RESEARCH ARTICLE

Fermented *Prunus mume* with Probiotics Inhibits 7,12-Dimethylbenz[*a*]anthracene and 12-O-Tetradecanoyl phorbol-13-acetate Induced Skin Carcinogenesis through Alleviation of Oxidative Stress

Jin-A Lee[1&], Jae-Hyung Ko[2&], Bock-Gie Jung[1], Tae-Hoon Kim[1], Ji-In Hong[1], Young-Seok Park[3], Bong-Joo Lee[1*]

Abstract

Maesil (*Prunus mume* Siebold & Zucc.), a member of the genus Rosaceae, has been reported to have antioxidative effects, as well as anticancer influence in many cancer lines. Thus, this present study was designed to investigate the inhibitory effect of fermented Maesil with probiotics against 7,12-dimethylbenz[*a*]anthracene (DMBA), 12-O-tetradecanoyl phorbol 13-acetate (TPA)-induced mouse skin carcinogenesis via its antioxidative potential. Mice were fed a diet containing fermented Maesil, containing either 1% (1% FM fed group) or 2% (2% FM fed group) along with probiotics following DMBA and TPA exposure. Continuous ingestion of the experimental feed markedly inhibited skin carcinogenesis, as evidenced by a marked decrease in papilloma numbers and epidermal hyperplasia as well as cellular proliferation and the percentage of proliferating-cell nuclear antigen positive cells. Also, the FM fed group showed an increase of total antioxidant capacity as well as an increased level of phase II detoxifying enzymes such as superoxide dismutase, concurrent with a decreased lipid peroxidation activity level. Taken together, these results suggest that fermented Maesil has the ability to suppress the development of DMBA-TPA induced skin carcinogenesis, via the reduction of lipid peroxidation, enhancing total antioxidant capacity and phase II detoxifying enzyme.

Keywords: *Prunus mume* - maesil - skin cancer - oxidative stress - inhibitory potential

Asian Pacific J Cancer Prev, **14 (5)**, 2973-2978

Introduction

Skin cancer is one of the most common malignancies and its incidence has been rising rapidly over the past several decades (Housman et al., 2003; Stern, 2010). The development of skin cancer is a multistage process that includes initiation, promotion and progression in experimental animal models and possibly in human cancer includes induction and propagation (Meeran et al, 2009). During the early tumor promotion stage of multistage carcinogenesis, the process is reversible but the initiation stage is irreversible and presumably unavoidable because of continuing exposure to carcinogenic chemicals and physical agents (Abel et al., 2009).

Skin is frequently exposed to sunlight and is always in contact with oxygen, resulting in the production of oxygen free radicals, more generally known as reactive oxygen species (ROS) (Inoue et al., 2003). Therefore, skin is continually subjected to oxidative stress which is mediated by ROS and this damage normally minimized by the action of non-enzymatic antioxidants and antioxidant enzyme systems (Halliwell et al., 2007). Despite this defense mechanism, a massive oxidant generation takes place at the same time along with a decrease in antioxidant protection which causes oxidative damage to lipids, proteins, carbohydrates and nucleic acids, ultimately leading to many diseases, e.g. aging, cardiovascular diseases and cancer (Reuter et al., 2010). In addition, many studies confirmed that many anticancer agents with diverse pharmacological and anti-oxidative activities are promising agents with the potential action to inhibit carcinogenesis (Klaunig et al., 2004). Especially, many naturally occurring dietary materials, such as fruits and vegetables, herbs and plants, which have phenolic compounds, showed anticarcinogenic or antimutagenic potential might be related to their antioxidative properties (Pan et al., 2008).

Maesil (*Prunus mume* Siebold & Zucc.) is a

*[1]Department of Veterinary Infectious Disease, College of Veterinary Medicine, Chonnam National University, Gwangju, [2]Kyonggido Livestock and Veterinary Service, Suwon, Kyonggi, [3]Department of Companion and Laboratory Animal Science, Kongju National University, Kongju, Chungnam, Korea [&]Equal contributors *For correspondence: bjlee@chonnam.ac.kr*

deciduous tree of the genus Rosaceae, which is now widely-distributed in Korea. The extracts of the Maesil fruit have been used as traditional herbal medicine for relief of fatigue, diarrhea, and fever (Choi et al., 2002; Jeong et al., 2006). Maesil contains abundant phenolic compounds, such as phenolic acids and flavonoids (Jeong et al., 2006; Kita et al., 2007), which are involved in the biological effects of antioxidant activity and free radical scavenging activity (Kim et al., 2005). These phenolic compounds obtained from Maesil also exhibited an inhibitory effect against human cancer cells (Jeong et al., 2006). Moreover, Maesil extract exerted antineoplastic effects in colon, breast and pancreatic cancer cells (Mori et al., 2007; Nakagawa et al., 2007; Okada et al., 2007). Also, probiotics have been demonstrated on have therapeutic potential in the treatment of skin disorders (Kirjavainen et al., 2003; Sawada et al., 2007). We have recently shown that dietary administration of fermented Maesil with probiotics delays the onset and suppresses the development of AD-like skin lesions in NC/Nga mice (Jung et al., 2010)

These observations prompted the present suggestion that fermented Maesil administered with probiotics may be a good candidate for the control of skin cancer through its antioxidative activity. Thus, the aim of this study was to demonstrate the effect of fermented Maesil with probiotics on the 7,12-dimethylbenz[*a*]anthracene (DMBA), 12-O-tetradecanoyl phorbol 13-acetate (TPA)-induced mouse skin carcinogenesis.

Materials and Methods

Animals

Specific pathogen-free (SPF) 6-week-old, female BALB/c mice were purchased from DBL (Daejeon, Korea). They were acclimatized for 1 week before use. All animals were housed in an air-conditioned room, maintained under standard conditions at 24±2°C and relative humidity of 50±10%, and allowed free access to their particular diet and tap water.

Fermented Maesil preparation

Fermented Maesil was prepared according to the method of Jung et al. (2007). Briefly, fruits were crushed, mixed thoroughly with non-fat rice bran, and the mixture was fermented with 1×10^7 colony forming units (cfu)/ml *Saccharomyces cerevisiae* (7928; Korea Collection for Type Cultures, Seoul, Korea) for 7 days at 25-30°C. The fermented products were further fermented with 1×10^7 cfu/ml each of *Bacillus subtilis* (KCTC 1666) and *Lactobacillus acidophilus* (KCTC 3155) for 5 days at 45-50°C. After fermentation, the products were air-dried for 20 hr at 20°C.

Experimental design

The mice were randomly divided into three groups of eight mice in each group. The control group received a commercial, nutritionally complete, extruded dry rodent feed (Superfeed, Gangwon, Korea) containing 22.1% crude protein, 8.0% crude ash, 5.0% crude fiber, 3.5% crude fat, 1.2% phosphorus, and 0.6% calcium. The experimental groups received the same extruded dry rodent feed supplemented with either 1% (w/w) fermented Maesil (1% FM group) or 2% (w/w) fermented Maesil (2% FM group). Experimental diets containing 1% and 2% fermented maesil were prepared as previously described (Lee et al., 2012). For carcinogenesis studies, supplemented diet containing fermented Maesil was provided to the mice starting 3 weeks before the initiation of DMBA treatment. The dorsal skin area of mice was shaved with electric clippers 2 days prior to the start of the experiment. All mice received two topical applications of 25 μg a suspension of DMBA (Sigma-Aldrich, St Louis, MO, USA) in 100 μl acetone over a 72 hr interval, followed by 4 μg of an acetone suspension in 100 μl TPA twice a week for 16 weeks. All animal procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University (Approval number: CNU IACUC-YB-2010-1).

Detection of papilloma growth

Animals of each treatment group were carefully examined twice weekly for counting and recording of the incidence and numbers of papilloma. Detection of papilloma was standardized in our laboratory; skin papilloma with a diameter exceeding 1 mm that persisted for at least two consecutive observations were defined as papilloma and were recorded. Papilloma that regressed after one observation was not considered for counting. Two different experts, who were blinded to the experimental groups, made the determinations of papilloma.

Measurement of epidermal thickness

All mice of each group were sacrificed at the end of the experiment, and the dorsal shaved areas of the skin were sliced into several parts and washed in phosphate buffered saline. The tissue slices were fixed with 10% buffered formalin and were embedded in paraffin. Tissue sections of 5 μm in thickness were cut and stained with hematoxylin and eosin (H&E). Epidermal hyperplasia was determined as the mean vertical epidermal thickness of 10 different locations by microscopic examination of skin tissue sections.

Detection of in situ cell proliferation

Cell proliferation in mouse skin was measured using a commercial PCNA staining kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, sections of skin tissues (5 μm) were deparaffinized with xylene and rehydrated with a series of graded alcohols. After dewaxing and rehydration, slide sections were placed in 10 mM/L citric acid buffer (pH 6.0) and were microwaved for antigen retrieval. Tissue sections were thern incubated with biotinylated mouse PCNA (proliferating-cell nuclear antigen) primary antibody at room temperature for 1 hr, and were then incubated with horseradish peroxidase-conjugated streptavidin at room temperature for 30 min. The PCNA-positive cells were visualized using a 3,3'-diaminobenzidine (DAB) substrate solution, and other cells were counterstained with hematoxylin. The proliferative index (PI) was determined by dividing the number of PCNA-positive cells by the

total cells counted and multiplying by 100.

Lipid peroxidation inhibitory activity in skin cytosol

Lipid peroxidation in the skin cytosol was estimated using a commercial TBARS assay kit (Cayman Chemical, Ann harbor, MI, USA). Thiobarbituric acid reacting substances (TBARS) content in the skin cytosol was used as an indicator of lipid peroxidation level in skin cytosol. Briefly, skin was homogenized in RIPA buffer and was sonicated. Skin homogenate was centrifuged at 1,600 x g for 10 min at 4°C. Supernatant was analyzed according to the manufacturer's instructions. The level of lipid peroxidation was expressed as μM MDA/mg of tissue.

Total antioxidant capacity in skin cytosol

The capacity of total antioxidants in the skin cytosol was estimated using a commercial ABTS antioxidant assay kit (Cayman Chemical, USA). The capacity of total antioxidants to prevent the oxidation of ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to $ABTS^{\bullet+}$ by metmyolobin is compared with that of trolox and is quantified as trolox equivalent antioxidant capacity (TEAC). Skin was homogenized in RIPA buffer and was then sonicated. The homogenate was centrifuged at 1,600x g for 10 min at 4°C. The resulting supernatant was analyzed according to the manufacturer's instructions. The concentration of antioxidant in the skin cytosol was inversely proportional to the ABTS radical formation. The level of total antioxidant capacity was expressed as μM Trolox/mg of tissue.

SOD activity level in skin cytosol

SODs are metalloenzymes that catalyze the dismutation of the superoxide anion to molecular oxygen and hydrogen peroxide, and thus, play a key role in the cellular antioxidant defense mechanism. For the detection of SOD activity in the skin cytosol, a commercial SOD Assay kit (Cayman Chemical) was used. Briefly, the skin tissue was collected, washed and homogenized in 20 mM HEPES (Sigma-Aldrich) buffer. The skin homogenate was centrifuged at 1,500x g for 5 min at 4°C, and the supernatant was analyzed according to the manufacturer's protocol. The level of SOD level was expressed as U/mg of tissue.

Statistical analysis

The data are expressed as mean±standard deviation (SD) and the mean of the different parameters were compared between groups by analysis of variance (ANOVA). Significant differences between the groups were evaluated by Tukey's multiple comparison test, using Minitab Statistical Software version 13.20 (Minitab, State College, PA, USA). $P<0.05$ was considered as the level of significance.

Results

Effects of fermented Maesil on DMBA- TPA-induced skin papilloma

Skin papilloma development began, and papilloma numbers were elevated at week in all groups of mice. However, after 17 weeks of fermented Maesil administration, papilloma numbers per mouse were significantly reduced in the 1% FM group (2.5±1.2) and 2% FM group (2.1±1.2) ($P<0.05$), compared to the control group (4.0±1.3) (Figure 1a).

Effects of fermented Maesil on epidermal hyperplasia

The vertical epidermal thickness of the skin was measured to assess the effects of fermented Maesil on epidermal hyperplasia. The vertical epidermal thickness of the skin was 36.5±6.0 μm in the control group of mice, but was reduced significantly ($P<0.001$) in the 1% FM group (25.5±4.1 μm) and 2% FM group (24.2±4.4 μm) (Figure 2d).

Effects of fermented Maesil on cell proliferation

Cell proliferation of the skin was measured by PCNA immunohistochemistry at week 17 to confirm that fermented Maesil could reduce cell proliferation in mice with skin carcinogenesis. Significant differences in the number of proliferative (PCNA-positive) cells in the skin tissues were observed. PI of the 1% FM group (27.3±3.8) ($P<0.001$) and 2% FM group (25.2±3.9) ($P<0.001$) was reduced in a dose-dependent manner compared to the PI of the control group of mice (34.6±5.1%) (Figure 3d).

Effects of fermented Maesil on lipid peroxidation level in skin cytosol

The lipid peroxidation levels of the 1% FM (1.06±0.44 μM MDA/mg tissue) and 2% FM group (0.97±0.57 μM

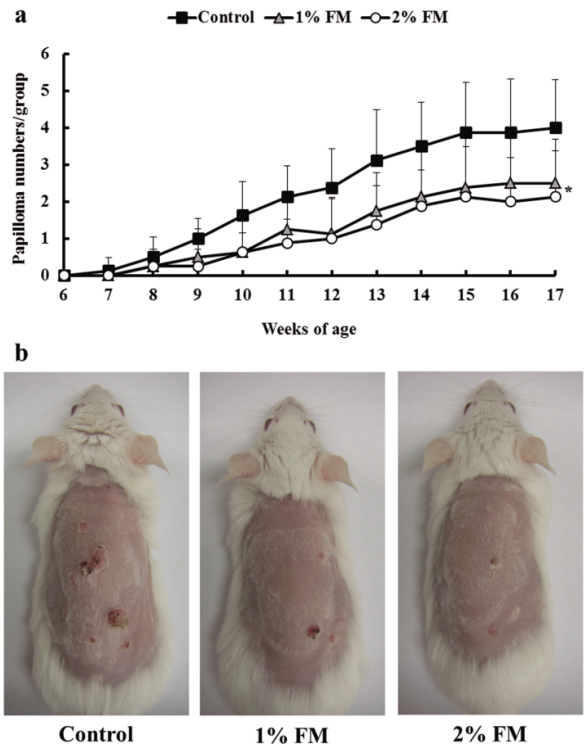

Figure 1. Inhibition of Skin Carcinogenesis by Fermented Maesil Administration. (a) Tumor multiplicity equates to an average number of papillomas per mouse. Values are the mean ± SD of eight mice. (b) Representative pictures of papilloma bearing BALB/c mice were shown. Photographs were taken at the end of the experiment

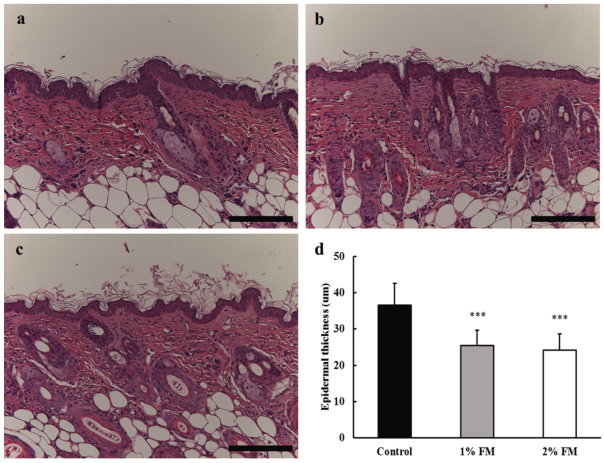

Figure 2. Inhibition of Epidermal Hyperplasia in Skin Carcinogenesis by Fermented Maesil Administration. H&E staining of a skin section obtained from (a) the control group, (b) the 1% FM group and (c) the 2% FM group. In each case, representative data are shown at ×200 magnification. (d) Changes of epidermal thickness in mice with skin carcinogenesis. Values are the mean±SD of eight mice. ***P<0.001 vs. the control group

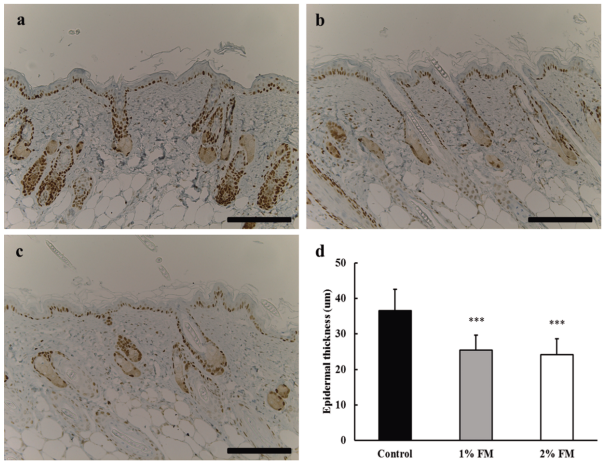

Figure 3. Inhibition of Cell Proliferation in Skin Carcinogenesis by Fermented Maesil Administration. PCNA immunohistochemical staining of a section of skin obtained from (a) the control group, (b) the 1% FM group and (c) the 2% FM group. Arrows indicate PCNA-positive cells. In each case, representative data are shown at ×200 magnification. (d) Changes of the PI in mice with skin carcinogenesis. Values are the mean±SD of eight mice. **P<0.01 vs control group; ***P<0.001 vs control group

MDA/mg tissue) were significantly decreased (P<0.01) compared to the level in the control group of mice (2.26±0.63 μM MDA/mg tissue) (Figure 4a).

Effects of fermented Maesil on total antioxidant capacity in skin cytosol

The total antioxidant capacity in the skin was significantly increased in the 1% FM group (333.2±78.8 μM Trolox/mg tissue) (P<0.001) and in the 2% FM group (385.5±44.9 μM Trolox/mg tissue) (P<0.001), compared to the control group of mice (116.3±30.6 μM Trolox/mg tissue) (Figure 4b).

Effects of fermented Maesil on SOD activity level in skin cytosol

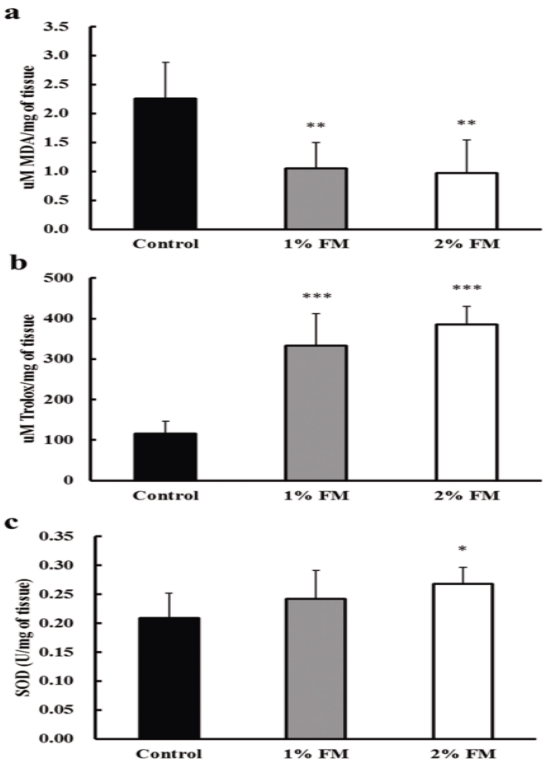

Figure 4. Effect of Fermented Maesil Administration on Antioxidant Capacity in Skin Carcinogenesis. (a) Lipid peroxidation level was measured at the end of the experiment by the colorimetric assay using an MDA equivalent method. (b) Total antioxidant activity was measured at the end of the experiment by colorimetric assay using ABTS radical. (c) Phase II detoxifying enzyme activity was determined by the level of SOD enzyme in skin cytosol. Values are the mean±SD of eight mice. *P<0.05 vs control group; **P<0.01 vs control group; ***P<0.001 vs .control group

SOD activity level in skin cytosol of the 2% FM group (0.26±0.03 U/mg of tissue) were significantly higher (P<0.05), compared to the level in the control group of mice (0.21±0.04 U/mg of protein) (Figure 4c). Although the SOD activity level in skin cytosol of the 1% FM group (0.24±0.05 U/mg of protein) showed an increasing tendency, this effect did not reach statistical significance.

Discussion

Skin cancer, the development of which is linked to progressive oxidative damage, is one of the most common public health problems in modern medicine (Hara-Chikuma et al., 2008). Therefore, a direct relationship has been reported between antioxidant activity and anticancer activity of these compounds involving the risk of oxidative damage-induced skin carcinogenesis (Ben-Dor et al., 2005; Kamaraj et al., 2009). Previously, we have shown that *O.humifusa*, a member of the Cactaceae family, has the ability to inhibit 7,12-dimethylbenz[*a*]anthracene and 12-O-tetradecanoylphorbol-13-acetate induced skin carcinogenesis in mice via the reduction of oxidative stress (Lee et al., 2012). Thus, the present study was designed to investigate the inhibitory effect of fermented Maesil with probiotics (FM) through its anti-oxidative effect in

skin carcinogenesis.

During the tumor promotion stage, one of the most common events after topical application of DMBA and TPA to mouse skin is a hyperproliferative response, such as epidermal hyperplasia and the promotion of PCNA-positive cells in the epidermis, as mediated by oxidative stress (Zhaorigetu et al., 2003; Hara-Chikuma et al., 2008). The production of squamous papilloma was also evident, along with a hyperproliferative effect in skin carcinogenesis. Therefore, the measurement of proliferating activity and detection of papilloma numbers has been routinely used to determine the grade of precancerous lesions during carcinogenesis (Parmar et al., 2010; Cibin et al., 2012). Throughout the present experiment, the administration of FM resulted in a marked inhibition of epidermal hyperplasia and PCNA-positive cells in a dose-dependent manner. Also, the administration of FM strongly suppressed skin carcinogenesis through a reduction of papilloma numbers.

The application of TPA is also closely related to the excessive production of ROS and consequently leads to oxidative stress which is characterized by increased levels of lipid peroxidation and decreased levels of ROS detoxification enzymes such as SOD (Arya et al., 2011). As a consequence of lipid peroxidation, malondiealdehyde (MDA) and other aldehydes are formed in the biological system which can cause chaotic cross-linkage between proteins and nucleic acids, resulting in an alteration in replication and transcription leading to tumor promotion (Das et al., 2004). Thus, enhanced levels of MDA and decreased activity of SOD enzymes suggested oxidative stress in mouse skin carcinogenesis (Cibin et al., 2012). In the present study, the level of MDA was significantly decreased in FM groups. Also, both total antioxidative capacity and the level of SOD activity were elevated in the FM group. These collective results indicated that fermented Maesil could effectively reduce the level of oxidative stress, which may be contributing factors to skin carcinogenesis. A similar observation also noticed that extracts of Maesil have anti-oxidative activities *in vitro* by radical scavenging activity and by the inhibition of oxidative DNA damage (Kim et al., 2005).

Taken together, these findings suggested that the oral administration of FM could reduce skin carcinogenic activity. These inhibitory effects on skin carcinogenesis could be involved in a reduction of the hyperproliferative effect and oxidative stress, which is associated with a decreased level of lipid peroxidation and an increased total antioxidative activity, along with an elevation of SOD level. Therefore, a fermented Maesil combination with probiotics may be a good candidate for the control of oxidative stress related skin tumorigenesis, in conjunction with detailed studies on molecular mechanisms of cellular anti-oxidant systems and anti-proliferative system.

Acknowledgements

This work was supported by a National Research Foundation of Korea grant No-2009-0071504, funded by the Korean government. The author(s) declare that there is no conflict of interests.

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