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Type of Cell Death Induced by Seven Metals in Cultured Mouse Osteoblastic Cells

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Abstract. *The use of dental metal alloys in the daily clinic makes it necessary to evaluate the cytotoxicity of eluted metal components against oral cells. However, the cytotoxic mechanism and the type of cell death induced by dental metals in osteoblasts have not been well characterized. This study investigated the cytotoxicity of seven metals against the mouse osteoblastic cell line MC3T3-E1. α-MEM was used as a culture medium, since this medium provided much superior proliferation of MC3T3-E1 cells over DMEM. Ag $(NH_3)_2F$ was the most cytotoxic, followed by CuCl>$CuCl_2$ >$CoCl_2$, $NiCl_2$>$FeCl_3$ and $FeCl_2$ (least toxic). None of the metals showed any apparent growth stimulating effect (so-called 'hormesis') at lower concentrations. A time course study demonstrated that two hours of contact between oral cells and Ag $(NH_3)_2F$, CuCl, $CoCl_2$ or $NiCl_2$ induced irreversible cell death. Contact with these metals induced a smear pattern of DNA fragmentation without activation of caspase-3. Preincubation of MC3T3-E1 cells with either a caspase inhibitor (Z-VAD-FMK) or autophagy inhibitors (3-methyladenine, bafilomycin) failed to rescue them from metal cytotoxicity. These data suggest the induction of necrotic cell death rather than apoptosis and autophagy by metals in this osteoblastic cell line.*

The inability of most human tissues and organs to regenerate after damage has been a profound frustration throughout the history to physicians, dentists and patients. Biocompatible metal-containing materials have provided options in many cases, but the reaction of the body to these materials is far from perfect (1, 2). Metal alloys have been used as restorative materials in dentistry. Gold (Au), silver (Ag) and palladium (Pd) are important components of alloys, causing infrequent allergic reactions in the oral cavity (2). An assay system has been recently established for the investigation of the interaction between metal and cultured cells (3). Using this technique, it has been recently reported that contact with a hydroxyapatite-coated titanium plate significantly enhanced the proliferation of mouse osteoblastic MC3T3-E1 cells, based on the increased cell number and the enhanced consumption of amino acids in the culture medium (4). Titanium surface roughness may affect gene expression (5) and osteoclastic differentiation (6). In contrast, contact with copper (Cu) plate induced rapid, non-apoptotic cell death characterised by a smear pattern of DNA fragmentation, minor caspase activation, loss of membrane barrier, cytoplasmic injury prior to the nuclear damage and vacuolisation, without the loss of cell surface microvilli in human promyelocytic leukemic cell line HL-60 (3). Contact with Cu plate similarly induced cytotoxicity and cysteine oxidation in human gingival fibroblasts, and the inclusion of Au at more than 10% in the alloy completely eliminated both the Cu-induced cytotoxicity and oxidation (Yamazaki, unpublished data). At present, it is not yet clear whether the cytotoxicity induced by a Cu plate is caused by the Cu ions released from the plate or direct contact with the plate surface. Furthermore, no detailed study of the comparison of cytotoxicity and the type of cell death induced by dental metals in osteoblasts has been reported. Therefore this study investigated the relative cytotoxicity of seven metals against MC3T3-E1 cells, and the type of cell death, by monitoring the expression of various apoptosis markers and the effect of inhibitors for apoptosis and autophagy.

It has been reported that many toxic substances such as metals, radiation and chemotherapeutic agents induce growth

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Key Words: Metals, cytotoxicity, osteoblastic cells, apoptosis, autophagy, necrosis.

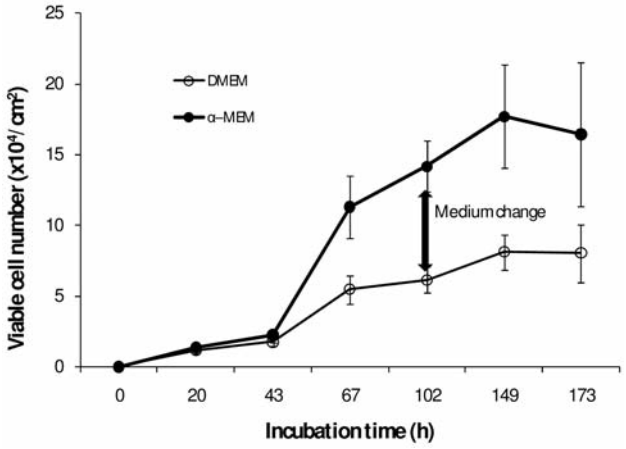

Figure 1. *Effect of two different media on the growth of MC3T3-E1 cells. MC3T3-E1 cells were incubated for different periods of times in either α-MEM or DMEM, and at the time indicated by the arrow, the medium was changed to a fresh one to maintain logarithmic growth. Each value represents the mean±S.D. of triplicate assays.*

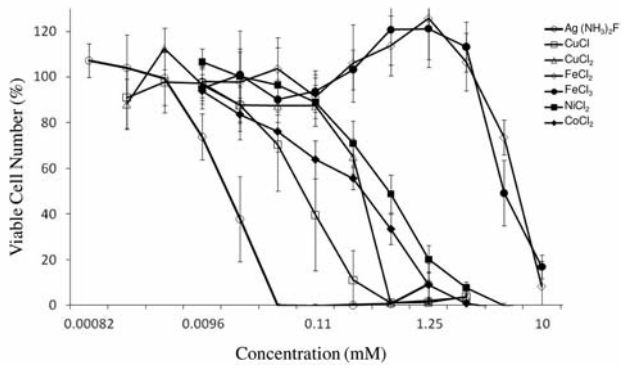

Figure 2. *Cytotoxicity of seven metals against MC3T3-E1 cells. MC3T3-E1 cells were incubated for 24 hours with the indicated concentrations of metal and then the relative viable cell number was determined by MTT method. Each value represents the mean±S.D. of triplicate assays. Reproducible results were obtained in further two independent experiments.*

stimulation at lower concentrations (so-called 'hormesis') (7). Therefore, this study also investigated whether dental metals induce such hormetic effect in MC3T3-E1 cells.

Materials and Methods

Materials. The following chemicals and reagents were used in the study: alpha minimum essential medium (α-MEM), Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); CuCl, $CuCl_2 \cdot 2H_2O$, $FeCl_2 \cdot 5H_2O$, $FeCl_3 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, bafilomycin (Wako Pure Chem Co., Tokyo, Japan); $Ag(NH_3)_2F$ (Bee Brand Medico Dental Co., Osaka, Japan); MTT [3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide], 3-methyladenine (Sigma-Aldrich, St. Louis, MO, USA); broad caspase inhibitor (Z-VAD-FMK) (Biomol, Enzo Life Science, Plymouth Meeting, PA, USA). Culture plastic dishes and plates (6-well, 96-well) were purchased from Becton Dickinson, Franklin Lakes, NJ, USA.

Cell culture. MC3T3-E1 mouse cells from the calvaria of C57BL/6 mice (8, 9) were sub-cultured as adherent cells in α-MEM supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate at 37˚C under a humidified 5% CO_2 atmosphere (4). Cells were washed with phosphate-buffered saline without Ca^{2+} and Mg^{2+} (pH 7.4) [PBS(–)] and detached by 0.25% trypsin-0.025% EDTA-2Na in PBS(–) for each experiment.

Determination of viable cell number. Cells were inoculated at 4×10^3/ml in 96-microwell plates and incubated for 48 hours to allow complete attachment. The cells were then incubated for the indicated times with fresh culture medium containing different concentrations of each metal. After incubation for a further 24 hours at 37˚C in 5% CO_2, the viable cell number was determined by the following colorimetric method, using the MTT reagent. In brief, cells were incubated for 4 hours with 0.2 mg/ml MTT in fresh DMEM medium with 10% FBS. DMEM/10% FBS was used since dissolving the MTT reagent in α-MEM produced a bluish colouring that interfered with the accurate determination of viable cells. The use of DMEM was only for the MTT assay, and all other experiments were carried out with α-MEM/10% FBS. The formazan formed during incubation was dissolved with 0.1 ml of dimethyl sulfoxide, and the absorbance at 540 nm of the lysate was determined by using a microplate reader (Multiskan, Biochromatic, Labsystem, Osaka, Japan).

Assay for DNA fragmentation. MC3T3-E1 cells (16×10^4) were seeded on 6-well plates and incubated for 48 hours to allow complete attachment. Cells were then incubated for a further 6 hours with the indicated concentrations of metals. After washing twice with PBS(–), cells were collected by scraping with a rubber policeman on ice and spin down in an Eppendorf tube. Cells were lysed with 50 μl lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate]. The solution was incubated with 0.4 mg/ml RNase A and 0.8 mg/ml proteinase K for 2 hours at 50˚C and then mixed with 50 μl NaI solution (40 mM Tris-HCl (pH 8.0), 7.6 M NaI, 20 mM EDTA-2Na) followed by 250 μl of ethanol. After centrifugation for 20 minutes at 20,000×*g*, the precipitate was washed with 1 ml of 70% ethanol and dissolved in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA-2Na]. Each sample (10-20 μl equivalent to 5×10^5 cells) was applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA-2Na). After staining with ethidium bromide, the DNA was visualized by UV irradiation, and photographed as described previously (10). DNA from apoptotic HL-60 cells induced by UV irradiation (6 J/m^2/min, 1 min) (11) were run in parallel as positive controls.

Assay for caspase-3 activation. MC3T3-E1 cells (2×10^6) were seeded on 80-mm dishes, and incubated for 24 hours to allow complete adherence. Cells were then incubated for a further 4 hours in fresh medium containing the indicated concentrations of metals. Cells were washed twice with PBS(–) and lysed with 200 μl of lysis solution. Cells were collected by scraping with a rubber policeman and transferred to an Eppendorf tube. After standing for 10 min on

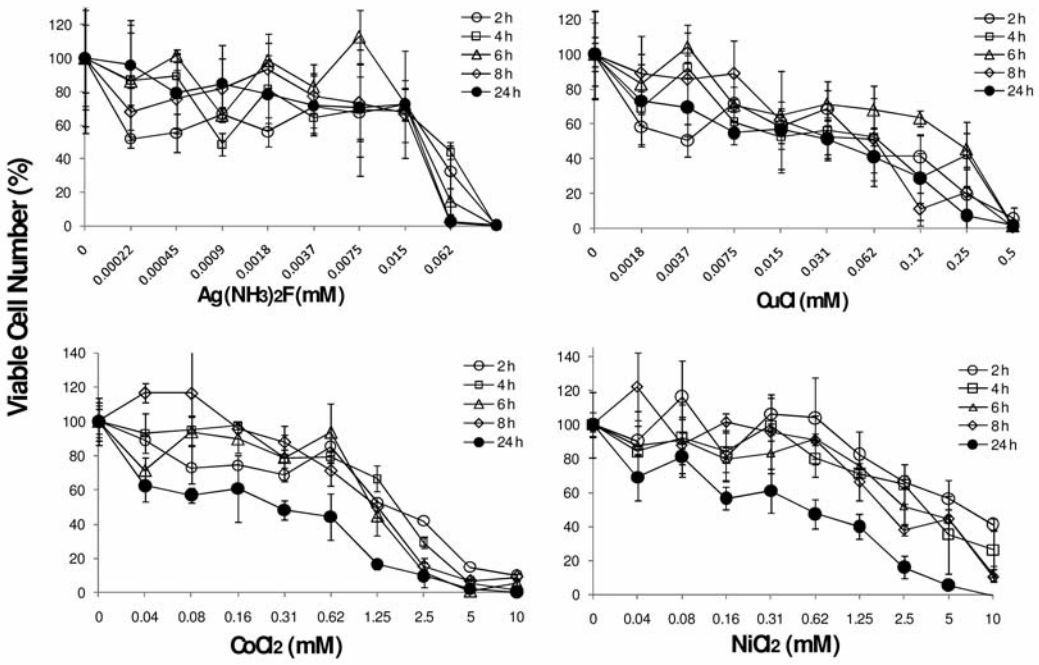

Figure 3. *Effect of exposure time to express the cytotoxicity by metals. MC3T3-E1 cells were incubated for 2, 4, 6, 8 or 24 hours with different concentrations of Ag $(NH_3)_2F$ (A), CuCl (B), $CoCl_2$ (C) or $NiCl_2$ (D), then replaced with fresh culture medium, and then further incubated for different times before MTT assay (Total incubation time=24 hours). Each value represents the mean±S.D. from three determinations.*

ice and centrifugation for 5 min at 10,000×*g*, the supernatant was collected. Lysate (50 µl, equivalent to 200 µg protein) was mixed with 50 µl lysis solution containing substrates for caspase-3 (DEVD-*p*NA (*p*-nitroanilide)). After incubation for 4 hours at 37˚C, the absorbance at 405 nm of the liberated chromophore *p*NA was measured by microplate reader as described previously (10).

Effect of apoptosis and autophagy inhibitors. MC3T3-E1 mouse cells (6×10^4) were inoculated on a 96-well plate, and incubated for 24 hours to allow complete adherence. Cells were then preincubated for 60 minutes with either 50 µM caspase inhibitor (Z-VAD-FMK), 10 mM 3-methyladenine or 100 nM bafilomycin, 60 minutes before the addition of each metal: 10-40 µM Ag $(NH_3)_2F$, 100-400 µM CuCl, 0.5-2 mM $CoCl_2$, 0.5-2 mM $NiCl_2$. Cells were then incubated for a further 24 hours, and then assayed for viable cell number by MTT methods.

Results

Superiority of α-MEM for the culture of MC3T3-E1 cells. First it was investigated which medium is suitable for the culture of MC3T3-E1 cells. MC3T3-E1 cells were found to grow much faster in α-MEM/10% FBS than in DMEM/10% FBS (Figure 1). Therefore, the subsequent experiments were carried out with α-MEM/10%FBS, except for MTT assay.

Cytotoxicity of seven metals. Cytotoxicity of seven metals was determined by incubating exponentially growing MC3T3-E1 cells with increasing concentrations of each metal. All metals reduced the cell viability in a dose dependent way, without showing any growth stimulating effect (hormetic response) at lower concentrations (Figure 2). Repeated experiments demonstrated that $Ag(NH_3)_2F$ was the most cytotoxic (CC_{50}=0.0096 µM), followed by CuCl (CC_{50}=0.049 µM)>$CuCl_2$ (CC_{50}=0.20 µM)>$CoCl_2$ (CC_{50}=0.25 µM), $NiCl_2$ (CC_{50}=0.25 µM)>$FeCl_3$ (CC_{50}=5 mM) and $FeCl_2$ (CC_{50}=6.8 mM) (least toxic). Time course study demonstrated that two hours' contact with Ag $(NH_3)_2F$, CuCl, $CoCl_2$ or $NiCl_2$ induced irreversible cell death (Figure 3). Further incubation with any of these metals enhanced the cytotoxicity only slightly, indicating the rapid decline of cell viability after metal treatment.

Type of cell death induced by metals. Figure 4 shows that Ag $(NH_3)_2F$ (A) and CuCl (B) induced smear pattern of DNA fragmentation in MC3T3-E1 cells, in contrast to internucleosmal DNA fragmentation observed in apoptotic HL-60 cells induced by UV irradiation. On the other hand, $CoCl_2$ (C) and $NiCl_2$ (D) produced neither internucleosomal nor smear pattern of DNA fragmentation. Figure 5 shows that Ag $(NH_3)_2F$ (A), CuCl (B) and $NiCl_2$ (D) did not activate caspase-3 [which has been reported to activate the caspase-activated DNase, so-called 'CAD' (12)], while $CoCl_2$ (C) activated capase-3 only slightly and to much lesser extent than that observed during the apoptosis HL-60 cells induced by UV irradiation.

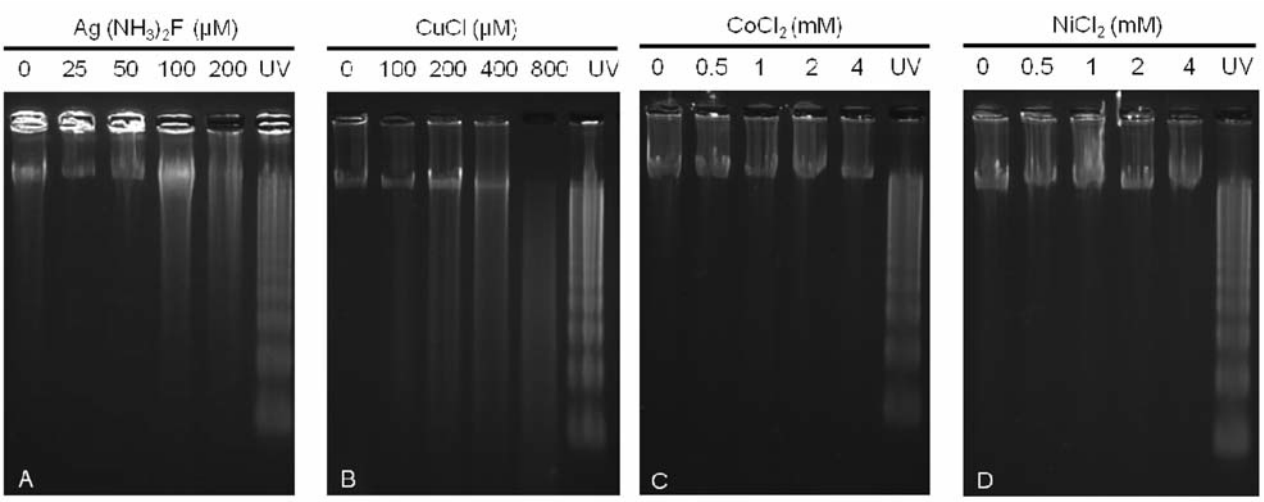

Figure 4. *Metals failed to induce internucleosomal DNA fragementation. MC3T3-E1 cells were incubated for 6 hours with the indicated concentrations of Ag $(NH_3)_2F$ (A), CuCl (B), $CoCl_2$ (C) or $NiCl_2$ (D), and then assayed for DNA fragmentation by agarose gel electrophoresis.*

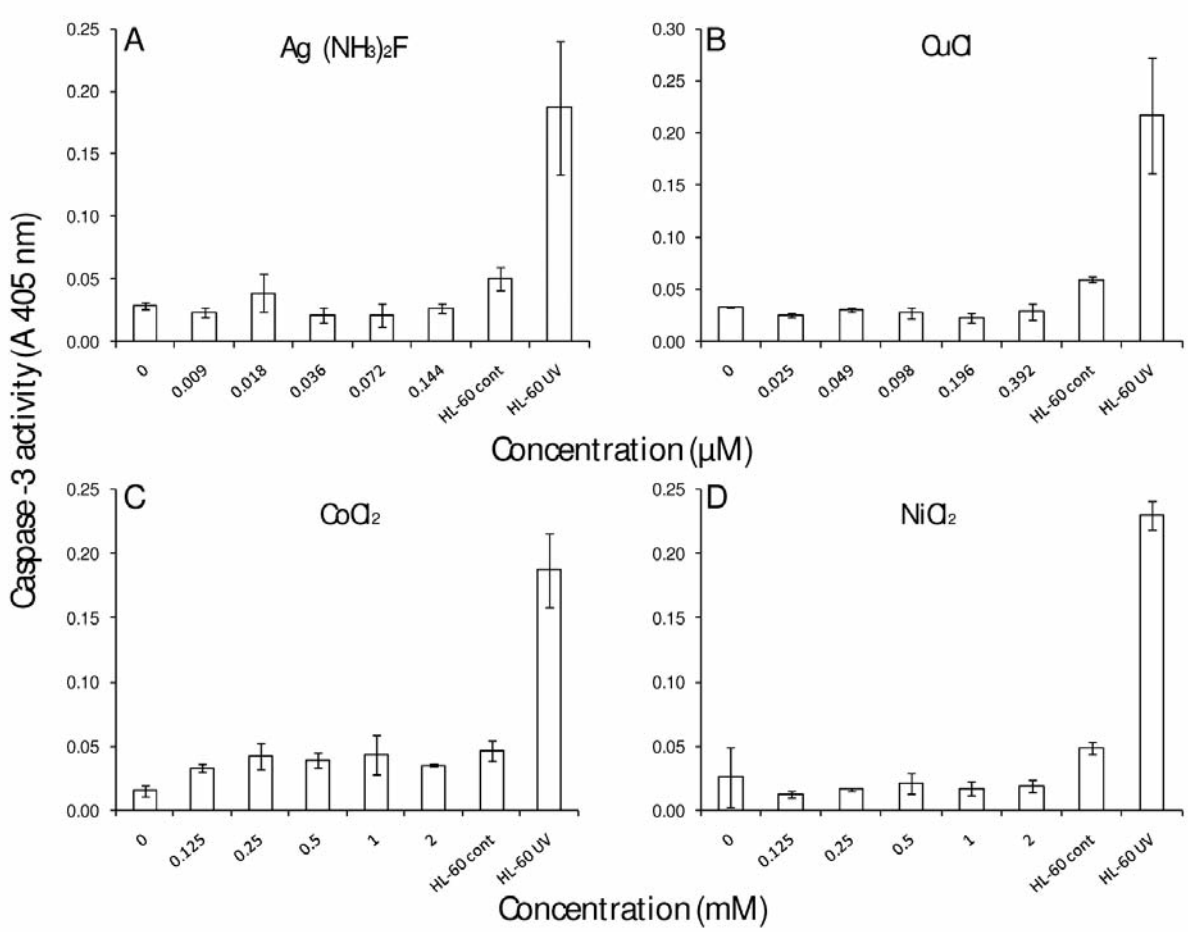

Figure 5. *Metals failed to activate caspase-3. Cells were incubated for 4 hours with the indicated concentrations of Ag $(NH_3)_2F$ (A), CuCl (B), $CoCl_2$ (C) or $NiCl_2$ (D), and then lysed for the assay of caspase-3. Apoptotic HL-60 cells induced by UV irradiation were used as positive control. Each point represents the mean±S.D. from triplicate assays.*

Effect of caspase and autophagy inhibitors. Caspase inhibitor (Z-VAD-FMK) and autophage inhibitors (3-methyladenine, bafilomycin) alone reduced the viability slightly. Preincubation with any of these inhibitors did not protect MC3T3-E1 cells from the cytotoxicity induced by 20-40 μM Ag $(NH_3)_2F$, 200-400 μM CuCl, $CoCl_2$ or 1-2 mM $NiCl_2$ (Figure 6). These inhibitors also failed to reverse the growth inhibition induced by slightly lower concentrations of metals: 10 μM $Ag(NH_3)_2F$, 100 μM CuCl, 500 μM $CoCl_2$ or 500 μM $NiCl_2$ (data not shown).

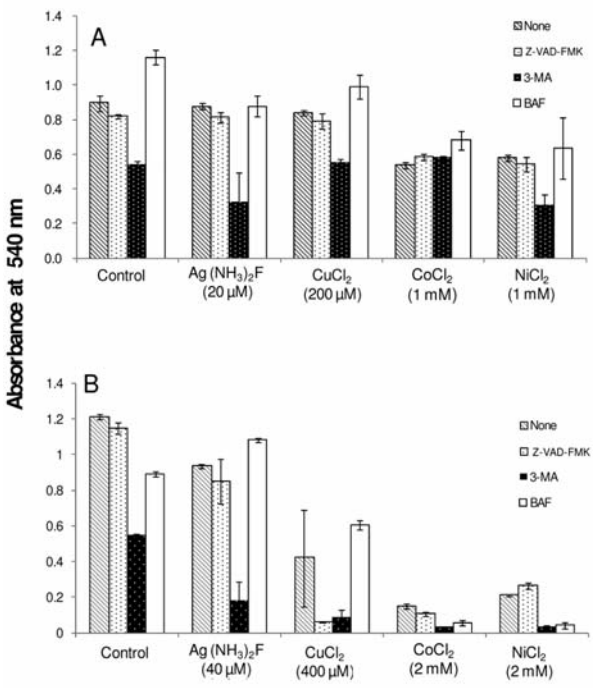

Figure 6. *Effect of several inhibitors on metal-induced cytotoxicity. Cells were pretreated for 1 hour without (control), and with caspase-3 inhibitor (Z-VAD-FMK, 50 μM), 3-methyl adenine (3-MA, 10 mM), or bafilomycin (BAF, 100 nM) and then Ag $(NH_3)_2F$ (20 or 40 μM), CuCl (200 or 400 μM), $CoCl_2$ (1 or 2 mM) or $NiCl_2$ (1 or 2 mM) (A, B) added. After incubation for 24 hours, viable cell number was determined by the MTT method. Each value represents the mean±S.D. from three determinations.*

Discussion

The present study demonstrated that seven metals induced irreversible necrotic cell death in MC3T3-E1 cells. This conclusion is based on the observations arising from this study that: (i) metals induced a smear pattern of DNA fragmentation without induction of internucleosomal DNA fragmentation (13), a biochemical hallmark of apoptosis; (ii) metals did not activate caspase-3; and (iii) the cytotoxicity or cell death induced by metals could not be reversed by apoptosis and autophagy inhibitors.

The present study demonstrated that $Ag(NH_3)_2F$ showed the highest cytotoxicity among the seven metals investigated, in agreement with previous reports (14). It was reported that silver materials, including silver nanoparticles, induced cell injury *via* reactive oxygen production (15-17) and mitochondrial damage (18, 19). These silver materials induced apoptotic cell death in mouse macrophage-like RAW264.7 cells (16) and mouse NIH3T3 fibroblasts (18), but no massive apoptosis or necrosis was found in normal human lung fibroblast cells (IMR-90) and human glioblastoma cells (U251) (19). In the present study, it was found that $Ag(NH_3)_2F$ induced necrotic cell death in MC3T3-E1 cells. These data suggest that the type of cell death induced by silver depends on the type of cells used. It was shown recently that elementary silver at low concentration exhibited no cytotoxicity but rather stimulated osteogenic maturation (20). Previous studies have suggested that the toxicity of silver towards bacteria was significantly greater than that towards cells (21). The osteogenic cell line MC3T3-E1 system (22) together with the cell contact system (4) may serve as new systems for evaluating the biocompatibility of various implant materials.

This study also found that $CoCl_2$ showed modest cytotoxicity by inducing little or no apoptosis. It has been reported that cobalt induces cytotoxicity *via* producing oxidised and nitrated proteins, increasing haeme oxygenase-1 and decreasing catalase activity, suggesting the modification of redox state (23). Toxic effects of cobalt have been reported in osteoblast-like cells (SaOS-2, MG-63) (24). On the contrary, cobalt has protected the human oral squamous cell carcinoma HSC-2, the human submandibular gland tumour HSG and the human gingival fibroblast HGF from the cytotoxicity of gallic acid, epigallocatechin gallate, curcumin and dopamine by stimulating their oxidation to inactive products (25). Further investigation of the transition between the adverse and beneficial effects of cobalt ions is necessary.

In the present study, none of the metals examined induced hormetic growth stimulation at lower concentrations in MC3T3-E1 cells, in contrast to a previous report (7). This suggests that the extent of hormetic response may depend on cell type. MC3T3-E1 cells showed a unique property of amino acid requirement. Their consumption rate of arginine was comparable with that of glutamine (major cellular energy in line with glucose), and arginine was depleted from the culture medium at 4 days after cell inoculation when logarithmic cell growth terminates (4). α-MEM/10% FBS contains higher arginine concentration (592 μM) (4) than DMEM/10%FBS (367 μM) (10), and this may explain in part the superiority of α-MEM over DMEM for the cultureof MC3T3-E1 cells.

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