Oral administration of phenolic antidiarrheic ingredients prevents ovariectomy-induced bone loss

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ABSTRACT

In the present study, we have attempted to evaluate the pharmacological actions of three major phenolic antidiarrheic ingredients, including 2-methoxyphenol (2MP), 2-methoxy-4-methylphenol (2M4MP) and 2-methoxy-4-ethyphenol (2M4EP), on the functionality and integrity of bone by in vitro and in vivo experimental techniques. Intermittent oral administration of 2M4MP and 2M4EP, but not 2MP, significantly prevented reductions of bone mineral density in total femur, distal femur and tibia, in addition to alterations of several osteoclastic parameters on histomorphometric analysis, when determined 28 days after ovariectomy in mice. All three phenolic ingredients examined significantly inhibited the developmental increase in the number of multinucleated cells positive to tartrate-resistant acid phosphatase staining in cultured mouse osteoclasts differentiated from bone marrow precursors in the presence of both macrophage-colony stimulating factor and receptor activator of nuclear factor-κB ligand, which occurred in a concentration-dependent manner at a concentration range of 1 μM–1 mM without inducing cell death. Moreover, both 2M4MP and 2M4EP at 1 mM not only prevented the cell death induced by 0.5 mM H_2O_2 in cultured rat calvarial osteoblasts, but also suppressed the generation of intracellular reactive oxygen species in osteoblasts exposed to H_2O_2, with a radical scavenging action as revealed by electron spin resonance analysis. These results suggest that particular phenolic antidiarrheic ingredients may prevent ovariectomy-induced bone loss through a mechanism related to the inhibition of osteoclastogenesis in association with an anti-oxidative property in osteoblasts.

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MEM, minimum essential medium
2MP, 2-methoxyphenol
2M4EP, 2-methoxy-4-ethyphenol
2M4MP, 2-methoxy-4-
methylphenol
MNC, multinucleated cell
NF-κB, nuclear factor-κB
PBS, phosphate-buffered saline
PI, propidium iodide
RANKL, receptor activator of
nuclear factor-κB ligand
ROS, reactive oxygen species
TRAP, tartrate-resistant
acid phosphatase

1. Introduction

Bone-forming osteoblasts and bone-resorbing osteoclasts are known to delicately regulate the bone formation and maintenance in bone tissues [1,2]. Imbalance between osteoblasts and osteoclasts leads to the pathogenesis as well as etiology of certain metabolic bone diseases including osteoporosis, Paget's disease and osteopetrosis [3,4]. The balancing mechanism atleast in part involves the endocrine control by estrogen and parathyroid hormone as well as the paracrine (autocrine) control by insulin-like growth factor and bone morphogenetic protein, respectively. The initiation of remodeling events and the subsequent proliferation and differentiation steps of osteoblasts must be tightly regulated, and the balance of osteoblast proliferation, differentiation and apoptosis could therefore determine the size of the osteoblast population at any given time. In particular, osteoblastic apoptosis plays a critical role during embryonic limb development, skeletal maturation, bone fracture healing and bone regeneration, in addition to a role in adult bone turnover [5,6].

Several independent lines of evidence indicate the possible usefulness of particular natural products containing polyphenol molecules for such beneficial treatments. For example, a protective action is shown against osteopenia and/or osteoporosis in experimental animals with catechin [7], rutin [8], green tea polyphenol [9], olive oil polyphenol [10] and apple polyphenol [11]. However, little attention has been paid to pharmacological properties of compounds with a single phenol moiety on both osteoblastogenesis and osteoclasto-

genesis to date. In the present study, therefore, we have focused on major ingredients with a single phenol moiety of a traditional antidiarrheic drug used for more than 100 years in Japan with regard to possible pharmacological properties beneficial for the prophylaxis as well as therapy of different bone diseases using both in vitro and in vivo experimental analyses. These include 2-methoxyphenol (2MP), 2-methoxy-4-methylphenol (2M4MP) and 2-methoxy-4-ethyphenol (2M4EP) (Fig. 1), which are all phenolic constituents present in the effective ingredient, wood creosote, of the traditional antidiarrheic drug.

2. Materials and methods

2.1. Materials

2MP, 2M4MP and 2M4EP were obtained from Tokyo Kasei (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM), alpha minimum essential medium (αMEM) and MEM were from Gibco BRL (Gaithersburgm, MD, USA). A cell counting kit was from Dojindo (Osaka, Japan). Dihydrodichlorofluorescein diacetate (DCFDA) was provided by Molecular probes (Eugene, OR, USA). Recombinant mouse macrophage-colony stimulating factor (M-CSF) and recombinant mouse receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) were purchased from R&D system International (Minneapolis, MN, USA). A Ficoll–Paque Plus and cycle sequencing kit was supplied by Amersham Pharmacia Biotech (Buckinghamshire, UK). Naphtol AS-MX phosphate, fast red violet Lurina–Bertani salt and
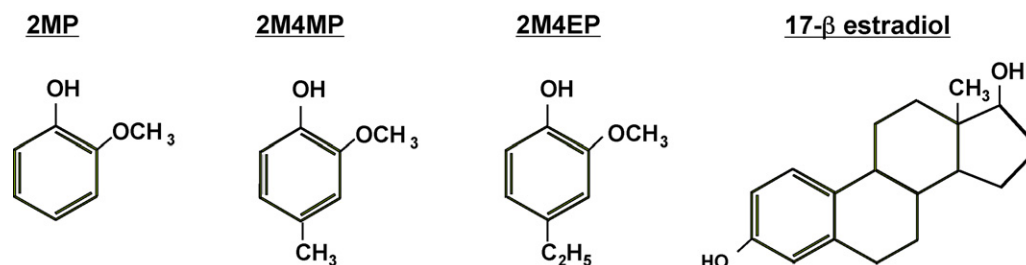

Fig. 1 – Chemical structures of three phenolic antidiarrheic ingredients in comparison with 17β-estradiol.

propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA). Other chemicals used were all of the highest purity commercially available.

2.2. Ovariectomy (OVX) and analysis of skeletal morphology

The protocol employed here meets the guideline of the Japanese Society for Pharmacology and was approved by the Committee for Ethical Use of Experimental Animals at Kanazawa University. All efforts were made to minimize animal suffering, to reduce the number of animals used and to utilize alternatives to *in vivo* techniques. Eight-week-old female ddY mice were subjected to OVX or sham operation as described previously [12]. Ovariectomized mice were subjected to the intermittent oral administration of 2MP, 2M4MP or 2M4EP, suspended in 0.5% carboxylmethyl cellulose on the day of the use, at 0.17 mg/kg three times a week. Animals were also subjected to the intraperitoneal injection of 17β-estradiol (βE2), dissolved in corn oil, once a week for 28 consecutive days at a dose of 5 μg/kg. Mice were killed by decapitation 28 days after operation, followed by the dissection of both femurs and tibiae and subsequent removal of adhering muscles around the bone for fixation with 70% ethanol. Bone mineral density was measured for bones isolated from mice 28 days after operation by single energy X-ray absorptiometry using a bone mineral analyzer (DCS-600R; Aloka Co., Tokyo, Japan). Histomorphometric analysis was carried out using femurs excised from mice 28 days after operation. For toluidine blue O staining, femur was fixed in 70% ethanol, embedded in glycolmethacrylate, and sectioned in 3 μm thickness. The specimens were subjected to histomorphometric analyses under a light microscope with micrometer, using a semiautomatic image analyzing system (Osteoplan II; Carl Zeiss, Thornwood, NY, USA).

2.3. Culture of primary osteoclasts

Primary osteoclast cultures were made from bone marrows according to the procedures previously described [13] with minor modifications. In brief, bone morrows were isolated from tibia and femur of Std-ddY male mice at 4 weeks old and cultured for 24 h with M-CSF at 10 ng/mL in αMEM containing 10% fetal bovine serum (FBS). After culturing for 24 h, non-adherent cells in supernatants were collected, followed by the lamination on Ficoll gradient and subsequent centrifugation at $500 \times g$ for 15 min. The non-adherent monocyte fraction was collected and suspended in MEM containing 10% FBS, M-CSF at 20 ng/mL and RANKL at 20 ng/mL. Cells were plated at a density of 1×10^5 cells/cm^2, followed by culturing in MEM containing 10% FBS, M-CSF at 20 ng/mL and RANKL at 20 ng/mL at 37 °C under 5% CO$_2$ for five consecutive days. For tartrate-resistant acid phosphatase (TRAP) staining, cultured cells were fixed with 10% formalin in phosphate-buffered saline (PBS) for 10 min, and then with ethanol–acetone (50:50, v/v) for 1 min at room temperature. Cells were incubated in acetate buffer (pH 5.0) containing naphtol AS-MX phosphate as a substrate and fast red violet LB salt as a dye in the presence of 50 mM sodium

tartrate. TRAP-positive cells with more than five nuclei were scored as TRAP-positive multinucleated cells (MNCs) for calculation of the number of matured osteoclasts. For PI staining to examine the morphology of dead cells, cells were washed in PBS and incubated with 5 μg/mL PI in PBS for 10–15 min. Nuclear morphology was observed using an epi-fluorescent microscope (IMT-2; Olympus) with G-excitation cubes for PI.

2.4. Lactate dehydrogenase (LDH) assay

Culture medium was collected 5 days after the sustained exposure to one of three phenolic compounds, followed by the immediate measurement of the release of LDH into culture medium. Usually, 0.5 mL of culture medium was added to 2 mL mixture of 0.1 mM β-NADH and 0.1 M potassium dihydrogen phosphate (pH 7.5). The activity of LDH was determined from the difference in the oxidation rate of NADH monitored by the absorbance at 340 nm before and after the addition of 1 mM sodium pyruvate.

2.5. Culture of primary osteoblasts

Osteoblasts were prepared from calvaria of 1- or 2-day-old Wistar rats by a sequential enzymatic digestion method as described previously [14]. In brief, calvaria were gently digested at 37 °C for 10 min with 0.1% collagenase and 0.25% trypsin, followed by the decantation of supernatants thus obtained. After repetition of these procedures five times, the last three fractionated cells were collected as primary osteoblastic preparations. Cells were plated at a density of 1×10^4/cm^2 to appropriate dishes, and then cultured at 37 °C for different periods under 5% CO$_2$. Culture medium was αMEM containing 10% FBS, 50 μg/mL ascorbic acid and 5 mM sodium β-glycerophosphate. Medium was changed every 2–3 days.

2.6. Determination of cell viability

Cell viability was determined using a cell counting kit according to the manufacturer's instructions as performed previously [15]. The cell counting kit used in this study is composed of a highly water soluble tetrazolium salt which is reduced to formazan dye by mitochondrial dehydrogenases. The kit might thus result in false positive viability because of a direct reaction between an anti-oxidative agent, including 2M4MP or 2M4EP, and tetrazolium salt to be reduced. To eliminate the possibility of such an artifact, all medium was replaced with DMEM without any other regents before the cell-counting kit was added. In addition, the background value obtained under cell-free conditions was invariably subtracted from the total value.

2.7. Determination of intracellular reactive oxygen species (ROS)

Osteoblasts cultured for 7 days were treated with hydrogen peroxide (H$_2$O$_2$) for 3 h in either the presence or absence of each phenolic compound, followed by the incubation with DCFDA at 10 μM in DMEM without FBS at 37 °C for 30 min in a

5% CO_2 incubator. In principle, DCFDA diffuses readily into cells, followed by the hydrolysis of ester groups by intracellular esterases and subsequent release of the dichloro derivative. This derivative is then oxidized to the fluorescent parent dye by intracellular ROS. Cells were then washed with PBS twice, followed by the determination of intracellular ROS under a confocal laser-scanning microscope (LSM 510; Carl Zeiss, Jena, Germany) with the excitation at 485 nm and the emission at 525 nm, respectively, as described previously [16].

2.8. Electron spin resonance (ESR) experiments

ESR spectroscopy was employed to detect the spectrum of H_2O_2 as published previously [17]. In brief, 0.5 mM H_2O_2 was incubated with 20 μM 5,5-dimethyl-1-pyrroline N-oxide (DMPO) in either the presence or absence of 2M4MP and 2M4EP at 1 mM. Immediately after mixing, the resulting solution was transferred to an ESR sample tube for the detection of the DMPO hydroxyl radical adduct (DMPO-OH) spectrum. ESR spectra of the reaction mixture were recorded at room temperature using ESR spectrometer (JES-TE300-II; JEOL, Tokyo, Japan) with 9.22 GHz magnetic field modulation.

2.9. Data analysis

Results are all expressed as the mean ± S.E. and the statistical significance was determined by the two-tailed and unpaired Students' t-test or the one-way analysis of variance ANOVA with Bonferroni/Dunnett post hoc test.

3. Results

3.1. Preventive effect on OVX-induced bone loss

To examine the possible pharmacological actions on OVX-induced bone loss, we conducted oral administration of three phenolic antidiarrheic ingredients to ovariectomized mice for subsequent investigation of the bone mineral density, in addition to different histomorphometric parameters. Ovariectomy-induced a significant reduction of bone mineral density in both total tibia (Fig. 2, left panel) and total femur (Fig. 2, middle panel), in addition to that in distal femur (Fig. 2, right panel), when determined by the single-energy X-ray absorptiometry at 28 days after operation. The intermittent oral administration of 2M4MP and 2M4EP, but not 2MP, significantly prevented the reduction of bone mineral density in total tibia, total femur and distal femur in ovariectomized mice (Fig. 2), while the administration of these three phenolic ingredients did not induce any marked behavioral abnormalities throughout the experimental periods (data not shown). The decrease by OVX was significantly prevented by the weekly intraperitoneal injection of βE2 used as a positive control.

In addition, the intermittent oral administration of 2M4MP and 2M4EP also apparently prevented the trabecular bone loss by OVX seen in cancellous bone when determined by both micro-computed tomography analysis and toluidine blue staining (data not shown). Moreover, histomorphometric analysis revealed that OVX significantly decreased several bone parameters, including BV/TV (bone volume/tissue volume), Tb.Th (trabecular thickness) and Tb.N (trabecular
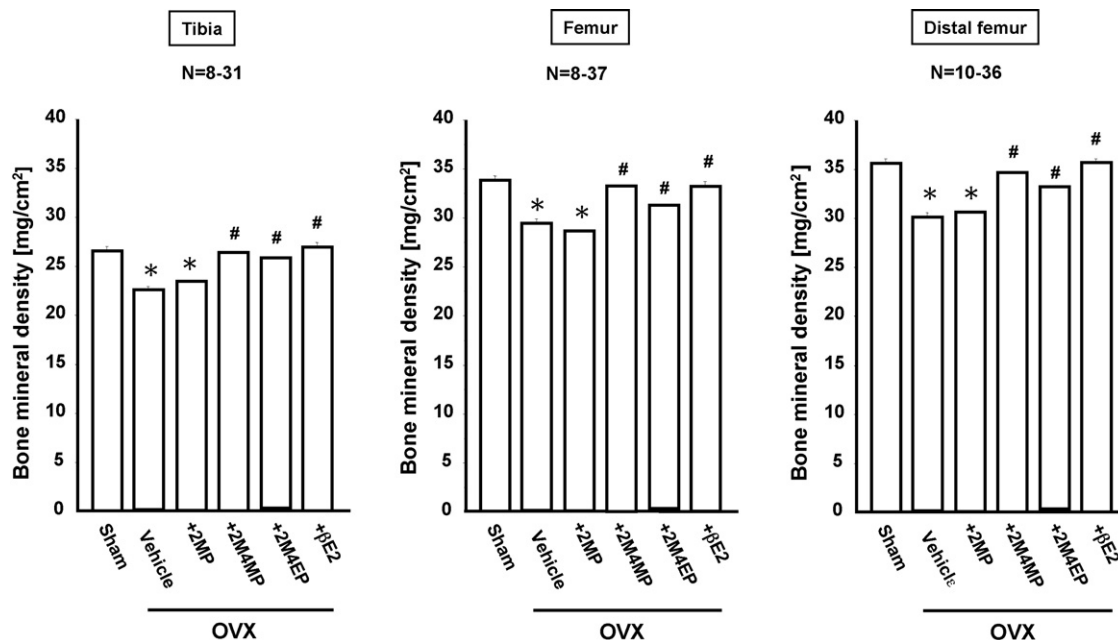

Fig. 2 – Bone mineral density. Eight-week-old female ddY mice were subjected to OVX or sham operation, followed by the oral administration of 2MP, 2M4MP or 2M4EP at 0.17 mg/kg three times a week, or the intraperitoneal injection of βE2 at 5 μg/kg once a week, for 28 consecutive days and subsequent determination of bone mineral density of both tibia and femur by single-energy X-ray absorptiometry. Values are the mean ± S.E. from 8 to 37 different experiments. *P < 0.05, significantly different from the control value obtained in sham-operated mice. #P < 0.05, significantly different from the value obtained in OVX mice.

Table 1 – Histomorphometric analyses

	Sham	OVX	OVX + 2M4MP	OVX + 2M4EP
BV/TV (%)	17.1 ± 0.59	6.2 ± 1.1*	11.6 ± 1.52#	11.3 ± 1.13#
Tb.Th (μm)	43.3 ± 2.03	30.0 ± 2.06*	40.47 ± 2.98#	41.4 ± 2.34#
Tb.N (mm^{-1})	3.53 ± 0.19	1.53 ± 0.19*	2.38 ± 0.22#	2.40 ± 0.26#
Tb.Sp (μm)	240.4 ± 24.0	693.2 ± 40.8*	406.2 ± 60.1#	481.3 ± 47.1#
Ob surface (%)	6.87 ± 0.69	8.92 ± 0.62	7.84 ± 0.93	7.90 ± 0.61
ES/BS (%)	4.08 ± 0.46	7.75 ± 0.44*	5.69 ± 0.41#	6.01 ± 0.39#
Oc no (mm^{-1})	0.64 ± 0.062	1.04 ± 0.072*	0.75 ± 0.048	0.79 ± 0.044#
Oc surface (%)	1.75 ± 0.21	2.89 ± 0.21*	2.06 ± 0.30#	2.16 ± 0.20#

Eight-week-old female ddY mice were subjected to OVX or sham operation, followed by the oral administration of 2MP, 2M4MP or 2M4EP at 0.17 mg/kg three times a week for 28 consecutive days and subsequent determination of different histomorphometric parameters. These included bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular separation (Tb.Sp), bone surface covered by osteoblasts (Ob surface), eroded surface (ES/BS), the number of osteoclasts on bone surface (Oc no) and bone surface covered by osteoclasts (Oc surface). Values are the mean ± S.E. from 3 to 4 different experiments.
* $P < 0.05$, significantly different from each control value obtained in sham-operated mice.
# $P < 0.05$, significantly different from the value obtained in OVX mice.

number), with a concomitant increase in the parameter Tb.Sp (trabecular separation), ES/BS (eroded surface), Oc no (the number of osteoclasts on bone surface) and Oc surface (bone surface covered by osteoclasts) (Table 1). However, OVX did not significantly affect the extent of bone surface covered by osteoblasts (Ob surface). Under these experimental conditions, the oral administration of either 2M4MP or 2M4EP significantly prevented OVX-induced alterations of histomorphometric bone parameters examined (Table 1).

3.2. Inhibitory effect on RANKL-induced differentiation in primary osteoclasts

Since the oral administration of either 2M4MP or 2M4EP significantly prevented the increases in osteoclastic parameters such as Oc no and Oc surface in ovariectomized mice *in vivo*, we at first investigated the possible effect of phenolic compounds on cellular differentiation of bone-resorbing osteoclasts to evaluate mechanisms underlying the prevention *in vitro*. For this purpose, hematopoietic precursor cells were isolated from bone marrows of murine tibia and femur, followed by the suspension in culture medium containing both M-CSF and RANKL. Semi-quantitative RT-PCR revealed that expression of mRNA was drastically increased for different osteoclastic marker genes during the cultivation for five consecutive days under the experimental conditions employed. These included RANK, carbonic anhydrase II, matrix metalloproteinase-9, c-fms, cathepsin K, calcitonin receptor and c-src in addition to TRAP (data not shown).

These murine primary osteoclasts were cultured in either the presence or absence of 2MP, 2M4MP and 2M4EP at concentrations of 1 μM–1 mM for five consecutive days, followed by TRAP staining and subsequent counting of the number of MNCs. Pavement-shaped and TRAP-positive MNCs were drastically increased during the cultivation with both M-CSF and RANKL for 5 days, while 2MP, 2M4MP and 2M4EP were all effective in markedly decreasing the number of pavement-shaped and TRAP-positive MNCs when exposed for five consecutive days in a concentration-dependent manner at concentrations over 10 μM (Fig. 3). In particular, sustained exposure to three compounds at 1 mM similarly resulted in a marked decrease by more than 80% in the number of

TRAP-positive MNCs. To examine the possible participation of cell death in the inhibition of osteoclastogenesis, primary osteoclasts were cultured in either the presence or absence of 2MP, 2M4MP and 2M4EP at a concentration of 1 mM for 5 days or H_2O_2 at 100 μM for 1 day, followed by the staining with PI and the determination of the activity of LDH released. Exposure to H_2O_2 led to a marked increase in the number of cells stained with PI, while PI-positive cells were not seen at all in osteoclasts exposed to either of 2MP, 2M4MP and 2M4EP for five consecutive days (data not shown). Moreover, sustained exposure to 2MP, 2M4MP or 2M4EP was ineffective in
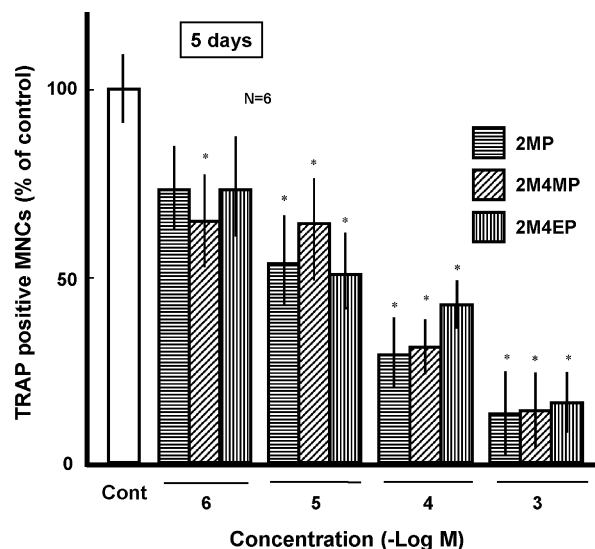

Fig. 3 – Differentiation of primary osteoclasts. Cells were cultured for 5 days with 20 ng/mL M-CSF and 50 ng/mL RANKL in either the presence or absence of 2MP, 2M4MP and 2M4EP at a concentration range from 1 μM to 1 mM. Cells were then fixed with 10% formalin, followed by the staining for TRAP and subsequent counting of the number of MNCs positive to TRAP staining. Values are the mean ± S.E. from six different experiments. *$P < 0.05$, significantly different from the control value obtained in the absence of phenolic compounds. Cont, control (0.1% DMSO).

significantly affecting the activity of LDH released during the culture of primary osteoclasts for five consecutive days (data not shown).

3.3. Protective effect on cell death by H_2O_2 in osteoblasts

In order to clarify the mechanism underlying the difference in the preventive effect of 2MP *in vivo* and *in vitro*, we next studied the possible effect of the three phenolic ingredients on the viability of bone-forming osteoblasts. Rat calvarial osteoblasts were cultured with 2MP, 2M4MP or 2M4EP at a concentration range from 1 μM to 1 mM for five consecutive days in the presence of 50 μg/mL ascorbic acid and 5 mM sodium β-glycerophosphate, followed by the determination of cell viability. As shown in Fig. 4A, none of three compounds significantly affected the cell viability at any concentrations used in cultured osteoblasts. Thus, osteoblasts were cultured for 7 days in the presence of 50 μg/mL ascorbic acid and 5 mM sodium β-glycerophosphate, followed by the brief exposure to H_2O_2 at different concentrations from 0.05 to 1 mM for 6 h in either the presence or absence of the three phenolic ingredients at a concentration range from 1 μM to 1 mM and subsequent determination of cell viability. Exposure to H_2O_2 alone led to a

significant decrease in cellular viability in a concentration-dependent manner at concentrations over 0.5 mM in cultured rat calvarial osteoblasts (Fig. 4B). Exposure to 0.5 mM H_2O_2 for 6 h resulted in a significant decrease in cell viability by more than 70%, indeed, while both 2M4MP and 2M4EP were effective in significantly preventing the cell death at 1 mM when exposed simultaneously with H_2O_2 (Fig. 4C). By contrast, no significant preventive effect was observed in cells treated with 0.5 mM H_2O_2 in the presence of 2MP at concentrations used.

3.4. Anti-oxidative effect on intracellular ROS

In order to further evaluate the protective property against cell death by H_2O_2, osteoblasts were cultured for 7 days, followed by the brief exposure to 0.5 mM H_2O_2 for 3 h in either the presence or absence of three ingredients at 1 mM. Cells were then incubated with DCFDA at 10 μM for 30 min for the determination of intracellular ROS levels on fluorescence image. The simultaneous addition of 2M4MP or 2M4EP at 1 mM apparently abolished the generation of intracellular ROS in osteoblasts exposed to 0.5 mM H_2O_2 for 3 h, while no marked reduction was found in cells treated with 2MP at 1 mM in the presence of 0.5 mM H_2O_2 (Fig. 5A).
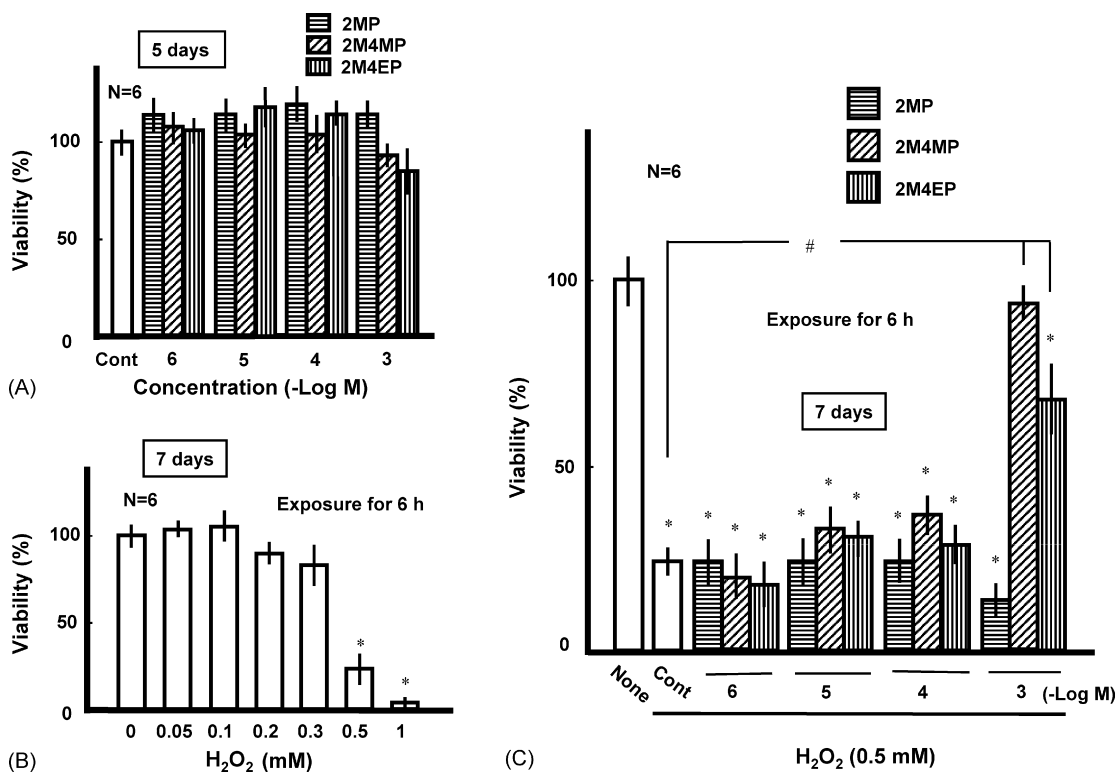

Fig. 4 – H_2O_2-induced cell death in cultured osteoblasts. (A) Osteoblasts were cultured with 2MP, 2M4MP or 2M4EP at a concentration range from 1 μM to 1 mM for five consecutive days in the presence of 50 μg/mL ascorbic acid and 5 mM sodium β-glycerophosphate, followed by the determination of cell viability by a cell counting kit. (B) Osteoblasts were cultured for 7 days, followed by the brief exposure to H_2O_2 at different concentrations of 0.05–1 mM for 6 h and subsequent determination of cell viability. (C) Osteoblasts were cultured for 7 days, followed by the brief exposure to H_2O_2 at 0.5 mM for 6 h in either the presence or absence of the three phenolic ingredients at a concentration range from 1 μM to 1 mM and subsequent determination of cell viability. Values are the mean ± S.E. from six different experiments. *$P < 0.05$, significantly different from the value obtained in cells not exposed to H_2O_2. #$P < 0.05$, significantly different from the value obtained in cells exposed to H_2O_2 alone. Cont, control (0.1% DMSO).
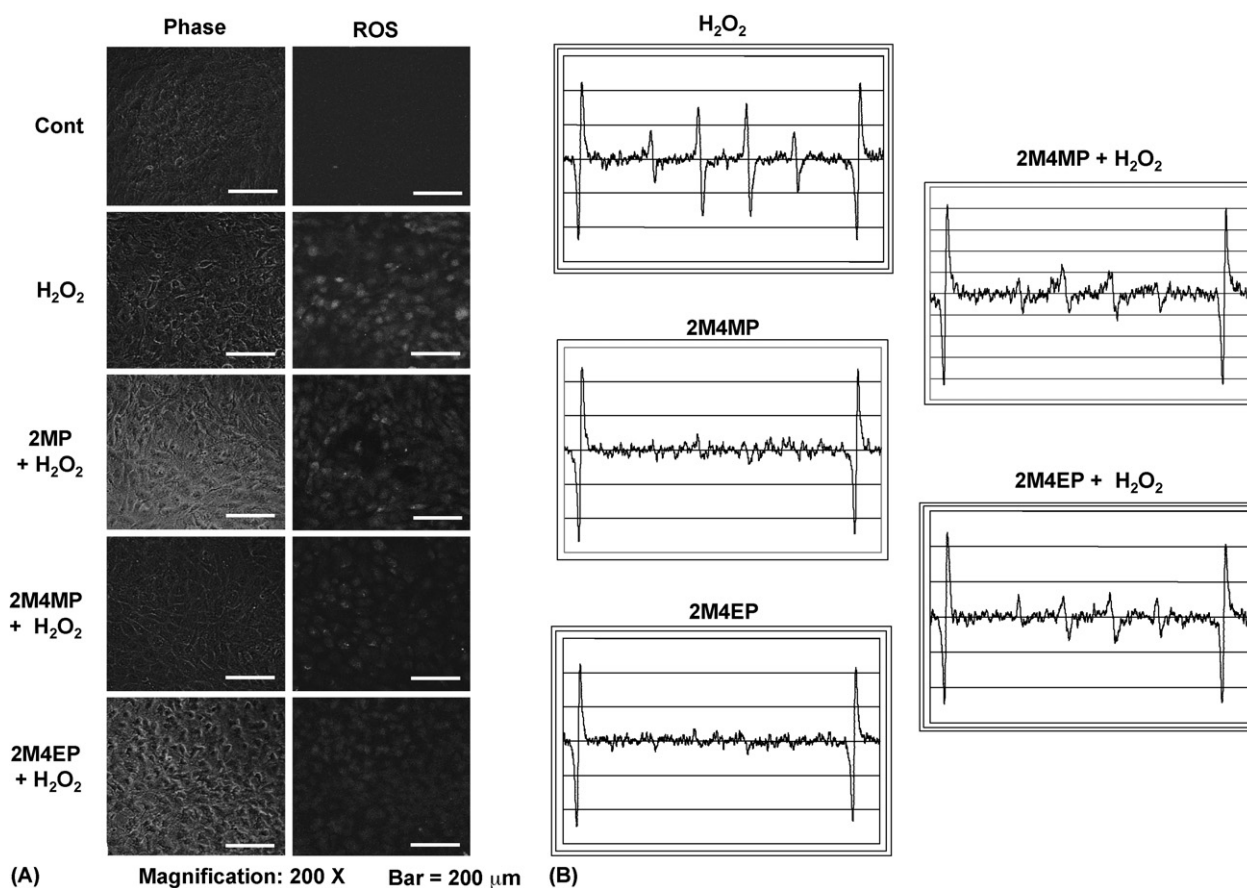
Fig. 5 – Generation of ROS in cultured osteoblasts. (A) Osteoblasts were cultured for 7 days, followed by the brief exposure to 500 μM H_2O_2 for 3 h in either the presence or absence of 2MP, 2M4MP and 2M4EP at 1 mM. Cells were then incubated with DCFDA at 10 μM for 30 min, for the subsequent determination of intracellular ROS under a confocal laser-scanning microscope. Typical pictures are shown here, with similar results in three independent determinations. (B) Hydrogen peroxide at 0.5 mM was incubated with DMPO in either the presence or absence of 2M4MP and 2M4EP at 1 mM, followed by the determination of DMPO-OH spectrum by ESR analysis. Typical ESR spectra are shown here, with similar results in three independent determinations. Cont, control (0.1% DMSO).

For evaluation of the anti-oxidative property, an attempt was made to determine whether the phenolic compounds have an ability to scavenge oxygen radicals. Hydrogen peroxide was incubated with DMPO in either the presence or absence of 2M4MP and 2M4EP, followed by the determination of DMPO-OH spectrum by ESR measurements. Incubation with H_2O_2 and DMPO generated a clear four-line ESR spectrum characteristic to DMPO-OH (Fig. 5B, left upper panel), whereas no clear spectrum was formed when DMPO was previously treated with 2M4MP (Fig. 5B, right upper panel) or 2M4EP (Fig. 5B, right lower panel). The formation of unique four-line DMPO-OH spectrum derived from H_2O_2 was apparently blocked by the addition of 2M4MP or 2M4EP, both of which exhibited no clear ESR spectrum characteristic to DMPO-OH alone (Fig. 5B, left middle and lower panels).

4. Discussion

The essential importance of the present findings is that the intermittent oral administration of the particular phenolic ingredients of a traditional antidiarrheic drug, such as 2M4MP and 2M4EP, significantly prevented the decrease in bone mineral density and the alternations of different osteoclastic parameters in histomorphometric analyses in ovariectomized mice in vivo. As the antidiarrheic drug has been usually used at a dose ranging from 400 to 900 mg/day for more than 100 years in Japan, the doses used in this in vivo analysis are not extraordinarily beyond the limit of the daily use in human beings. Moreover, both phenolic ingredients markedly prevented RANKL-induced osteoclastic differentiation as well as ROS-induced osteoblastic cell death in vitro. Several phenolic compounds including 2MP (=guaiacol), eugenol and phenol, are shown to enhance the uptake of [^{3}H]thymidine in the murine osteoblastic cell line MC3T3-E1 cells [18], while 2MP is shown to decrease the level of hydroxyl radical generated by the Fenton reaction [19]. Although these previous studies have referred to a stimulatory effect of 2MP on osteoblastic cell proliferation, no direct evidence for the prevention by both 2M4MP and 2M4EP against the OVX-induced bone loss in association with the cytoprotective property in osteoblasts and the inhibitory action on cellular differentiation in

osteoclasts is available in the literature to date. To our knowledge, furthermore, this is the first direct demonstration of a radical scavenging action of both 2M4MP and 2M4EP with a simple single phenol structure as revealed by ESR analysis. The evaluation of the structure–activity relationship gives rise to the importance of methyl and ethyl residues on the position 4 in a phenol ring for cytoprotective properties against the osteoblastic cell death by ROS.

There is accumulating evidence for close correlations amongst oxidant stress, antioxidant levels and bone mineral density [20,21]. For instance, H_2O_2 has been suggested to be the ROS responsible for the bone loss induced by estrogen deficiency, while OVX significantly decreases the endogenous contents of reduced glutathione in addition to enzyme activity of glutathione reductase in bone marrows [21]. Administration of antioxidants such as N-acetyl cysteine and ascorbic acid, increases tissue levels of reduced glutathione as well as abolishes OVX-induced bone loss, whereas L-buthionine-(S,R)-sulphoximine, a specific inhibitor of glutathione synthesis, causes substantial bone loss [22]. In addition, OVX-induced bone loss is indeed prevented by the administration of pegylated catalase, which strongly suggests the involvement of H_2O_2 in mechanisms underlying the bone loss induced by OVX [23]. The present differential properties among 2MP, 2M4MP and 2M4EP on H_2O_2-induced osteoblastic death, generation of intracellular ROS and RANKL-induced osteoclastogenesis give support to an idea that both the prevention of bone loss and the reduction of osteoclastic parameters *in vivo* would be mediated not only by the inhibition of osteoclastogenesis but also by the protection against osteoblastic cell death through an anti-oxidative mechanism related to the direct scavenging action *in vitro*. Both 2M4MP and 2M4EP significantly protected against the cytotoxicity of H_2O_2 in cultured osteoblasts *in vitro* as well as the bone loss in ovariectomized mice *in vivo*, indeed, while the three phenolic ingredients were similarly effective in preventing the RANKL-induced osteoclastogenesis *in vitro*. This means that the inhibition of osteoclastogenesis may be insufficient for 2MP to prevent the bone loss in ovariectomized mice. Considering the fact that the unique ESR spectrum of hydroxyl radical was not seen in the presence of the phenolic ingredients with protective properties *in vitro* and *in vivo*, moreover, it is likely that the scavenging action of H_2O_2 is atleast in part responsible for the prevention of bone loss in ovariectomized mice. More work including studies on nitric oxide and prostaglandins is undoubtedly necessary for the full clarification of the protective properties of particular phenolic antidiarrheic ingredients in mechanisms associated with bone remodeling.

An apparent discouraging aspect that considerably high concentrations were required for the effective protection by both 2M4MP and 2M4EP could be accounted for by taking into consideration the fact that cell death was artificially induced by the exposure to exogenous H_2O_2 at 0.5 mM for 6 h in cultured osteoblasts *in vitro*. One possible speculation is that both effective phenolic ingredients would be protective against ROS such as H_2O_2 at a molar stoichiometry of 2:1. Structural comparison of the phenolic ingredients with βE2 argues in favor of an idea that 2M4MP and 2M4EP, rather than 2MP, could be a potential mother compound toward the innovative strategy for development of a drug useful for the

prophylaxis and/or treatment of the bone loss relevant to postmenopausal osteoporosis in human beings without estrogenic and carcinogenic properties. Although the final conclusion should await the further biochemical and pharmacological profiling, the possible involvement of estrogenic activities in the protection against OVX-induced bone loss *in vivo* is not feasible for 2M4MP or 2M4EP.

On the other hand, osteoclastogenesis is a multi-step process dependent on the intimate cellular interaction of myeloid pre-osteoclastic precursors with either osteoblasts or stromal cells under the influence by a wide range of local factors such as M-CSF [24,25] and RANKL [26,27]. Cellular differentiation and function could be stimulated by ROS through intracellular signaling molecules essential for the osteoclast formation including NF-κB and c-Jun amino terminal kinase in osteoclasts, while antioxidants would inhibit osteoclastic differentiation in addition to NF-κB activation [28–30]. The present findings that all three phenolic ingredients examined significantly inhibited the RANKL-induced differentiation without inducing cell death in mouse primary osteoclasts thus give rise to the possibility that both 2M4MP and 2M4EP may atleast in part inhibit osteoclastogenesis through the direct radical scavenging action as antioxidants. The exact mechanism underlying the inhibition of osteoclastogenesis by 2MP, however, is not clarified so far. By taking into consideration the effectiveness amongst three phenolic ingredients on osteoblastogenesis and osteoclastogenesis *in vitro*, accordingly, the *in vivo* prevention of OVX-induced bone loss would predominantly involve the *in vitro* protection against osteoblastic cell death by ROS rather than the *in vitro* inhibition of osteoclastogenesis under the conditions employed. Namely, it should be noted that 2MP was similarly ineffective in preventing the H_2O_2-induced cell death *in vitro* or the OVX-induced bone loss *in vivo*, but effective in significantly inhibiting the RANKL-induced osteoclastic differentiation *in vitro*.

From the data cited above, it appears that 2M4MP and/or 2M4EP could be a key mother compound protective against the bone loss induced by OVX through a direct scavenging action of ROS without significantly affecting osteoblastic viability *in vitro*. These results suggest that particular phenolic antidiarrheic ingredients would be also effective in preventing the abnormality and malfunction associated with the possible prolonged generation of ROS seen in different pathological situations such as ageing and inflammation in a variety of cells, in addition to both osteoblasts and osteoclasts. As this traditional antidiarrheic drug has been used without any significant untoward side effects in human beings for more than 100 years in Japan, future elucidation of underlying mechanisms would give us a clue for the discovery and development of a novel drug useful for the therapy and treatment of a variety of disorders associated with the sustained generation of ROS in human beings.

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