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RESEARCH ARTICLE

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Inhibitory Effect of *Agrocybe Chaxingu (Chaxines)* on RANKL-Induced Osteoclast Differentiation in RAW246.7 Cells: Possible Prevention of Osteoporosis

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ABSTRACT

Objectives: Receptor-activator of nuclear factor-κB ligand (RANKL) is an essential stimulating factor for inducing osteoclast differentiation, feasibly resulting in osteoporosis. The bioactive extract of *Agrocybe chaxingu* mushroom, CHX, has been shown to have osteoclastic inhibitory activity. Accordingly, we investigated if CHX would inhibit or disrupt RANKL-induced osteoclast differentiation, implying possible prevention of osteoporosis.

Materials and Methods: Monocyte/macrophage RAW264.7 cells (RAW cells) were employed as our experimental model and treated with varying concentrations of RANKL alone or with CHX for 5 days. Formation of osteoclasts was then assessed using tartrate-resistant acid phosphatase (TRAP) assay, counting stained cells as osteoclasts under a light microscope. The inhibitory mechanism of CHX was also explored by examining the RANKL-mediated signaling pathways, oxidative stress (OXS), antioxidant enzymes, and apoptosis.

Results: RANKL (100 ng/mL)-induced osteoclast differentiation in RAW cells was significantly (~20%) inhibited with 10 μg/mL of CHX. This was accompanied by the down-regulation of two key signaling pathways and activation of antioxidant enzymes that likely led to the reduction in OXS. Moreover, CHX ultimately induced undifferentiated or RANKL-unresponsive cells to apoptosis, indicated by the modulation of apoptotic regulators. These findings may then account for a disruption of osteoclast differentiation with CHX.

Conclusion: CHX appears to have the inhibitory effect on RANKL-induced osteoclast differentiation in RAW cells. It is thus plausible that CHX may have potential clinical implications in osteoclast-mediated bone diseases/disorders, including osteoporosis.

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Introduction

Osteoporosis can be simply described as an *imbalance* between bone formation by osteoblasts and bone resorption (breakdown) by osteoclasts [1]. Osteoblasts are osteogenic cells located on the bone surface and mainly responsible for *forming* bones, while osteoclasts are terminally differentiated multinucleated cells derived from hematopoietic monocyte/macrophage cells responsible for *breaking down* bones [1,2]. In the *normal* bone remodeling process, osteoblasts and osteoclasts work cooperatively to constantly replace old bone with new bone [2,3]. However, in osteoporosis, this fine balance between osteoblasts and osteoclasts is broken (by any causes) and then *osteoclasts* become *more active* and *more rapidly break down bones* than forming new bones by osteoblasts [2]. For instance, such an imbalance could be likely caused by an estrogen deficiency in post-menopausal osteoporosis. Once an estrogen deficiency sets in, osteoclastic (bone) resorption becomes far more active than osteoblastic (bone) formation, thereby leading to a net loss of bone [4]. A hormone replacement therapy is currently being used for post-menopausal women but its long-term effect is not clear and some reports showed the potential risks for breast cancer and heart attack [4,5]. Because of these concerns, non-hormonal therapy or other safer options are also taken into consideration.

Nevertheless, it is theoretically possible that osteoporosis could be prevented by reducing the osteoclast formation through receptor-activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation of monocyte/macrophage – *no (excessive) osteoclasts are formed, no osteoporosis develops*. It is known that the osteoclastogenic process starts with "precursor" cell, i.e. monocyte/macrophage, which is terminally differentiated to (mature) osteoclast through RANKL-induced signaling pathway [6]. Hence, if this signaling pathway was specifically disrupted/blocked, the RANKL-initiated sequential signals will not reach at the nucleus for activation/initiation of osteoclast differentiation (osteoclastogenesis). Although *this* is certainly an oversimplified scheme, it is yet a logical approach to inhibit osteoclast formation by targeting such RANKL-induced differentiation.

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We currently have several therapeutic options for osteoporosis [4], but the *safer* and *more effective* modalities need to be promptly established. We then came across an edible *Agrocybe chaxingu* mushroom with abundant nutritional components, including amino acids, vitamins, minerals etc. [7,8]. Its bioactive extract, CHX [9], has been also isolated and a number of studies revealed its medicinal/pharmacological properties, such as osteoclastic inhibitory activity as well as antitumor, antioxidant, hypoglycemic, Cox-1/2 inhibitory activities etc. [10-14]. Particularly, its *osteoclastic inhibitory activity* [10] could be rather significant to inhibit/disrupt RANKL-induced osteoclast differentiation, preventing excessive bone resorption and subsequently the onset of osteoporosis.

Accordingly, we investigated if CHX might have inhibitory activity against RANKL-induced osteoclast differentiation. We employed monocyte/macrophage RAW264.7 cells (RAW cells) as an excellent experimental model in this study because they express endogenous RANK (a receptor for RANKL) and can be differentiated to osteoclasts with RANKL stimulation [6,15]. Our main objective was to address if CHX would inhibit/disrupt RANKL-induced osteoclast differentiation in RAW cells and also explore its possible inhibitory mechanism, focusing on the RANKL-mediated signaling pathways, antioxidant enzymes, oxidative stress (OXS), and apoptosis. More details are described and the interesting findings are also discussed herein.

Materials and Methods

Cell culture

Macrophage/monocyte RAW264.7 cells (RAW cells) were purchased from the American Type Culture Collection (ATCC; Manassas, VA). They were cultured in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) in an incubator. For experiments, RAW cells were treated with RANKL alone or with CHX for 5 days since they have been shown to be usually differentiated to osteoclasts with RANKL in 3-6 days [6]. Cells were subjected to MTT assay for determining cell viability and tartrate-resistant acid phosphatase (TRAP) assay for assessing osteoclastic phenotype/formation as described below.

MTT (cell viability) assay

RAW cells seeded in the 6-well plate were treated with varying concentrations of RANKL (0-100 ng/mL) alone or with CHX (0-10 µg/mL) for 5 days. Cell viability was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay following the vendor's protocol (Sigma-Aldrich, St. Louis, MO). One mL of MTT reagent (1 mg/mL) was added to each well in the plate that was then incubated for 3 h in an incubator. After discarding MTT, dimethyl sulfoxide (DMSO) was added to the plate and absorbance of samples was read in a microplate reader. Cell viability was expressed by the % of sample readings (OD) relative to the control reading (100%) as follows: Cell viability (%) = [OD of sample/OD of control] x 100.

Tartrate-resistant acid phosphatase (TRAP) assay

How many osteoclasts were actually formed from RAW cells with RANKL alone or RANKL+CHX was assessed using the TRAP assay kit (Kamiya Biomedical Co., Seattle, WA). The procedures essentially followed the manufacturer's protocol. Briefly, cells treated with RANKL alone or with CHX for 5 days in the 6-well plate were fixed with 10% formalin neutral buffer for 5 min and washed thoroughly with water. Chromogenic substrate dissolved in tartrate-containing buffer was added to the plate, which was then incubated at 37 °C for 1 h. TRAP-stained (dark-red) multinucleated (≥3 nuclei) cells were scored as osteoclasts, which were counted under a light microscope, and expressed by the number of osteoclasts/well.

Lipid peroxidation (LPO) assay

To assess the severity of OXS during RANKL-induced osteoclast differentiation, cells treated with RANKL or with RANKL+CHX for 6 h were all subjected to LPO assay. It has been shown that OXS usually took place at the early phase and a 6-h exposure was sufficient to properly monitor the severity of OXS, which was determined by measuring the amount of malondialdehyde (MDA) formed as a by-product of OXS [16]. A rule of thumb is that "the *greater* MDA formed, the *greater* OXS". Following the vendor's protocol (ABCAM, Cambridge, MA), the amounts of MDA formed in those cells were measured by µM in reference to the MDA standard and converted to arbitrary values relative to controls (1.0).

Enzymatic assay for caspase-7 and caspase-9

Caspase-7 (Csp-7) and caspase-9 (Csp-9) colorimetric assay kits were purchased from Boster Bio (Pleasanton, CA) and BioVision (Waltham, MA), respectively. Enzymatic activities were quantified following each manufacturer's protocol that was principally similar. Briefly, an equal amount (100 µg) of cell lysates was incubated with the reaction buffer containing the substrates, Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp-p-nitroaniline) for Csp-7 or Ac-LEHD-pNA (acetyl-Leu-Glu-His-Asp-p-nitroaniline) for Csp-9, at 37 °C for 2 h. Absorbance of the reaction mixture was then determined at 405 nm using a microplate reader. Activities of Csp-7 and -9 were calculated and expressed by the fold-increase in treated samples relative to controls (1.0).

Western blot analysis

How RANKL or RANKL+CHX would modulate RANKL-mediated signaling pathways, antioxidant enzymes, and apoptosis was examined using Western blots. Briefly, an equal amount of cell lysates (10 µg) from control and treated cells was resolved by 10% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and transferred to a nitrocellulose membrane (blot), which was blocked with 3% non-fat milk overnight. The blot was first incubated for 90 min with the primary antibodies against the two signaling pathways, nuclear factor-κB (p-NF-κB) and mitogen-activated protein kinases (MAPK), including nuclear factor of activated T-cells cytoplasmic 1 (NFATc1), c-fos, cathepsin K (Cat.K), tartrate-resistant acid phosphatase (Trap), p-NF-κB, and MAPK (p-p38), two antioxidant enzymes, heme oxygenase-1 (HO-1) and NADPH:quinone reductase (NQO-1), or two apoptotic regulators, bcl-2 and Bax (Santa Cruz Biotechnology, Santa Cruz, CA). After discarding those primary antibodies, the blot was extensively washed and incubated with the appropriate secondary antibody conjugates

(Santa Cruz Biotechnology) for 30 min. Once antibodies were discarded, those specific immunoreactive proteins described above were detected by chemiluminescence (Kirkegaard and Perry Laboratories, Gaithersburg, MD) on an X-ray film (autoradiography).

Statistical analysis

All data are presented as the mean ± SD (standard deviation), and statistical differences between groups are assessed with either one-way analysis of variance (ANOVA) or the unpaired Student's t test. Values of $p < 0.05$ are considered to indicate statistical significance.

Results

Effects of RANKL or CHX on cell viability in RAW cells

We first examined if RANKL or CHX would be cytotoxic or have any adverse effects on RAW cells. As cell viability determines the number or percent (%) of viable cells following RANKL or CHX treatment, the reduced cell viability (number) would indicate their possible cytotoxic effects on cells. RAW cells were treated with varying concentrations of RANKL (0-100 ng/mL) or CHX (0-10 µg/mL) for 5 days and cell viability was determined by MTT assay. The results showed that RANKL or CHX at any given concentrations had little effects on cell viability (Figure 1A and B), suggesting that they are non-cytotoxic to cells.

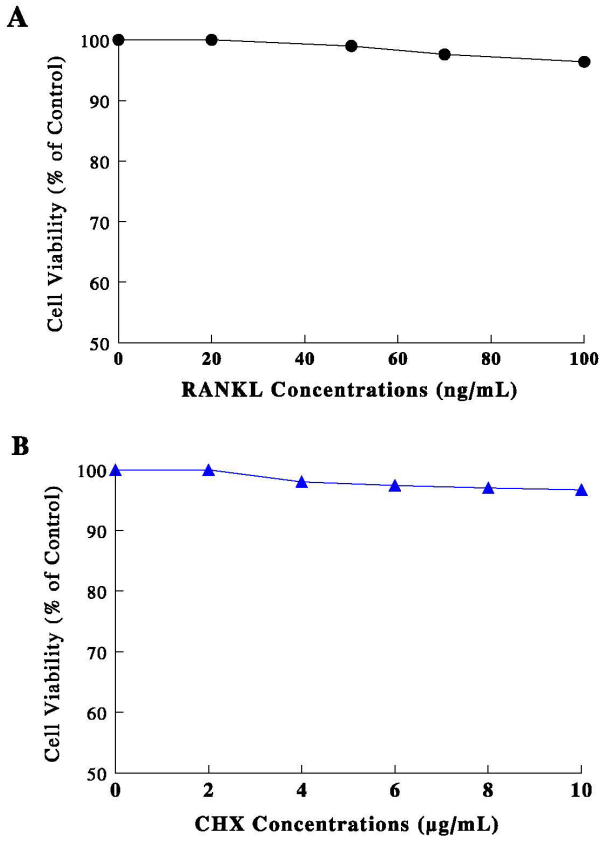

Figure 1: Effects of RANKL or CHX on RAW cell viability. RAW cells were treated with varying concentrations of RANKL (0-100 ng/mL) (**A**) or CHX (0-10 µg/mL) (**B**) for 5 days and cell viability was determined by MTT assay. Cell viability was then expressed by the % of viable cell numbers in agent-treated cells relative to that in control cells (100%). All data represent mean ± SD (standard deviation) from three independent experiments.

Induction of RAW cell differentiation to osteoclasts

We next examined if RAW cells would be differentiated to osteoclasts by RANKL, as it has been reported elsewhere [6]. To obtain a maximal effect of RANKL on RAW cells, the highest concentration (100 ng/mL) of RANKL was tested on cells for various days (0-5 days), and the number of osteoclasts formed was quantitated by TRAP assay [17]. As shown in Figure 2, no osteoclast differentiation was seen up to 2 days but osteoclasts then became apparent in 3-5 days. These results thus demonstrate that RANKL (100 ng/mL) can indeed induce differentiation of RAW cells to osteoclasts at a peak of 5 days.

Inhibitory effect of CHX on RANKL-induced osteoclast differentiation

Now, whether CHX would disrupt/inhibit RANKL-induced osteoclast differentiation in RAW cells was examined next. Cells were treated with 100 ng/mL of RANKL each with 5 or 10 µg/mL of CHX for 5 days and subjected to TRAP assay. As shown in Figure 3, the number of osteoclasts induced by RANKL (100 ng/mL) was marginally (~8%) down with CHX (5 µg/mL) and significantly (~20%) reduced with CHX (10 µg/mL). Statistical analysis confirmed that the reduction in RANKL-induced differentiation with CHX (10 µg/mL) was statistically significant ($p < 0.05$). Thus, these specific concentrations of RANKL (100 ng/mL) and CHX (10 µg/mL) were used in the rest of our study.

Inhibition of RANKL-mediated NF-κB and MAPK signaling pathways with CHX

To explore the inhibitory mechanism of CHX on RANKL-induced osteoclast differentiation, its effects on the two signaling pathways, NF-κB and MAPK [18-20], were examined after RAW cells were treated with RANKL alone or with CHX for 5 days. Western blot analysis (Figure 4) revealed that the expressions of six selected regulators, NFATc1, c-fos, Cat.K, Trap, p-NF-κB, and p-p38, were all significantly up-regulated or activated with RANKL alone (compared to controls), suggesting a steady progress in osteoclast differentiation [18-20]. However, such a progress was disrupted/inhibited with CHX, indicated by the down-regulated or reduced expressions of all these regulators. Therefore, these results suggest that a disruption or blocking of the two RANKL-mediated signaling pathways with CHX may primarily account for the inhibition of RANKL-induced osteoclast differentiation/formation.

Reduction in OXS and activation of antioxidant enzymes with CHX

As RANKL has been shown to induce OXS to facilitate osteoclast differentiation [18,21], this possibility was then tested. The severity of OXS induced by RANKL was assessed by lipid peroxidation (LPO) assay [16], measuring the amounts of malondialdehyde (MDA) formed through OXS – "*The more MDA formed, the greater OXS*". It is also known that OXS usually takes place at the early phase and a 6-h exposure/treatment should be sufficient to monitor the status of OXS. RAW cells exposed to RANKL alone or with CHX for 6 h were subjected to LPO assay. The amount of MDA formed with RANKL alone was a ~1.7-fold higher than that in control; however, such an elevated MDA level was reduced by ~30% with CHX (Figure 5A). These results thus suggest that RANKL alone can induce severe

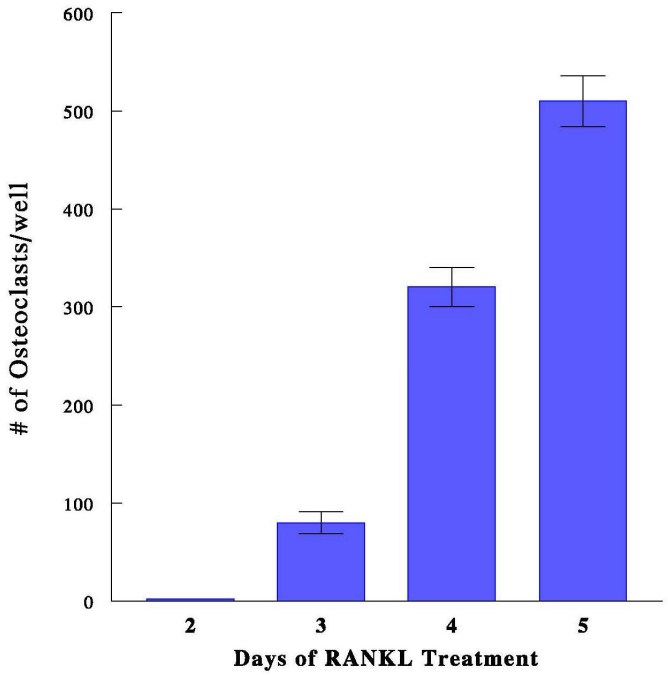

Figure 2: RANKL-induced osteoclast differentiation. RAW cells were treated with 100 ng/mL of RANKL for various time points (0-5 days), and the number of osteoclasts differentiated/formed was determined by TRAP assay. The data are mean ± SD from three separate experiments.

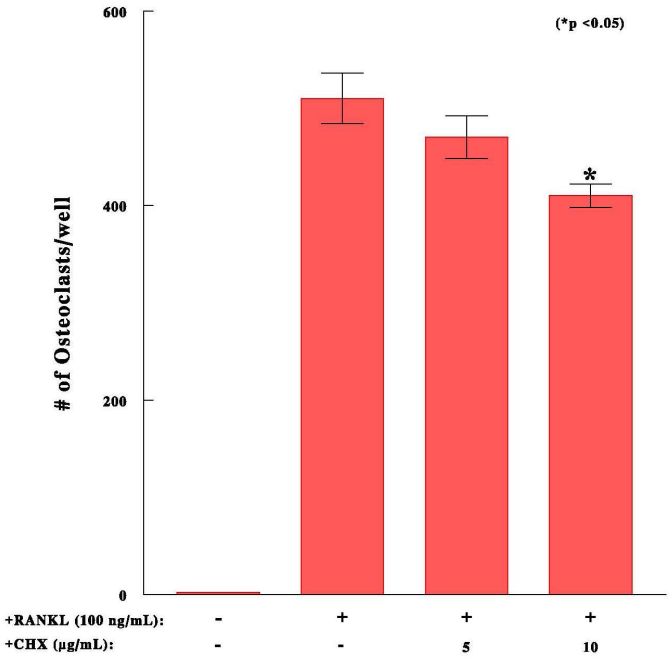

Figure 3: Inhibitory effects of CHX on RANKL-induced osteoclast differentiation. RAW cells were treated with RANKL (100 ng/mL) alone or in the presence of CHX (5 or 10 μg/mL) for 5 days, and the number of osteoclasts formed was determined by TRAP assay (*p < 0.05 compared to 100 ng/mL RANKL-treated cells).

OXS on RAW cells, which could be yet significantly reduced with CHX.

To understand how CHX reduces OXS, we then examined activities of two antioxidant enzymes, heme oxygenase-1 (HO-1) and NADPH: quinone reductase (NQO-1), which were known to immediately respond to OXS [22,23]. RAW cells treated with RANKL alone or with CHX for 5 days were subjected to Western blots. Such analysis revealed that both HO-1 and NQO-1 expressions were significantly enhanced with RANKL+CHX (compared to control) (Figure 5B), indicating their activation with CHX. However, no such activation was seen with RANKL (compared to control). Thus, activation of these antioxidant enzymes with CHX may then abolish/reduce OXS, partially disrupting the RANKL-induced osteoclastogenic process.

Induction of apoptosis with CHX

Lastly, it was important to address what the fate of RAW cells following the treatment of RANKL alone or RANKL+CHX would be. Strictly speaking, not all cells would be differentiated to osteoclasts and the question is "what" may happen to the rest of "undifferentiated" or "RANKL-unresponsive" cells – whether they would undergo apoptosis (programmed cell death) [24]. RAW cells were treated with RANKL alone or RANKL+CHX for 5 days and analyzed for two apoptotic regulators, bcl-2 and Bax, using Western blots. We found that bcl-2 expression in RANKL-treated cells was apparently *lesser* than that in control, but it was significantly down-regulated or reduced with RANKL+CHX (Figure 6). In contrast, Bax expression was apparently *increased* in RANKL-treated cells and significantly up-regulated or enhanced with RANKL+CHX (Figure 6). Since this pattern of *reduced* bcl-2 but *increased* Bax expressions indicates induction of apoptosis because bcl-2 is an anti-apoptotic regulator while Bax is a pro-apoptotic regulator [25].

In addition, we also performed enzymatic assay to quantify a possible difference in apoptosis as Western blots are qualitative analysis. Cells treated with RANKL or RANKL+CHX for 5 days

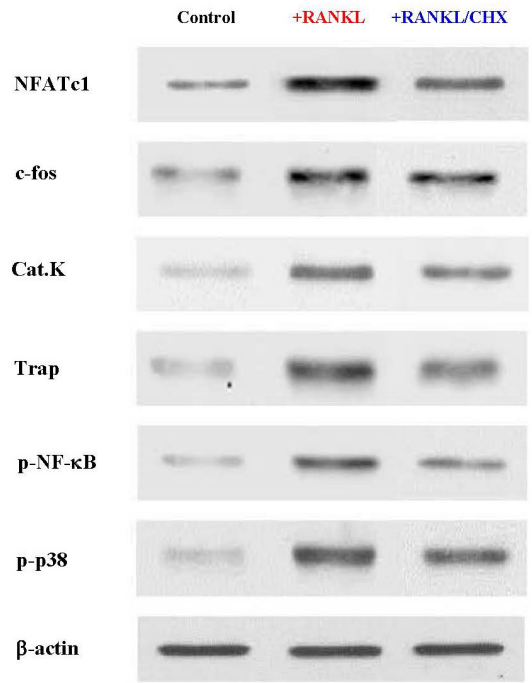

Figure 4: Effects of CHX on six regulators/factors involved in NF-κB and MAPK signaling pathways. Cells treated with RANKL (100 ng/mL) alone or with CHX (10 μg/mL) for 5 days were subjected to Western blot analysis. Autoradiographs of NFATc1, c-fos, Cat.K, Trap, p-NF-κB, and p-p38 in control, +RANKL-, or +RANKL/CHX-treated cells are shown for comparison. Additionally, β-actin is also shown as an internal loading control.

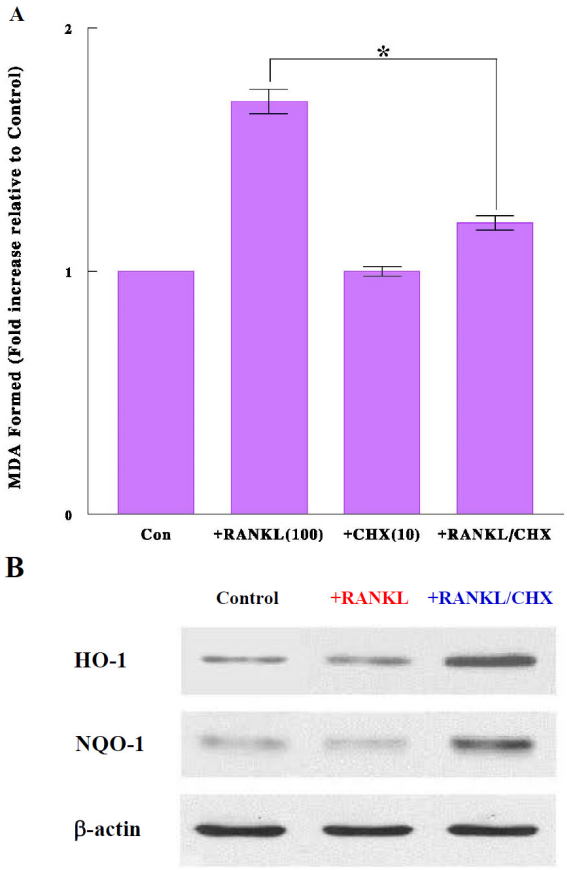

Figure 5: A) Effect of CHX on RANKL-induced OXS. Cells were exposed to RANKL alone, CHX alone or RANKL/CHX for 6 h and subjected to LPO assay. MDA formed in each experimental condition was calculated and expressed by the fold-increase relative to control (1). All data are mean ± SD from three independent experiments (*p < 0.05 compared to RANKL-treated cells). (**B**) Activation of antioxidant enzymes (HO-1 and NQO-1) with CHX. Cells treated with RANKL alone or RANKL/CHX for 5 days were analyzed for antioxidant enzymes using Western blots. Autoradiographs of HO-1 and NQO-1 in control, +RANKL-, or +RANKL/CHX-treated cells are shown for comparison. Beta (β)-actin is also shown as a loading control.

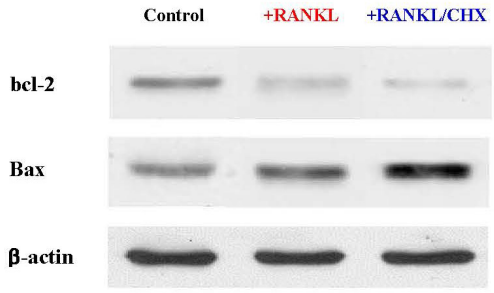

Figure 6: Induction of apoptosis with CHX. Cells treated with RANKL alone or RANKL/CHX for 5 days were analyzed for two apoptotic regulators, bcl-2 and Bax, using Western blots. Autoradiographs of bcl-2 and Bax in control, +RANKL-, or +RANKL/CHX-treated cells are shown for comparison. Beta (β)-actin is also shown as a loading control.

Table 1: Activation of Caspase-7 (Csp-7) and Caspase-9 (Csp-9).

	Control	+RANKL	+RANKL/CHX
Caspase-7	1.0	1.7	2.5*
Caspase-9	1.0	1.5	2.8*

Enzymatic activities for caspase-7 and caspase-9 were calculated and expressed by a fold-increase relative to control cells (1.0). Activation of both caspases with +RANKL/CHX indicates induction of apoptosis (*P < 0.05 compared to control).

were assayed for activities of two apoptotic caspases, caspase-7 (Csp-7) and caspase-9 (Csp-9) [26], using enzymatic assays as described in Methods. Table 1 shows that compared to control cells, activities of both Csp-7 and Csp-9 were ~1.6- and ~2.6-fold higher in RANKL-treated and RANKL+CHX-treated cells, respectively. Such statistically significant ($p < 0.05$) *activation* of these caspases is indicative of cells undergoing apoptosis [26]. On the other hand, although no significant *statistical* difference was seen in their activities between control and RANKL-treated cells ($p = 0.06$), those *values* are yet apparently *higher* in RANKL-treated cells (than those in control), implying induction of apoptosis to a lesser extent. Therefore, these results suggest that undifferentiated cells (with either RANKL or RANKL+CHX) are destined for apoptosis, although significantly more cells appear to undergo apoptosis in RANKL+CHX-treated cells than those in RANKL-treated.

Discussion

To find a safer and effective modality for preventing osteoporosis, we studied an interesting mushroom extract, CHX, to address if it might disrupt or inhibit RANKL-induced osteoclast differentiation, which is known to play an essential role in osteoporosis [2,3]. This osteoclast differentiation has been extensively studied and well elucidated. As osteoclasts are responsible for a breakdown (resorption) of bone, once a balance between bone formation (by osteoblasts) and bone resorption (by osteoclasts) was broken, due to an excessive amount of osteoclasts being formed, it tilts towards bone resorption, feasibly leading to those osteoclast-mediated bone diseases/disorders, including osteoporosis, rheumatoid arthritis, osteoarthrosis, Paget's disease etc. [1,27]. Hence, it is plausible that the disruption or inhibition of RANKL-induced osteoclast differentiation would significantly reduce the number of osteoclasts formed, presumably preventing the development of those potential diseases.

In the meantime, we have been working on a variety of natural products from fruits, flowers, vegetables, mushrooms, seeds etc. [28-32] and recently came across the bioactive mushroom extract, CHX, which was reported to have osteoclastic inhibitory activity [10]. As we believe that natural products are generally safe and have few side effects, we investigated if CHX would disrupt/inhibit RANKL-induced osteoclast differentiation in RAW cells. Our study indeed showed that RANKL (100 ng/mL)-induced differentiation was significantly inhibited with CHX (10 µg/mL) (Figure 3).

To explore the possible inhibitory mechanism of CHX on RANKL-induced osteoclast differentiation, we first examined if any regulators involved in the two signaling pathways, NF-κB and MAPK [18-20], would be modulated with CHX. Such studies showed that six regulators tested, NFATc1, c-fos, Cat.K, Trap, p-NF-κB, and p-p38, were all up-regulated with RANKL but also significantly *down*-regulated with CHX, indicating that CHX may disrupt/inhibit these two pathways mediated by RANKL. This finding is significant and may primarily account for the inhibition of osteoclast differentiation with CHX because these pathways are essential for successfully carrying out osteoclast differentiation [18-20].

It was true that RANKL was capable of inducing severe OXS during the differentiation processes as reported [21,22]. However, such RANKL-induced OXS was significantly (~30%) reduced with CHX, subsequently disrupting the course of osteoclast differentiation, because OXS is required for its successful completion [22]. This OXS reduction then seems to be attributed to activation of antioxidant enzymes (HO-1 and NQO-1) with CHX. In general, these enzymes would be inactivated by RANKL stimulation (to sustain a high OXS level) [22] but their minor activation with RANKL has been also reported [18] as seen in this study (Figure 5B). Hence, CHX exhibited significant activation of HO-1 and NQO-1, leading to the OXS reduction. This may also account for how RANKL-induced osteoclast differentiation was inhibited.

To address if CHX may have potential clinical implications, we examined if RAW cells would undergo apoptosis. It is true that not all cells treated with RANKL would become osteoclasts [33] but peculiar to see what happens to the rest of undifferentiated or RANKL-unresponsive cells. Our study showed that those undifferentiated cells did undergo apoptosis, indicated by the results of two different analyses performed. One method was Western blot analysis, i.e. *qualitative* analysis, which revealed the down-regulation of bcl-2 (anti-apoptotic) with the up-regulation of Bax (pro-apoptotic) in RANKL+CHX-treated cells, indicating induction of apoptosis [25]. Some minor changes in bcl-2 and Bax were also seen in RANKL-treated cells, but how far apoptosis was expanded is uncertain (due to its qualitative analysis) and it is fair to assume that *limited* apoptosis was yet taking place there. The other method was enzymatic assay, i.e. *quantitative* assay, which showed significant ($p < 0.05$) activation of Csp-7 and Csp-9 with RANKL+CHX (compared to control), indicating induction of apoptosis [26]. Such activation to a lesser extent (a ~1.6-fold increase yet) was also seen with RANKL, although no statistical difference ($p = 0.06$) was found. This yet implies that a minor apoptotic event might have occurred in those undifferentiated cells. It should be also noted that differentiated cells or osteoclasts induced by RANKL have been reported to undergo apoptosis shortly after they were made [27,33]. Although the specific signaling pathways (Fas/FasL and TRAIL/DR4) is believed to be primarily involved in such osteoclast apoptosis [27], the exact mechanism has not been fully elucidated. Hence, it cannot rule out the possibility that a few or some osteoclasts could have also undergone apoptosis in our study, although the overall results would not be affected but remain the same. Taken together, these results suggest that CHX appears to induce undifferentiated cells (and osteoclasts) to apoptosis while RANKL itself may induce them to *limited* apoptosis, although the number of cells induced to apoptosis would be certainly higher in undifferentiated cells than in osteoclasts.

Now, a significance of apoptosis has been often implied in clinical utility. Briefly, apoptosis is also considered as "cell suicide", as opposed to necrosis or "cell murder" caused typically by chemotherapy. It is a highly organized biochemical death process *without* rupturing cells to release toxic materials that can cause secondary injury/inflammation to the surrounding cells/tissues [34], manifesting "side effects". Hence, only cells determined to commit suicide would quickly and quietly die out without creating any side effects during apoptosis. It is then considered that any drugs, chemicals, biologicals etc. ultimately inducing *apoptosis* are generally safe with few side effects and more suitable to patients who need medications. Therefore, CHX capable of inducing apoptosis could be used in patients without side effects, implying its potential clinical utility.

Conclusion

In the present study, RANKL (100 ng/mL)-induced osteoclast differentiation was significantly inhibited with CHX (10 μg/mL) in 5 days. This was also accompanied by a disruption of the two RANKL-mediated signaling pathways, NF-κB and MAPK, primarily accounting for the reduction in osteoclast formation. Additionally, RANKL-induced OXS was significantly reduced by antioxidant enzymes (HO-1 and NQO-1) activated with CHX. This may further account for the inhibitory mechanism of CHX against osteoclast differentiation. Moreover, CHX appears to ultimately induce apoptosis mostly in undifferentiated or RANKL-unresponsive cells. Thus, it is conceivable that CHX may have potential clinical implications for preventing osteoporosis, other osteoclast-mediated bone diseases/disorders, or simply maintaining normal bone homeostasis. Further studies are indeed warranted.

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