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Research Article

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The zinc composition potently enhances the anti-inflammatory effect of the oyster-derived antioxidant 3,5-dihydroxy-4-methoxybenzyl alcohol in RAW264.7 mouse macrophages

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Short title: Potent anti-inflammatory effects of DHMBA and zinc

Abstract

Antioxidants play an important role in treating various diseases. DHMBA is a new antioxidant derived from oysters. Studies have shown that AHMBA has anti-inflammatory effects in mouse macrophages in vitro, suggesting a pharmacological usefulness. Additionally, oysters contain zinc, which also has antioxidant properties. These two factors may therefore have potent anti-inflammatory effects. This study investigates whether the combination of DHMBA and zinc is potent enough to act as an anti-inflammatory agent in RAW264.7 macrophages *in vitro*. DHMBA significantly suppressed proliferation and stimulated cell death in RAW264.7 cells at comparatively lower concentrations (1 or 10 μM), resulting in decreased cell numbers. Zinc sulfate at 10 and 100 μM did not affect the proliferation or death of RAW264.7 cells. Interestingly, the potential effects of suppressing cell growth and stimulating cell death were observed at comparatively lower concentrations of DHMBA (0.1 or 1 μM) and zinc (10 or 100 μM). Combining DHMBA and zinc decreased the expression levels of key proteins that promote cell growth, such as Ras, PI3K, Akt, MAPK, and mTOR. Furthermore, the combination exhibited potent effects in suppressing cytokine production and osteoclastogenesis stimulated by lipopolysaccharide treatment in RAW264.7 macrophages. Culturing with both DHMBA (0.1 μM) and zinc (10 μM) mechanistically reduced NF-κB levels in macrophages. Thus, the suppressive effects of DHMBA or zinc were potentiated by combining the two. These findings suggest the potential for a combined antioxidant effect. This study presents a novel approach to the prevention and treatment of inflammatory conditions by combining the antioxidants DHMBA and zinc.

Keywords: 3,5-dihydroxy-4-methoxybenzyl alcohol, DHMBA, zinc, antioxidant, inflammation, macrophages

1 Introduction

The compound 3,5-dihydroxy-4-methoxybenzyl alcohol (DHMBA) was firat identified in the Pacific oyster *Crassostrea* gigas [1-3]. It is a more potent peroxyl radical scavenger than other well-known antioxidants, such as vitamins E and C, resveratrol, and gallic acid [4, 5]. DHMBA may be useful as a novel pharmacological tool. In recent years, its usefulness in inhibiting adipogenesis activity, suppressing inflammatory macrophage activation, and stimulating osteoblastogenesis *in vitro* has been demonstrated [6-8]. Furthermore, DHMBA exhibits anti-cancer activity in several types of cancer cell by targeting various signaling pathways [9, 10]. This antioxidant may have therapeutic applications in the treatment of various diseases.

Zinc is an essential trace element that plays a key role in maintaining a variety of bodily functions. It acts as a cofactor for various enzymes involved in antioxidant activity, and plays a pivotal role in regulating cellular functions [11]. Zinc is commonly used as a dietary supplement. Notably, the metal is found in significant quantities in oysters, among other dietary sources [12, 13]. We were therefore interested in the interaction between DHMBA and zinc. Notably, we focused that zinc has been reported to have an inhibitory effect on inflammation [14, 15]. The aim of this study was conducted to elucidate the

comparative effects of DHMBA alone, zinc alone, and both DHMBA and zinc together.

Inflammation is a complex biological response of body tissues to harmful stimuli, involving a protective response. This process is based on the effects of various factors, including immune cells, blood vessels, and molecular mediators [16]. RAW264.7 mouse macrophages are used as a model for inflammatory conditions *in vitro*. Macrophages are characterized by their metabolic and phagocytic functions [17]. This study investigates whether a composition with DHMBA and zinc at comparatively lower concentrations exhibits potent suppressive action on the inflammatory effects of RAW264.7 mouse macrophages *in vitro*. The results of this study could inform the development of useful strategies for the pharmacological application of these antioxidants, which have anti-inflammatory properties.

2 Materials and Methods

2.1 Reagents

Dulbecco's modification of Eagle's medium (DMEM) supplemented with 4.5 g/L glucose, L-glutamine, and sodium pyruvate and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin; 1% P/S) was purchased from Corning (Mediatech, Inc. Manassas, VA, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Zinc sulfate, lipopolysaccharide (LPS), and all other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Reagents were diluted with 100% ethanol or phosphate buffer (PBS) was stored at -20℃ until use.

2.2 3, 5-dihydroxy-4-methoxybenzyl alcohol

3,5-Dihydroxy-4-methoxybenzyl alcohol (DHMBA) is an amphipathic phenolic compound that was originally isolated from the Pacific oyster (*Crassostrea* gigas) [1, 2]. Chemical structure of DHMBA has been reported previously [1, 2] and this was presented as supplemental data in this report. We used the synthesized DHMBA with 100% purity was obtained from the Watanabe Oyster Laboratory, Inc. (Hachioji, Tokyo, Japan) [5]. DHMBA was dissolved in 100% ethanol and stored at -20℃ until used.

2.3 RAW264.7 macrophages

Macrophages are a monocyte/macrophage cell line originating from a cell lineage transformed by the *Abelson leukemia* virus, derived from BALB/c mice [18]. RAW264.7 mouse macrophages were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in DMEM containing 10% FBS, and 1% P/S.

2.4 Colony formation assay

RAW264.7 macrophages ($1x10^3$ cells per 2 mL per well in 6-well plates) were cultured in DMEM containing 10% FBS and 1% P/S at 37℃ in a 5% CO_2 atmosphere. Visible clones formed on the plates after 10 days. The colonies were then washed with PBS and fixed with methanol at room temperature. The colonies were then stained with

0.1% crystal violet (1 mL). After PBS washing, the plate was air-dried at room temperature. Colonies containing more than 50 cells were counted using a Nikon TMS microscope (Tokyo, Japan).

2.5 Assays of cell proliferation and death

To determine the comparative effects of DHMBA and zinc on cell growth, RAW264.7 cells ($1x10^5$/mL per well in 24-well plates) were cultured in DMEM (containing 10% FBS and 1% P/S) with either vehicle (final concentration of 1% ethanol), DHMBA (final concentrations of 0.1, 1, 10, 50, or 100 μM), or zinc sulfate (final concentrations of 0.1, 1, 10, 50, or 100 μM) for 3 days on reaching subconfluence, in a water-saturated atmosphere under 5% CO_2 and 95% air at 37ºC [19, 20]. In separate experiment to determine the effects of the mixture of a DHMBA and zinc mixture on RAW264.7 macrophage growth, the cells were cultured in DMEM (containing 10% FBS and 1% P/S) either with vehicle, DHMBA (0.1 or 1 μM), or zinc (10 or 100 μM) for 3 days. After culturing, the cells attached to the dish were counted as described in the section of cell counting.

In order to study the effects of DHMBA and zinc on cell death, RAW264.7 macrophages ($1x10^5$ cells per mL per well in 24-well plates) were cultured in DMEM (10% FBS) 3 days. Once subconfluence was reached, the cells were cultured in DMEM (10% FBS) with either vehicle or DHMBA (0.1, 1, 10, 50, or 100 μM), or zinc (0.1, 1, 10, 50, or 100 μM) for an additional 24 hours [21]. In other experiments, to determine the

effects of a mixture of DHMBA and zinc mixture on RAW264.7 macrophage death, the cells were cultured in DMEM (10% FBS) for an additional 24 hours, with either vehicle, 0.1 or 1 μM DHMBA, or 10 or 100 μM zinc.

After culturing, macrophages were detached from each well by adding a solution of 0.05% trypsin plus EDTA in Ca^{2+}/Mg^{2+}-free PBS (Thermo Fisher Scientific, Waltham, MA, USA), as previously reported [19-21]. Viable cells were mixed with 0.5% trypan blue staining solution, and the cells were counted under a microscope (Olympus MTV-3) using a hemocytometer (Sigma-Aldrich, St. Louis, MO, USA) and a cell counter (Line Seiki H-102P, Tokyo, Japan). The results are presented as the mean ± standard deviation (SD) from a total of 8 wells with different cell preparations.

2.6 Western blot analysis

RAW264.7 macrophages ($1x10^6$ cells per well in 10 mL of medium) were cultured in DMEM (10% FBS), with either vehicle (1% ethanol) or with DHMBA (1 μM) plus zinc (100 μM) for 3 days. We have previously described the methods of Western blot analysis [22]. Supernatant proteins (40 μg per lane) from the cell lysate were separated by SDS-PAGE (12%). The transferred membranes were immunoblotted using specific antibodies, which were obtained from Cell Signaling Technology (Danvers, MA). These included antibodies against Akt, Ras, PI3-kinase p110α, MAPK, phosphorylated MAPK, mTOR, caspase-3, cleaved caspase-3, as well as an antibody against β-actin. The immunoblotting membranes were incubated by using horseradish peroxidase-conjugated secondary antibodies from Cell Signaling Technology, Inc.). After incubation, the

membranes were developed using X-ray film using a photo scanner of the film, the bands were quantified using ImageJ2 software (National Institutes of Health, Bethesda, MD, USA). A total of three blots from independent experiments were scanned.

2.7. Assay of reactive oxygen species (ROS) production

The ROS production assay used the previously method [23]. The ROS Assay Kit (MAK144-1, Sigma-Aldrich) with the fluorescent substrate 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) were used to determine the intracellular ROS [23]. Macrophages (2.5×10^4 cells per 100 µL in 96-well plates) were cultured either with vehicle (1% ethanol), DHMBA (0.1 µM), or zinc sulfate (10 µM) for 24 hours in an atmosphere of 5% CO_2 and 95% air at 37 °C. After culturing, the plate was analyzed using a fluorescence plate reader. Fluorescence intensity was measured at Ex/Em = 490/520 nm in endpoint mode. The results are presented as a percentage of the control group for each treatment. To determine the effects of the combined DHMBA (0.1 µM) and zinc sulfate (10 µM) on cell number, another experiment was conducted. Macrophages (1×10^5 cells per mL in 24-well plates) were cultured in DMEM containing 10% FBS and 1% P/S, either with 0.1 µM DHMBA, 10 µM zinc sulfate, or both for 24 hours.

2.8 Assay of cytokine production

To determine cytokine production in macrophages, the cells ($1x10^5$/mL per well) were cultured in a 24-well plate containing DMEM (10% FBS) for 3 days on reaching

subconfluence [5]. Macrophages were then cultured for an additional 5 hours after the treatment with either with vehicle (1% ethanol), 0.1 μM DHMBA, or 10 μM zinc sulfate, a combination of 0.1 μM DHMBA and 10 μM zinc sulfate, or LPS (100 ng/ mL). After incubation, the medium was collected for cytokine analysis, and then the cells were detached from each culture dish to determine cell number as described in "cell counting". TNF- α or IL-6 concentrations in the medium were analyzed using ELISA kits for mouse TNF-α and IL-6, purchased from Cayman Chemical (Ann Arbor, MI, USA), according to the manufacturer's instructions. The production of cytokine is presented as picograms (pg) of cytokine secreted into the culture medium (mL).

2.9 Osteoclastogenesis assay

RAW264.7 macrophages (1 x 10^5 cells/1 mL per well in 24-well plates) were cultured in DMEM (10% FBS). Macrophages were cultured either with vehicle, 0.1 μM DHMBA, 10 μM zinc sulfate, or a combination of both compounds, with or without LPS (100 ng/mL) for 3 days. After culturing, the cells were fixed and stained for tartrate-resistant acid phosphatase (TRACP), a marker enzyme of osteoclasts [24]. TRACP-positive stained cells were counted as osteoclastic cells under a 40x microscope (Olympus MTV-3; Olympus Corporation, Tokyo, Japan). To determine TRACP-staining-positive osteoclastic cells, one field per well was photographed and measured using ImageJ2 software. The number of TRACP-positive osteoclastic cells in a well was counted under a microscope.

2.12 Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical significance was estimated using GraphPad InStat version 3 for Windows XP (GraphPad Software Inc., La Jolla, CA, USA). A Student's t-test was used to calculate statistical significance between the 2 groups. Multiple comparisons were performed using one-way analysis of variance (ANOVA) with a Tukey-Kramer post hoc test for parametric data. A p-value of <.05 was considered statistically significant.

3 Results

3.1 Combined effects of DHMBA and zinc on the growth of macrophages

First, we investigated the effects of DHMBA and zinc sulfate on the growth of RAW264.7 macrophages. The cells were cultured in a medium containing either with vehicle (1% ethanol), DHMBA (0.1, 1, 10, 50, or 100 μM) or zinc (0.1, 1, 10, 50, or 100 μM) for 3 days after reaching subconfluence (Figures 1A and 1B. Macrophage growth was repressed by culturing with DHMBA (1, 10, 50, or 100 μM). Culturing with zinc sulfate did not significantly affect cell growth. Furthermore, we investigated the effects of combining DHMBA (0.1 or 1 uM) and zinc sulfate (10 or 100 uM) at lower concentrations on cell growth by culturing the cells for 3 days (Figure 1C). A significant decrease in cell growth was observed with the combination of 0.1 uM DHMBA and 10 or 100 uM zinc sulfate, neither of which had a significant effect alone. Notably, DHMBA and zinc at a comparatively lower concentrations were found to exhibit potent repressive

effects on macrophage growth *in vitro*. Mechanistically, culturing with the combined 0.1 uM DHMBA and 10 or 100 uM zinc slfate reduced the expression levels of Ras, PI3K, Akt, MAPK, phospho-MAPK, and mTOR, all of which are involved in cell promotion.

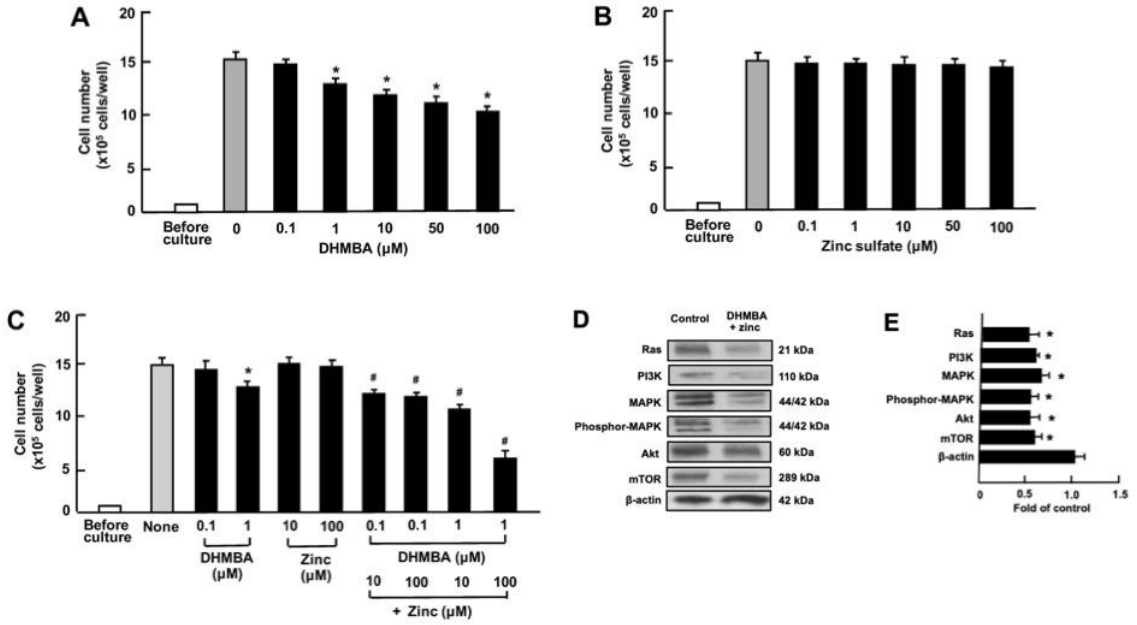

Figure 1. Effect of DHMBA and zinc sulfate on the growth of RAW264.7 macrophage *in vitro*. (Figures A and B): Cells ($1x10^5$ cells/mL per well in 24-well plates) were cultured for 3 days after reaching subconfluence with either vehicle (1% ethanol), DHMBA (Figure A), or zinc sulfate (Figure B) with final concentrations of 0.1, 1, 10, 50, or 100 μM, respectively. Figure C: Cells ($1x10^5$ cells/mL per well) were cultured for 3 days with either vehicle (1% ethanol), DHMBA (0.1 or 1 μM), or zinc sulfate (10 or 100 μM) either alone or in combination. After culturing, the number of cells attached to the dish was counted. Data are presented as the mean ± SD of 8 wells with different cell preparations. *$p<0.001$ versus the control group (grey bar). #$p<0.001$ versus the DHMBA group or the zinc sulfate alone group. Figures D and E show that to determine the levels of proteins related to cell growth, cells ($1x10^6$ cells/10 mL medium in 100 mm dishes) were cultured for 3 days in DMEM containing DHMBA (1 μM) and zinc sulfate (100 μM). After culturing, 40 micrograms of cell lysate protein per lane were separated by SDS-PAGE (12%) for Western blotting using specific antibodies. (D) Representative data are shown. (E) The band is shown as a fold of the control. Data are presented as the mean ± SD of the values obtained from four dishes with different cell preparations. *$p<0.001$ versus control (gray bar).

3.2 Combined effects of DHMBA and zinc on the death of macrophages

Next, we investigated the effects of DHMBA and zinc sulfate on the viability of macrophages *in vitro*. The cells were cultured for 3 days after reaching subconfluence. They were then cultured for an additional 24 hours in the presence of either vehicle (1% ethanol), DHMBA (0.1, 1, 10, 50, or 100 μM; see Figure 2A) or zinc sulfate (0.1, 1, 10, 50, or 100 μM; see Figure 2B). Macrophage growth was suppressed by culturing with DHMBA at concentration of 1, 10, 50, or 100 μM. Culturing with zinc sulfate (0.1, 1, 10, 50, or 100 μM) did not significantly affect cell death (see Figure 2B). Furthermore, we examined the combined effects of DHMBA (0.1 or 1 μM) and zinc (10 or 100 μM) on cell death (see Figure 3C). The effects of DHMBA (0.1 or 1 μM) on cell death were significantly increased by the addition of zinc (10 or 100 μM) (Figure 2C). Mechanistically, culturing with a combination of 1 μM DHMBA and 100 μM zinc significantly increased the expression levels of caspase-3 and cleaved caspase-3, which are associated with apoptosis. These results suggest that lower concentrations of DHMBA and zinc in combination stimulate potent macrophage death.

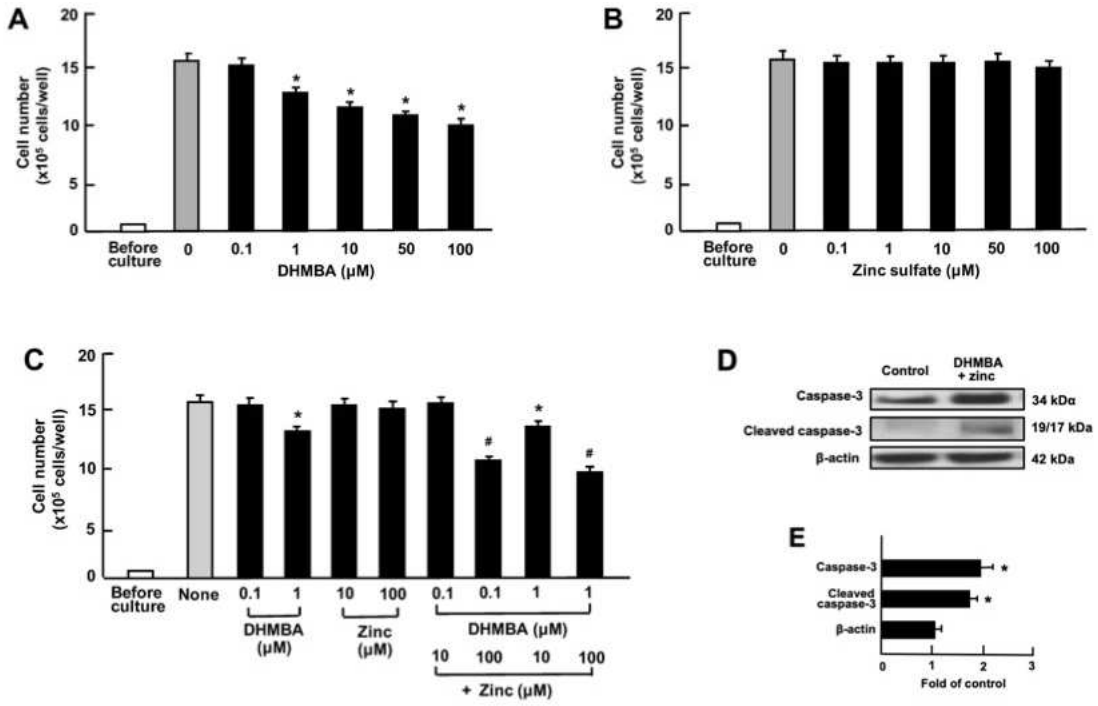

Figure 2. Effect of DHMBA and zinc sulfate on macrophage death *in vitro*. (Figures A and B): Cells ($1x10^5$ cells/mL per well) were cultured for 3 days and then cultured for an additional 24 hours with DHMBA (Figure A), or zinc (Figure B) at final concentrations of 0.1, 1, 10, 50, or 100 μM, respectively. Figure C: Cells ($1x10^5$ cells/mL per well in 24-well plates) were cultured for 3 days, and then they were cultured for an additional 24 hours either with vehicle, DHMBA (0.1 or 1 μM) and zinc sulfate (10 or 100 μM), with or without combined DHMBA (0.1 or 1 μM) or zinc sulfate (10 or 100 μM). After incubation, the number of cells attached to the dish was counted. Data are presented as the mean ± SD of 8 wells with different cell preparations. *$p<0.001$ versus the control group (gray bar). #$p<0.001$ versus the DHMBA group or the zinc sulfate alone group. To determine the levels of proteins related to cell death, cells ($1x10^6$ cells/10 mL medium in 100 mm dishes) were cultured for 3 days in DMEM, and then the cells were cultured for an additional 24 hours with DHMBA (1 μM) and zinc sulfate (100 μM). After culturing, 40 micrograms of cell lysate protein per lane were assayed for Western blotting using specific antibodies. Representative data are shown in Figure D. (Figure E) The band is shown as a fold of the control. Data are presented as the mean ± SD of the values obtained from four dishes with different cell preparations. *$p<0.001$ versus control (gray bar).

3.3 Effects of combined DHMBA and zinc on the growth and death with caspase-3 inhibitor of macrophages

Furthermore, we examined the combined effects of DHMBA and zinc on the growth and death of RAW264.7 macrophages cultured with a caspase-3 inhibitor (see Figure 3). The suppressive effects of the combined DHMBA (1 μM) and zinc (10 or 100 μM) on cell growth were not significantly altered by culturing with the caspase-3 inhibitor (10 μM) (Figure 3A). However, the stimulatory effects of the combined DHMBA (1 μM) and zinc (10 or 100 μM) on the death of macrophages were significantly blocked by culturing with a caspase-3 inhibitor (Figure 3B). These results suggest that the suppressive effects of the combined DHMBA and zinc on cell growth are exhibited via a mechanism that is independent of the enhanced death of macrophages when cultured with the combined DHMBA and zinc.

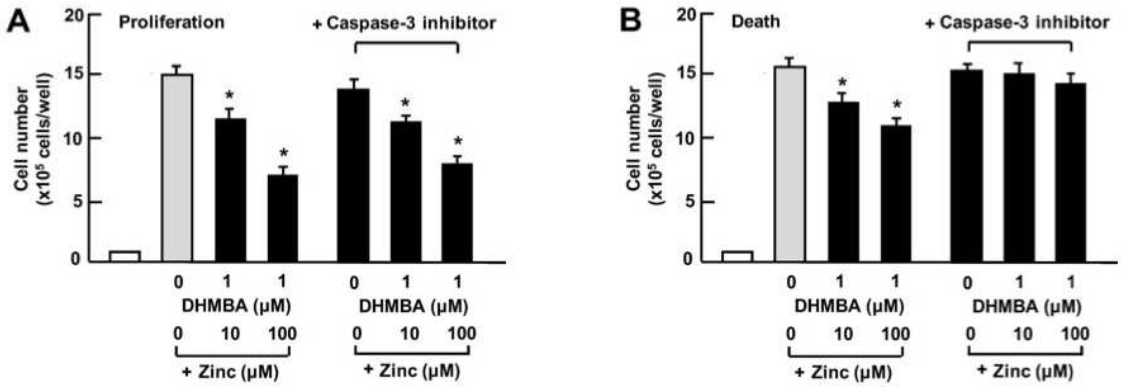

Figure 3. Effect of the combined DHMBA and zinc sulfate treatment on macrophage proliferation and death with or without a caspase-3 inhibitor *in vitro*. (Figure A): Cells ($1x10^5$ cells/mL per well) were cultured with the combined DHMBA (1 μM) and zinc sulfate (10 or 100 μM) with or without a caspase-3 inhibitor (10 μM) for 3 days. (Figure B) After reaching subconfluence after 3 days of a culturing, the cells were cultured for an additional 24 hours with the combined DHMBA (1 μM) and zinc sulfate (10 or 100 μM), with or without the caspase-3 inhibitor (10 μM). After culturing, the number of cells attached to the dish was counted. Data are presented as the mean ± SD of 8 wells with different cell preparations. *$p<0.001$ versus the control group (gray bar). #$p<0.001$ versus the DHMBA group or the zinc sulfate alone group.

3.4 Effects of the combined DHMBA and zinc on colony formation of macrophages

We demonstrated that a lower concentration of the combined DHMBA and zinc exhibited a potent suppressive effect on the proliferation of macrophages and a stimulatory effect on their death, indicating repressed cell growth. We also investigated whether this combination suppresses the colony formation of macrophages. The cells were cultured with the combined DHMBA (1 μM) or zinc sulfate (10 or 100 μM) for 9 days. Colony formation was significantly inhibited when the cells were cultured with the combined DHMBA (1 μM) and zinc sulfate (10 or 100 μM) (see Figure 4). These results further support the idea that a combination of lower concentrations of DHMBA and zinc sulfate potently suppresses the growth of macrophages.

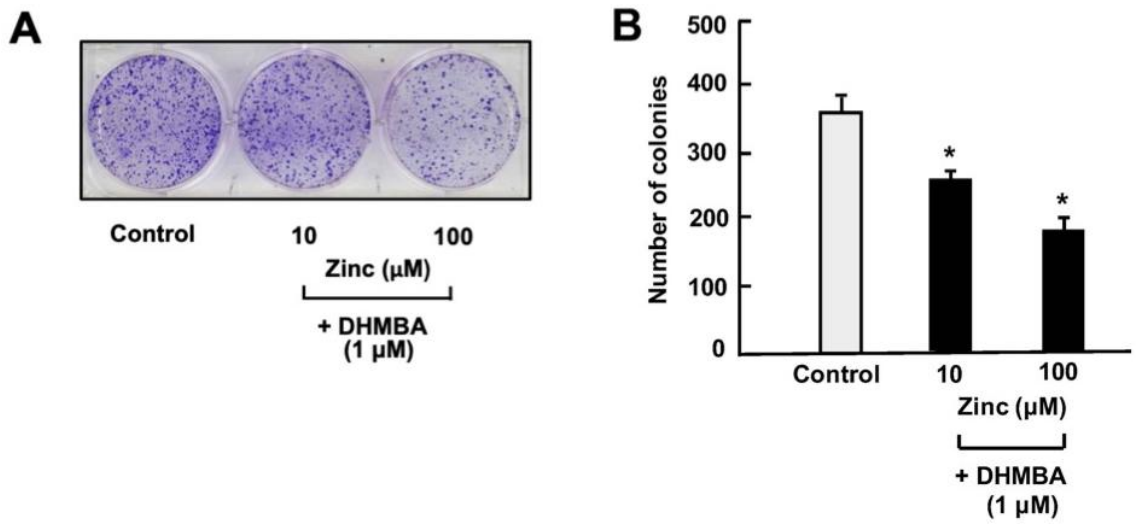

Figure 4. Effects of the combined DHMBA and zinc sulfate on the colony formation of macrophages *in vitro*. Cells ($1x10^3$ cells/2 mL per well) were cultured in DMEM containing 10% FBS either with vehicle (1% ethanol) or the combined DHMBA (1 μM) or zinc sulfate (10 or 100 μM) for 9 days. Figure A shows a photograph of the crystal violet-stained plates is shown. Figure B shows the number of colonies. Data are presented as the mean ± SD from a total of 6 wells with different cell preparations. *$p<0.001$ versus control (gray bar).

3.5 Combined effects of DHMBA and zinc on ROS production in macrophages

DHMBA is a potent antioxidant. We examined the influence of lower combined concentrations of DHMBA and zinc on ROS production in macrophages. Culturing the cells with DHMBA (0.1 μM), zinc (10 μM) or a combination of DHMBA (0.1 μM) and zinc (10 μM) for 24 hours had no effect on cell number (see Figure 5A). The cells were cultured either with vehicle, DHMBA (0.1 μM), zinc (10 μM), or DHMBA (0.1 μM) plus zinc (10 μM). The combined presence of DHMBA (0.1 μM) and zinc (10 μM) significantly blocked ROS production in macrophages. Culturing with either DHMBA (0.1 μM) or zinc (10 μM) alone did not have affect ROS production (Figure 5B). ROS production is regulated via nuclear factor kappa B (NF-κB). The expression levels of NF-κB were decreased with the combined treatment of DHMBA (0.1 μM) and zinc (10 μM) (Figures 5C and 5D). Thus, the antioxidant activity of DHMBA at lower concentrations was found to be enhanced by zinc.

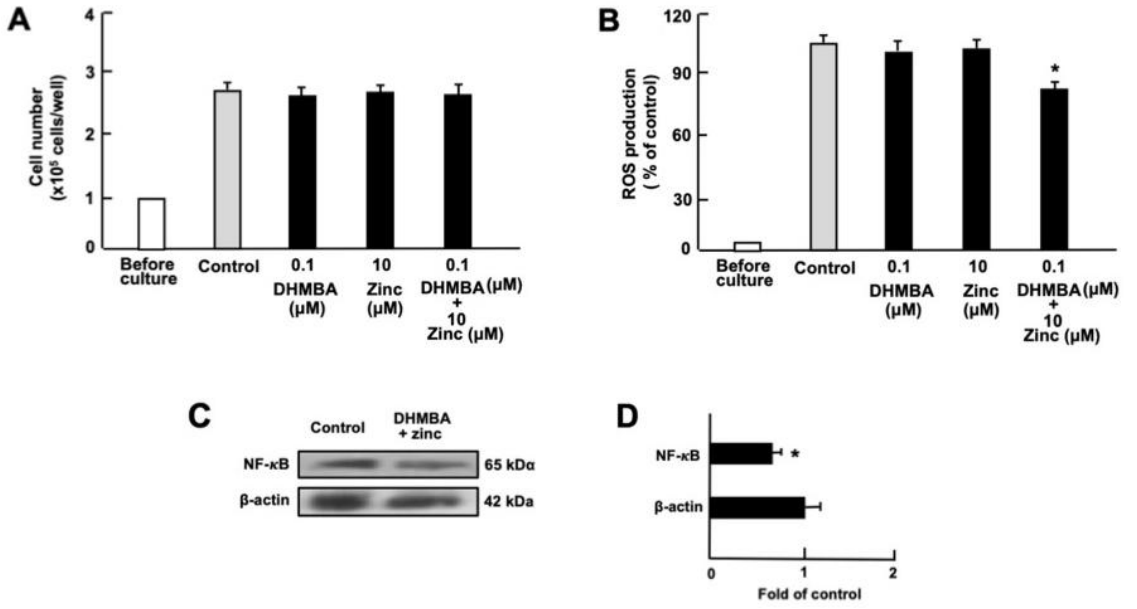

Figure 5. Culturing with DHMBA suppresses reactive oxygen species (ROS) production in macrophages. Figure 5A shows the effect of combining DHMBA (0.1 μM) and zinc

(10 μM) on cell numbers. The cells ($1x10^5$ cells/mL per well) were cultured in DMEM containing 10% FBS and 1% P/S, either with vehicle (1% ethanol), DHMBA (0.1 μM), zinc (10 μM) or a combination of DHMBA and zinc for 24 hours. Figure 5B shows the effects of DHMBA and zinc on ROS production in cells. The cells ($2.5x10^4$ cells/100 μL per well) were cultured in DMEM containing 10% FBS and 1% P/S for 24 hours, either with vehicle (1% ethanol), DHMBA (0.1 μM), zinc (10 μM), or DHMBA (0.1 μM) plus zinc (10 μM). After culturing, ROS levels were measured using an assay kit. ROS production is presented as a fold change relative to the control (%). Data are presented as the mean ± SD from a total of 8 wells with different cell preparations. *p<0.001 versus control (white bar). To determine the levels of proteins related to cell death, cells ($1x10^6$ cells/10 mL medium) were cultured for 3 days in DMEM, and then the cells were cultured for an additional 24 hours with DHMBA (1 μM) and zinc sulfate (100 μM). After culturing, cell lysate protein (40 micrograms per lane) were assayed by Western blotting (Figure C) Representative data are shown. (Figure D) The band is shown as a fold change relative to the control. Data are presented as the mean ± SD of the values obtained from four dishes with different cell preparations. *p<0.001 versus control (gray bar).

3.6 Effects of the combined DHMBA and zinc on cytokine production in macrophages

Macrophages produce inflammatory cytokines when stimulated with lipopolysaccharide (LPS) [25]. This production is related to increased levels of NF-κB expression. We then examined whether a combination of DHMBA and zinc influences cytokine production in LPS-stimulated RAW264.7 macrophages (see Figure 6). After culturing cells that had reached subconfluence for 3 days, they were cultured with DHMBA (0.1 μM), zinc (10 μM), or DHMBA (0.1 μM) plus zinc (10 μM) for 24 hours. This culturing did not affect the number of cells in the combined treatment group. Culturing with either DHMBA (0.1 μM) or zinc (10 μM) did not significantly affect TNF- α (Figure 6A) or IL-6 (Figure 6B) production in cells cultured with or without LPS (100 ng/mL). However, the combination of DHMBA (0.1 μM) and zinc (10 μM) inhibited TNF- α (Figure 6A) and IL-6 (Figure 6B)

production in cells cultured with or without LPS stimulation. These results suggest that lower concentrations of combined DHMBA and zinc suppress cytokine production by inflammatory RAW264.7 macrophages in vitro.

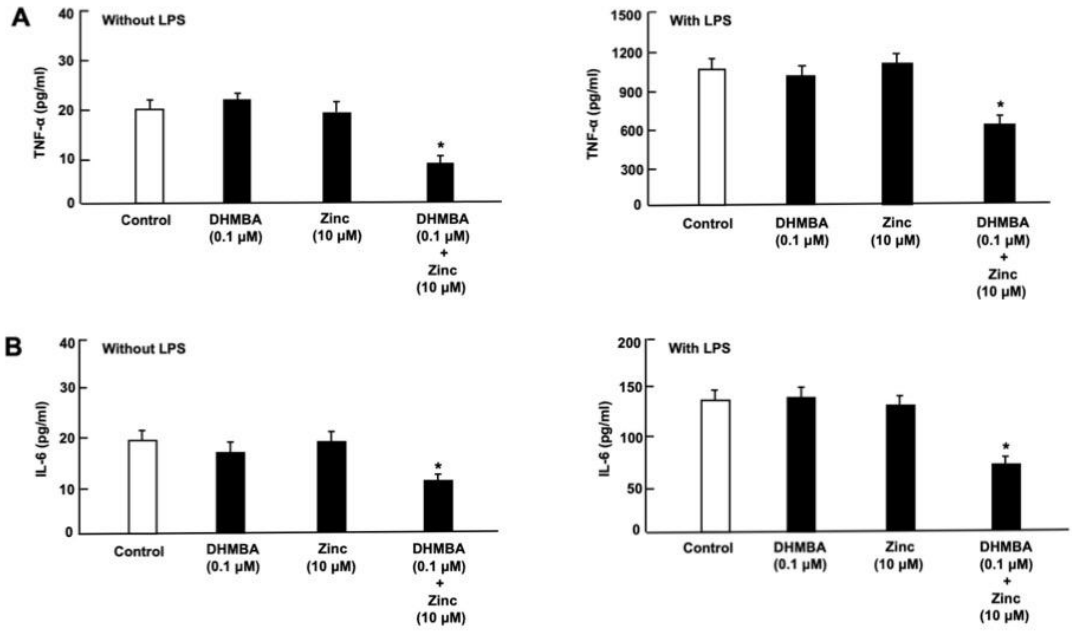

Figure 6. Effect of the combined DHMBA and zinc on the production of inflammatory mediators by macrophages with or without lipopolysaccharide (LPS). The cells ($1x10^5$/mL per well) were cultured in a 24-well plate using DMEM for 3 days on reaching subconfluence. Then, the cells were cultured for an additional 5 hours after the addition of either PBS (control), DHMBA (0.1 μM), zinc (10 μM), or a combination of DHMBA (0.1 μM) and zinc (10 μM) together, with or without LPS (100 ng/ mL). After incubation, the medium was collected for cytokine assay. The concentrations of TNF- α (Figure A) or IL-6 (Figure B) in the culture medium were determined using an ELISA kit. Data are presented as the mean ± SD of the values obtained from 8 wells in a total of 2 replicate plates by using different cell preparations. *p<0.001 versus the control group (white bar).

3.7 Combined effects of DHMBA and zinc on osteoclastogenesis in inflammatory macrophages

Macrophages are commonly used as model cells in studies of osteoclastogenesis

[24, 26]. Osteoclasts originate from the monocyte-macrophage lineage [27]. LPS has been shown to stimulate the osteoclastogenesis in RAW264.7 cells by activating the NF-κB pathway [25, 27]. Here, we investigated whether a combination of 0.1 μM DHMBA and 10 μM zinc influences their osteoclastogenesis in macrophages cultured with 100 ng/mL LPS. Culturing the cells with LPS for 3 days enhanced osteoclastogenesis of the RAW264.7 cells (Figure 7A). This enhancement was suppressed by culturing with the combination of DHMBA (0.1 μM) and zinc (10 μM); DHMBA or zinc alone had no effect (Figure 7B). These results suggest that a combination of lower concentrations of DHMBA and zinc exhibits a potent suppressive effect on LPS-stimulated osteoclastogenesis enhanced in macrophages.

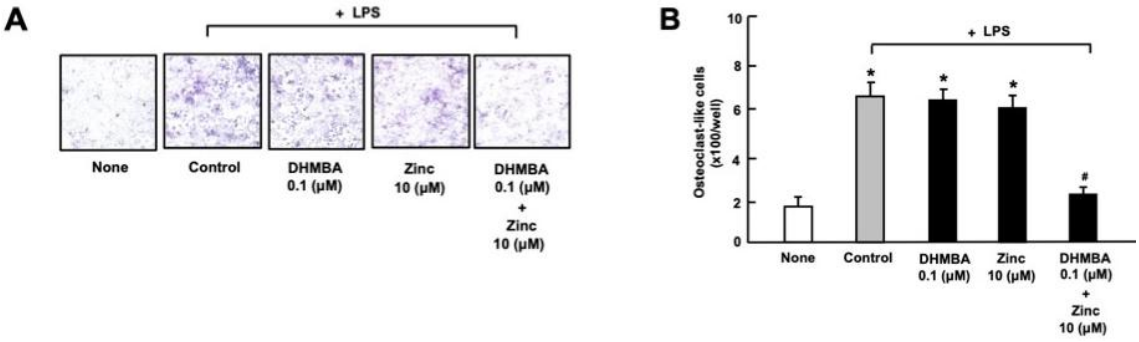

Figure 7. Effect of the combined DHMBA and zinc use on osteoclastogenesis in RAW264.7 macrophages cultured with lipopolysaccharide (LPS) *in vitro.* (Figure A) Cells (1 x 10^5 cells/1 mL per well) were cultured in DMEM either with vehicle (1% ethanol), DHMBA (0.1 μM), zinc (10 μM), or a combination of DHMBA (0.1 μM) and zinc (10 μM) with or without LPS (100 ng/mL) for 3 days. After culturing, the cells that had adherent to the plates were fixed and stained for tartrate-resistant acid phosphatase (TRACP), an osteoclastic marker enzyme. TRACP-positive, red stained cells were counted as osteoclast-like cells under a microscope (40x). Figure A shows a photomicrograph of TRACP-stained cells. Figure B shows the results, presented as the mean ± SD of the values obtained from a total of 6 wells in total with different cell preparations. Data are presented as the mean ± SD of the values obtained from 6 wells using different cell preparations. *p<0.001 versus control (white bar). $^{\#}p$<0.001 versus LPS (gray bar).

4. Discussion

Oxidative stress can lead to various diseases, such as obesity, diabetes, osteoporosis, and cancer [28-31]. Antioxidants play a crucial role in preventing and treating these diseases [13]. DHMBA, a potent antioxidant found in the Pacific oyster (*Crassostrea* gigas), is a novel compound [1-3]. Zinc is an essential trace metal and antioxidant that is plentiful in oysters and is commonly used as a dietary supplement. In the current study, we compared the effects of DHMBA and zinc on RAW264.7 macrophages *in vitro*. We found that comparatively lower concentrations of DHMBA and zinc combined to suppress inflammatory biomarkers, including cell growth, ROS production, cytokine production, and osteoclastogenesis, more effectively than either compound alone when cultured with LPS stimulation. These findings could be useful for preventing and treating inflammatory conditions.

A combination of DHMBA (0.1 or 1 μM) and zinc (10 or 100 μM) at comparatively lower concentrations was found to potently block the proliferation and stimulate the death in RAW264.7 cells. Interestingly, DHMBA at 0.1 μM, which had no effect on cell proliferation or death, exhibited synergistic effects when combined with zinc at 10 and 100 μM. Mechanistically, the combination of DHMBA and zinc decreased the expression levels of proteins that promote cell growth, such as Ras, PI3K, Akt, MAPK, and mTOR. Furthermore, the combination of DHMBA and zinc increased the expression levels of caspase-3 and cleaved caspase-3 in RAW264.7 cells. These enzymes contribute to cell death signaling pathways, leading to apoptosis. Thus, the combination of DHMBA and zinc at lower concentrations exerts a synergistic effect on macrophage growth.

Oxidative stress may be caused by excessive ROS production in cells. Antioxidant defences counteract ROS production. Culturing with lower concentrations of the combined DHMBA (0.1 μM) and zinc (10 μM), which had no significant effect, was found to inhibit ROS production in RAW264.7 macrophages. ROS production is regulated via NF-κB signaling pathways. Culturing with the combined DHMBA (0.1 μM) and zinc (10 μM) was found to decrease the expression levels of NF-κB at a mechanistic level..

Culturing with LPS increases cytokine production in RAW264.7 macrophages. NF-κB signaling plays an important mechanistic role in this process. Culturing with a combination of DHMBA (0.1 μM) and zinc (10 μM) at concentrations lower than those required for a significant effect when used alone, was found to potently inhibit TNF-α and IL-6 production in RAW264.7 macrophages. These results imply that the combination of DHMBA and zinc is effective in reducing inflammation via inflammatory cytokines.

Inflammatory conditions lead to bone loss by enhancing osteoclastogenesis [32, 33]. RAW264.7 macrophages become inflammatory when differentiated via NF-κB, which is linked to TNF-α signaling [34]. Culturing with a combination of 0.1 μM DHMBA and 10 μM zinc potently suppresses osteoclastogenesis in RAW264.7 macrophages stimulated with LPS. This suppression did not occur when culturing with DHMBA (0.1 μM) or zinc (10 μM) alone. These results suggest that a lower concentration of the DHMBA and zinc combination exerts a synergistic suppressive effect on osteoclastogenesis in inflammatory RAW 264.7 macrophages in vitro. This combination

could therefore be an effective treatment for inflammatory osteoclastic bone resorption.

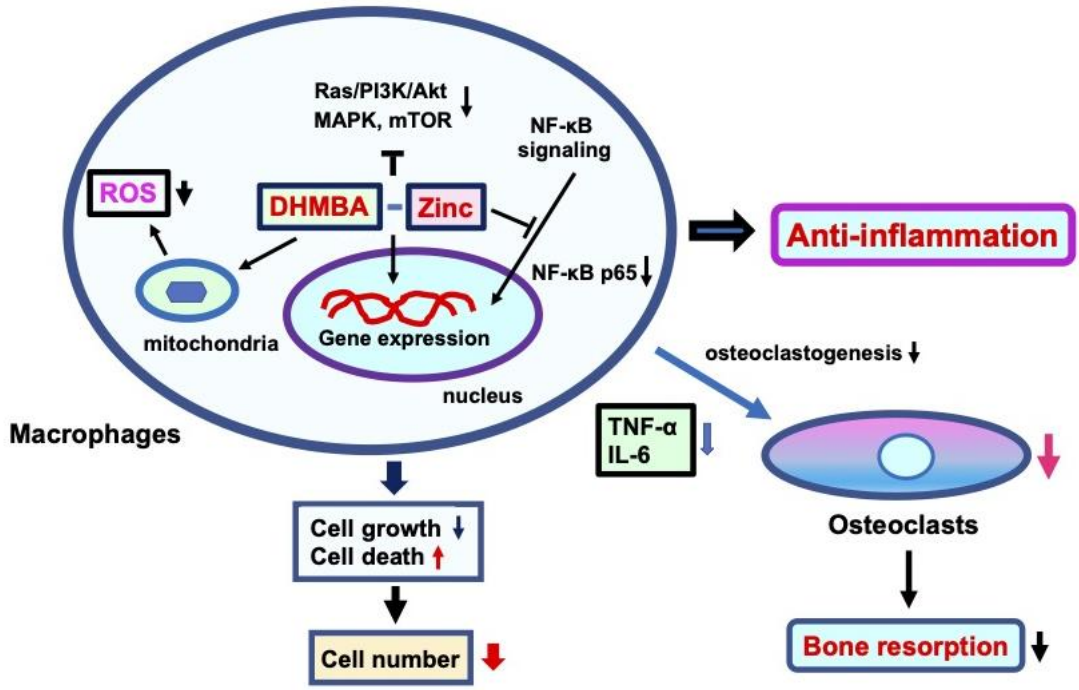

Figure 8. This is the hypothetical mechanism by which the combination of DHMBA and zinc potently suppresses the activity of inflammatory macrophages. The combination of DHMBA and zinc exhibits strong synergistic effects on these cells. The combination inhibits the growth of macrophages and stimulates their death, resulting in a fewer cells in vitro. This effect may be mediated by an underlying mechanism that regulates various proteins and signalling pathways involved in cell proliferation, as well as molecules related to programmed cell death (apoptosis). The combined treatment inhibits the production of reactive oxygene species (ROS) and inflammatory mediators (TNF-α and IL-6), which are involved in inflammatory conditions. Furthermore, it suppresses NF-κB expression levels in cells and inhibits lipopolysaccharide (LPS)-stimulated osteoclastogenesis by inducing the differentiation of macrophages from the M0 to M1 phenotype. A combination of DHMBA and zinc at lower concentrations may be useful as an anti-inflammatory agent. Abbreviations: DHMBA: 3,5-dihydroxy-4-methoxybenzyl alcohol, ROS: reactive oxygen species.

Figure 8 shows the hypothetical mechanism by which the combination of DHMBA and zinc potently suppresses the activity of inflammatory macrophages. This combination of DHMBA and zinc has a synergistic suppressive effect on various inflammatory biomarkers in RAW264.7 macrophages in vitro. This mechanism may be complex. DHMBA may protect the mitochondrial metabolic pathway involved in oxidative stress [1-3, 35]. Zinc is an antioxidant that plays a crucial role as a cofactor in biochemical processes, including superoxide dismutase and other zinc-requiring enzymes, metallothionein, and metal transcription factor-1, among others, which protect against oxidative stress. Zinc has been shown to suppress NF-κB activation by TNF-α in macrophages. It has been speculated that the combination of DHMBA and zinc influences various biochemical processes involved in oxidative stress, exhibiting potent antioxidant effects at lower levels. These effects are comprehensive. The detailed molecular mechanism remains to be elucidated. This may be important in demonstrating complementary effects.

In conclusion, we investigated the effects of the potent antioxidant DHMBA and the essential trace element zinc on inflammatory RAW264.7 macrophages *in vitro*. We found that comparatively lower concentrations of DHMBA and zinc, when used together, had a strong suppressive effect on various inflammatory biomarkers. These results suggest a new strategy for preventing and treating inflammatory conditions by utilising these antioxidants.

Authorship contribution statement

Masayoshi Yamaguchi: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, resources, software, validation, visualization, writing - original draft, writing - review & editing.

Kenji Yoshiike: Investigation, methodology, resources, software, supervision, validation, visualization, writing - review & editing.

Hideaki Watanabe: Investigation, methodology, resources, software, validation, visualization, writing - review & editing.

Mitsugu Watanabe: Fundraising, investigation, methodology, resources, validation, visualisation, writing - review & editing.

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Declaration of competing interests

All authors declare that they have no known competing financial interests or personal relationships that could be perceived as influencing the work reported in this paper.

Ethical approval and informed consent

This article does not include any human or animal studies performed by any of the authors. All experimental protocols used databases or in vitro cell culture.

Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Consent for publication

Not applicable.

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