2006]. Dietary AP significantly increased the survival of the mutant mice, extending their mean lifespan by 29%. Dietary AP also suppressed the progression of cardiac dilatation and fibrosis in the H/M-*Sod2*$^{-/-}$ mice. *In vitro* experiments revealed that AP treatment strongly suppressed the production of hydrogen peroxide induced reactive oxygen species (ROS) in C2C12 myoblast cells and endogenous superoxide production in Mn-SOD-deficient cells. Furthermore, dietary AP suppressed ROS production in Mn-SOD-deficient cardiomyocytes and oxidative DNA damage *in vivo*. These results indicate that dietary AP improved the survival and pathology of short-lived mice with cardiomyopathy by suppressing mitochondrial superoxide production.**

***Keywords*- Apple Polyphenol; Procyanidin; Oxidative Stress; Manganese-Superoxide Dismutase; Antioxidant**

I. INTRODUCTION

A disturbance in the balance between the production of reactive oxygen species (ROS) and the antioxidant defense system causes oxidative stress, which leads to tissue injury and dysfunction [1]. It has been proposed that the accumulation of oxidative damage accelerates ageing [2]. Moreover, in the heart, the generation of ROS and the age-related loss of antioxidant capacity might be involved in age-related cardiac dysfunction [3-5].

Manganese-superoxide dismutase (Mn-SOD) is an antioxidant enzyme that locates in mitochondria and catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. We previously reported that conditional knockout mice that specifically lacked Mn-SOD in their cardiac and skeletal muscle developed progressive dilated cardiomyopathy (DCM) [6]. This report showed that mitochondrial superoxide caused by impairment of the antioxidant defense system was involved in cardiac dysfunction.

Antioxidants are widely used in dietary supplements for maintaining good health and preventing disease. Several studies have demonstrated that dietary polyphenols from fruits and vegetables prevent oxidative stress *in vivo* [7, 8]. The polyphenols extracted from apples (AP) mainly contain procyanidins, which account for approximately 65% of AP [9]. AP are contained in the edible part of the apple and so are easy to routinely ingest. Several physiological functions of AP have been reported, such as anti-allergy [10], anti-tumor [11], and anti-obesity effects [12] as well as inhibitory effects on triglyceride absorption [13]. We recently demonstrated that PC from apples had Sir2-dependent anti-ageing effects in worms (*Caenorhabditis elegans*) [9]. Moreover, we demonstrated that PC suppressed amyloid-β protein aggregation [14]. However, the preventative effects of AP intaking against aging phenomena caused by endogenous chronic oxidative stress remain to be fully evaluated. This study investigated the effects of the chronic intake of AP on heart/muscle-specific Mn-SOD deficient (H/M-*Sod2*$^{-/-}$) mice. We herein report that dietary AP regulates mitochondrial ROS production and ameliorates the phenotypes of H/M-*Sod2*$^{-/-}$ mice.

II. MATERIALS AND METHODS

A. Compounds

The AP were obtained according to the methods reported by Ohnishi-Kameyama et al. [15]. They were prepared from immature apples (*Malus pumila* Mill. cv. Fuji), and their

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components were analyzed according to the method of Shoji *et al.* [16]. The detailed experimental procedures involved in the preparation and analysis of the AP are previously reported [9]. Procyanidins (PC) accounted for 63.8% of AP.

B. Animals

H/M-*Sod2*$^{-/-}$ mice were generated by crossbreeding *Sod2*$^{flox/flox}$ mice with muscle creatine kinase promoter-Cre transgenic mice using in vitro fertilization techniques [6]. Four groups of mice were used for this study: H/M-*Sod2*$^{-/-}$ mice, H/M-*Sod2*$^{-/-}$ mice that were treated with AP (H/M-*Sod2*$^{-/-}$+AP), *Sod2*$^{flox/flox}$ (Control) mice, and *Sod2*$^{flox/flox}$ mice that were treated with AP (Control+AP). AP were administered to the mice throughout their lives; i.e., the AP-treated mice with allowed ad libitum access to drinking water containing 0.1% or 0.5% AP. Tamoxifen-inducible Mn-SOD-deficient mice (*Rosa26-CreERT2*$^{c/w}$, *Sod2*$^{flox/flox}$) were produced by crossbreeding *Sod2*$^{flox/flox}$ mice with ROSA26 promoter-CreERT2 transgenic mice [17]. All mice were housed in plastic cages within a pathogen-free barrier facility (23.5 ± 0.5℃) under a 12 h light cycle (08:00-20:00). All animal care and experimentation procedures were approved by the Animal Care and Use Committee of the Tokyo Metropolitan Institute of Gerontology (approval number: IACUC 99-16; approval date: September 9, 2007) and the Institutional Animal Care and Use Committee of Chiba University (approval number: IACUC 99-16; approval date: September 9, 2007).

C. Blood Analyses

For the measurement of creatine phosphokinase (CPK), blood samples were obtained from the inferior vena cava. Serum CPK activity was measured with the Cica Liquid CK test (Kanto Chemical Co., Inc.).

D. Measurement of Anti-Oxidative Effect on C2C12 Cells

Mouse myoblast C2C12 cells (RCB0987, Riken BioResource Center, Japan) were pre-incubated for 30 min with 0.1 mg/mL or 1.0 mg/mL AP, washed thoroughly with PBS twice, and then incubated for 1 h with media containing an additional 200 μmol/mL H_2O_2. The control cells were incubated in the absence of H_2O_2. The cells were thoroughly washed with PBS and then resupplied with fresh H_2O_2-free medium. We confirmed that the AP showed no cytotoxicity at 10 mg/mL when incubated with the cells for 1 h. The intracellular ROS level was measured using a fluorescent probe, 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA, Invitrogen, Life Technologies Corporation). CM-H_2DCFDA is a nonspecific detector of intracellular ROS such as H_2O_2, hydroxyradical, and hydroperoxides. CM-H_2DCFDA was dissolved in DMSO at a concentration of 1 mmol/L. The cells were incubated with HANKS buffer containing CM-H_2DCFDA (10 μmol/L) in a dish for 20 min at 37℃. After being incubated, the cells were washed three times with buffer and then photographed using a Leica DFC300 FX camera (Leica Co., Ltd.) and the software application Leica IM50 v4.0. The intracellular fluorescence intensity was calculated using the software application Leica QWin Plus v3.5.

E. Isolation of Mouse Embryonic Fibroblasts (MEF)

The MEF were isolated from tamoxifen-inducible Mn-SOD-deficient embryos at E14.5 by dissociation in 0.25% trypsin-EDTA (Invitrogen) at 37˚C for 30 min. The MEF were cultured in α-MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 unit/ml penicillin, and 0.1 mg/ml streptomycin. 4-OH-tamoxifen (1 μmol/L, Calbiochem) was added to delete the *Sod2* gene on culture days 0 and 3. On Days 3 and 6, we confirmed the loss of Mn-SOD protein by Western blotting, and the presence of the deletion allele as well as the absence of the flox allele by genomic PCR (data not shown). The intracellular ROS level was measured on culture Day 6.

F. Isolation of Cardiomyocytes

Left ventricular (LV) myocytes were isolated from the female mice at the age of 5 months, as described previously [18]. Briefly, the hearts of open-chested mice anaesthetized with urethane (1.5 g/kg, i.p.) were quickly removed after the intravenous administration of 300 U/kg heparin sodium (Novo-Heparin™, Mochida Pharmaceutical Co., Ltd.). After the hearts had been rapidly washed in cool Tyrode solution (143 mmol/L NaCl, 5.4 mmol/L KCl, 0.5 mmol/L NaH_2PO_4•$2H_2O$, 0.33 mmol/L $MgCl_2$•$6H_2O$, 1.8 mmol/L $CaCl_2$, 5.5 mmol/L glucose, and 5.0 mmol/L HEPES; adjusted to pH 7.4 with NaOH), they were mounted onto a Langendorff perfusion apparatus and retrogradely perfused via the coronary circulation for 15 min with oxygenated Tyrode solution warmed to 37 ± 0.5℃. Then, the hearts were perfused for 15 min with oxygenated Ca^{2+}-free Tyrode solution warmed to 37 ± 0.5℃, before being perfused for 10-15 min with oxygenated Ca^{2+}-free Tyrode solution containing 0.26 mg/mL collagenase (Wako Pure Chemical Industries) warmed to 37 ± 0.5℃. This was followed by perfusion with 50 mL Kraft-Brühe (KB) solution (70 mmol/L KOH, 50 mmol/L L-glutamic acid, 40 mmol/L KCl, 20 mmol/L taurine, 20 mmol/L KH_2PO_4, 3.0 mmol/L $MgCl_2$•$6H_2O$, 10 mmol/L glucose, 1.0 mmol/L EGTA, and 10 mmol/L HEPES; adjusted to pH 7.4 with KOH). Then, the LV apex tissue was shaved and minced in KB solution, before being filtered through a 70 μm nylon mesh (Cell Strainer, BD Biosciences). The isolated myocytes were stored in KB solution at 4℃ and used within 12 hr of their dissociation.

G. Measurement of Intracellular ROS

A fluorescent probe, dihydroethidium (DHE), was used for the assessment of intracellular ROS formation in MEF from the *Rosa26-CreERT2*$^{c/w}$, *Sod2*$^{flox/flox}$ mice. This is a specific detector of the intracellular superoxide anion [19]. After a burst of superoxide production, DHE is oxidized to ethidium and incorporated into nuclear DNA. DHE was dissolved in DMSO at a concentration of 10 mmol/L. Before being incubated with DHE, the MEF were pre-treated for 72 hr with or without AP followed by treatment with 10μmol/L DHE for 10 min at 37℃. After incubation, the cells were

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washed with PBS. Their fluorescence intensity was assessed using a flow cytometer (EPICS ALTRA, Beckman Coulter, Inc.). CM-H_2DCFDA was used for the assessment of intracellular ROS formation in isolated cardiomyocytes. CM-H_2DCFDA (final conc.: 10 μmol/L) was added to KB solution containing cardiomyocytes. After incubation at 37℃ for 10 minutes, the myocytes were washed with KB solution. Data were acquired for 10,000 cells for each group. The DHE or DCF positivity rate of the cells was calculated for each group.

H. Determination of Oxidative DNA Damage

The measurement of cardiac 8-oxo-2'-deoxyguanosine (8-oxodG) levels was performed as described previously [20].

I. Histological Studies

For histological analysis, the heart tissues were immersed in 10% buffered formalin. The fixed tissues were dehydrated, embedded in paraffin, sectioned into 4 μm slices, and stained with hematoxylin-eosin (HE). Myocardial sections were stained with azan to evaluate the degree of fibrosis. Images were obtained using a Pixera Pro600EX camera attached to a VANOX-S microscope (Olympus Corporation) with a 10x objective, and the fibrotic area was quantitatively analyzed with Qwin Plus V3 (Leica). The collagen volume percentage is expressed as the mean of all fields examined for each animal.

J. Statistical Analysis

We used the SPSS ver. 11.5 software (SPSS, USA). The standard errors and p values of the survival data were calculated using the Log-rank test. Differences were considered significant at $p < 0.05$. Statistical comparisons were performed by one-way analysis of variance (ANOVA). Multiple comparisons between groups were performed with Tukey's test as a post-hoc test.

III. RESULTS

In order to investigate whether oxidative stress plays a pathological role in the development of heart failure, we previously generated H/M-*Sod2*$^{-/-}$ mice. The mutant mice developed DCM associated with excess superoxide formation [6, 20]. In this study, they displayed a markedly reduced survival rate (Figures 1A, 1B). The maximum lifespan of the H/M-*Sod2*$^{-/-}$ mice was about at the age of 22 weeks. The mutant mice showed progressive body weight loss (Figure 1C) and heart dilatation (Figure 1D). To examine the effects of dietary AP on symptoms caused by oxidative stress, AP were administered to the H/M-*Sod2*$^{-/-}$ mice throughout their lives; i.e., they were allowed ad libitum access to drinking water containing 0.1% AP. Dietary AP extended the lifespan of the H/M-*Sod2*$^{-/-}$ mice (Figures 1A, 1B). The maximum lifespan of the H/M-*Sod2*$^{-/-}$ mice treated with AP was about at the age of 29 weeks. Thus, the intake of AP significantly extended their mean lifespan by 29% with water alone ($p < 0.0001$). Dietary AP also significantly delayed their progressive body weight loss (Figure 1C) and cardiac dilatation (Figure 1D).

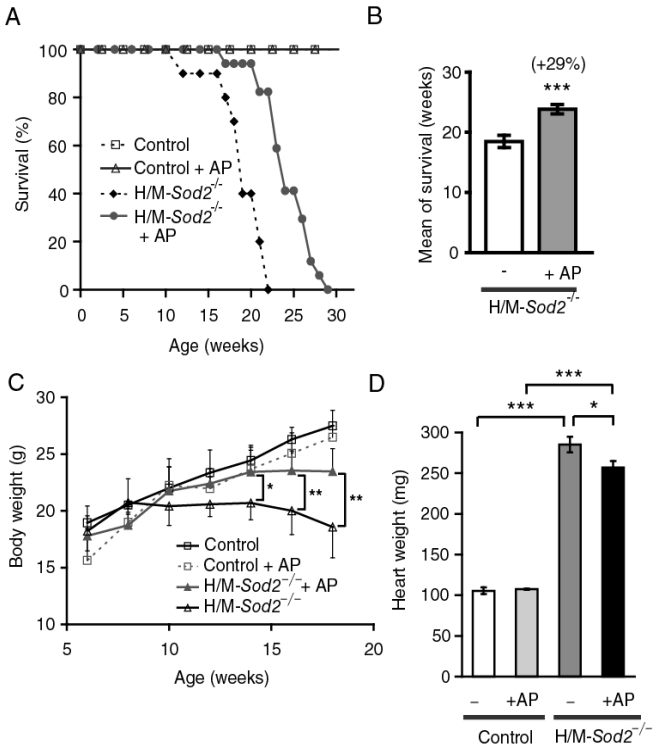

Fig. 1 AP extend the lifespan of H/M-*Sod2*$^{-/-}$ mice

A, **B**, Survival curves and the mean lifespan of male H/M-*Sod2*$^{-/-}$ mice. The maximum lifespan of the H/M-*Sod2*$^{-/-}$ mice was about 22 weeks. The intake of AP extended their mean lifespan by 29% compared with water alone. Control, n = 10; Control+AP, n = 10; H/M-*Sod2*$^{-/-}$, n = 10; H/M-*Sod2*$^{-/-}$+AP, n = 17. Values are mean ± SE, ***$p < 0.0001$.

C, Body weights of male H/M-*Sod2*$^{-/-}$ mice. Dietary AP delayed the progressive weight loss of the H/M-*Sod2*$^{-/-}$ mice. Values are mean ± SEM, n = 7. Differences between groups, **$p < 0.01$, *$p < 0.05$.

D, Heart weights of 18-week-old female H/M-*Sod2*$^{-/-}$ mice. The hearts of the H/M-*Sod2*$^{-/-}$ mice were heavier than those of the control mice. Dietary AP decreased their heart weight. Values are mean ± SEM. Control, n = 8; Control + AP, n = 9; H/M-*Sod2*$^{-/-}$, n = 15; and H/M-*Sod2*$^{-/-}$ + AP, n = 15. Differences between groups, ***$p < 0.001$, *$p < 0.05$.

In order to examine the effects of dietary AP on cardiac histopathology caused by oxidative stress, we measured the plasma CPK levels of the H/M-*Sod2*$^{-/-}$ mice, which reflect their cardiac pathology and heart histopathology. Dietary AP ameliorated the CPK levels of the H/M-*Sod2*$^{-/-}$ mice (Figure 2A). HE and azan staining of transverse sections of LV walls revealed myocardial degeneration, cardiomyocyte disarray, and vacuolization with irregular myofilaments and pleomorphic nuclei (Figure 2B). Dietary AP also ameliorated the heart histopathology and fibrosis of the H/M-*Sod2*$^{-/-}$ mice (Figures 2B, 2C).

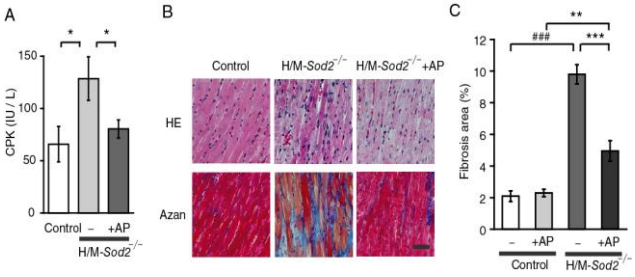

Fig. 2 AP ameliorate the heart histopathology of H/M-*Sod2*$^{-/-}$ mice

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A, Serum CPK concentration of H/M-*Sod2*$^{-/-}$ mice. Serum CPK has been reported to be a blood biomarker that reflects cardiac pathology. The H/M-*Sod2*$^{-/-}$ male mice treated with AP displayed reduced serum CPK levels. Values are mean ± SEM, n = 6~7. Differences between groups, *p < 0.05.

B, HE and azan stained transverse sections of LV walls. LV walls from male H/M-*Sod2*$^{-/-}$ mice showed myocardial degeneration, cardiomyocyte disarray, and vacuolization with irregular myofilaments and pleomorphic nuclei. Azan staining revealed diffuse myocardial fibrosis (blue). AP administration ameliorated the symptoms of the H/M-*Sod2*$^{-/-}$ mice. The scale bar represents 10 μm.

C, The fibrotic areas of the LV walls from the H/M-*Sod2*$^{-/-}$ mice treated with AP were significantly decreased compared with those of the control mice. Values are mean ± SEM, n = 4. Differences between groups, $^{\#\#\#}$p < 0.0001, ***p < 0.001, **p < 0.01.

In order to examine the anti-oxidative effects of AP *in vitro*, we measured the intracellular ROS levels of C2C12 cells that had been treated with H_2O_2 using the fluorescent probe CM-H_2DCFDA. As a result, we found that preincubation with AP significantly suppressed the production of ROS in C2C12 cells treated with H_2O_2 in a dose-dependent manner (Figures 3A, 3B).

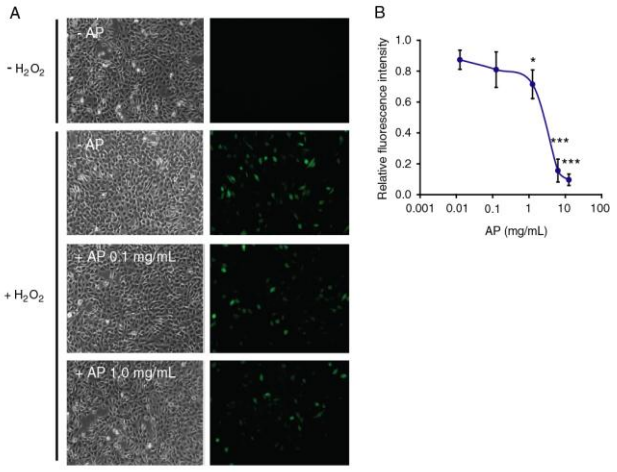

Fig. 3 AP have anti-oxidant effects *in vitro*

A, Anti-oxidative effect of AP on mouse C2C12 myoblast cells. The cells were preincubated for 30 min with 0.1 mg/mL or 1.0 mg/mL AP and then incubated for another 1 h with 200 μmol/mL H_2O_2. The cells were incubated with 10 μM CM-H_2DCFDA for 20 min at 37℃. After the incubation, the cells were photographed using a fluorescence microscopy imaging system. ROS production was induced in the cells treated with H_2O_2, as assessed by DCF fluorescence.

B, Relative fluorescence intensity of C2C12 myoblast cells treated with AP. AP treatment decreased the production of ROS. Values are mean ± SEM, n = 3. ***p < 0.001, *p < 0.05 vs. AP 0 μg/mL.

Next, in order to examine the anti-oxidative effects of AP against intrinsic ROS, we measured the intracellular ROS level of tamoxifen-induced Mn-SOD deficient MEF using the fluorescent probe DHE. As a result, we found that preincubation with AP suppressed the production of ROS in Mn-SOD-deficient MEF (Figures 4A, 4B). We also measured the intracellular ROS levels of cardiomyocytes isolated from H/M-*Sod2*$^{-/-}$ mice using the fluorescent probe CM-H_2DCFDA. Accordingly, we found that dietary AP suppressed the production of ROS by Mn-SOD deficient cardiomyocytes (Figures 4C, 4D). Furthermore, to examine the *in vivo* anti-oxidative effects of dietary AP, the amount of DNA 8-oxodG incorporation in the heart cell nuclei of the H/M-*Sod2*$^{-/-}$ mice was measured. Dietary AP tended to decrease the 8-oxodG/10^6 dG ratio of the nuclear fractions of the H/M-*Sod2*$^{-/-}$ mice (Figure 4E).

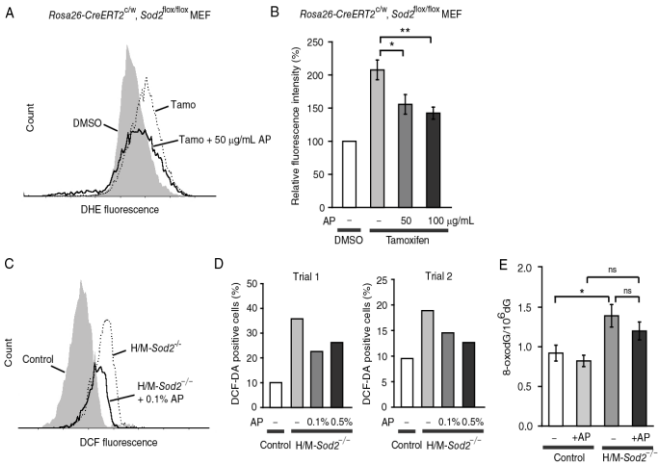

Fig. 4 AP have anti-oxidant effects *in vivo*

A, Frequency histogram of tamoxifen-induced Mn-SOD deficient MEF treated with DHE. After being incubated with 10 μmol/L DHE at 37℃ for 30 minutes, the fluorescence intensity of the cells was assessed using a flow cytometer. Data for 10,000 MEF were acquired for each group.

B, Proportion of DHE fluorescence positive MEF. ROS production was induced in the MEF from the tamoxifen-induced Mn-SOD deficient mice. Preincubation with 50 μg/mL or 100 μg/mL AP decreased the production of ROS. Values are mean ± SEM, n = 8. **p < 0.01, *p < 0.05 vs. MEF treated with tamoxifen and AP 0 μg/mL.

C, Frequency histogram of isolated cardiomyocytes treated with CM-H_2DCFDA. After being incubated with 10 μmol/L CM-H_2DCFDA at 37℃ for 10 minutes, DCF fluorescence intensity was assessed using a flow cytometer. Data for 10,000 cardiomyocytes were acquired for each group.

D, Proportion of DCF fluorescence positive cardiomyocytes in the H/M-*Sod2*$^{-/-}$ mice. ROS production was induced in isolated myocytes in the H/M-*Sod2*$^{-/-}$ mice, as assessed by DCF fluorescence. The intake of 0.1% or 0.5% AP decreased the production of ROS.

E, Level of 8-oxodG in the cardiac cell nuclei of the H/M-*Sod2*$^{-/-}$ mice. The proportion of 8-oxodG/10^6 dG in their nuclear fractions is shown. The nuclear oxidant level was increased in the H/M-*Sod2*$^{-/-}$ mice, and the intake of AP decreased it. Values are mean ± SEM, n = 13~14. Differences between groups, *p < 0.05.

IV. DISCUSSION

Polyphenols belong to a family of plant secondary metabolites and are known to have anti-oxidant properties. Polyphenols have several physiological actions, as well as having a protective effect against cardiomyopathy [21-26]. In the present study, to examine the effects of AP on murine cardiomyopathy caused by endogenous oxidative stress, we consecutively administered AP to a mouse model of DCM. As a result, we found that dietary AP significantly prolonged the lifespan of the short-lived mice. AP also delayed the progressive body weight loss displayed by these mice and suppressed the progression of cardiac dilatation and cardiac fibrosis. The lifespan extension and delay in body weight loss might represent preventive effects of AP against progressive DCM, and the suppression of cardiac fibrosis might aid the maintenance of several cardiac functions.

It has been reported that some polyphenols, such as resveratrol, tea catechins, and quercetin, have preventive

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effects against cardiomyopathy in murine hearts after ischemia/reperfusion [21-26]. In the case of exposure to acute oxidative stress, these effects might be achieved by the antioxidant effects of polyphenols. In this study, the anti-oxidative effects of AP on acute and chronic exposure to oxidative stress were determined. First, *in vitro* experiments revealed that AP pretreatment suppressed the production of hydrogen peroxide-induced ROS in cultured cells. Free radical scavenging and metal sequestration by complex formation might underlie the anti-oxidative effects of AP. AP also suppressed endogenous superoxide production in Mn-SOD-deficient MEF. Furthermore, dietary AP suppressed ROS production in Mn-SOD-deficient cardiomyocytes and reduced oxidative DNA damage *in vivo*. We previously reported that the administration of an SOD/catalase mimetic, EUK-8, significantly ameliorated the cardiac pathology and dysfunction of H/M-*Sod2*$^{-/-}$ mice by suppressing mitochondrial oxidative stress [20, 27]. Taken together with our results, AP suppresses mitochondrial superoxide production in a preventive manner.

In this study, AP had a potent *in vivo* anti-oxidative effect in a mouse model of DCM and extended the lifespan of the mice. We have also shown that the oral administration of AP reduced the frequency of ventricular arrhythmia in a rapid-pacing protocol (unpublished data), suggesting that AP might extend lifespan via anti-arrhythmia effects in DCM model mice. Recently, we revealed that AP also showed *sir2*-dependent anti-ageing effects leading to lifespan extension in a nematode [9]. Taken together, we propose that the beneficial effects of AP on lifespan not only involve a direct radical scavenging action but also the modulation of arrhythmia and the expression of the mammalian SIR-2 homologs sirtuins in murine cardiomyocytes. Further studies are necessary to elucidate the molecular mechanisms responsible for the effects of AP.

The bioavailability of AP has been reported previously [28]. Shoji *et al*. revealed that apple PC, which range in length from dimers to pentamers, were detectable in rat plasma after their oral administration. In a study of ROS production in cardiomyocytes isolated from H/M-*Sod2*$^{-/-}$ mice, the anti-oxidative effects of 0.1% AP were found to be comparable to those reported for an AP dose of 0.5%. Thus, an AP dose of AP 0.1% might be sufficient to produce anti-oxidative effects. In conclusion, dietary AP prevent murine cardiomyopathy caused by endogenous oxidative stress in H/M-*Sod2*$^{-/-}$ mice, and hence, prolong their lifespan.

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