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Menadione-induced cytotoxicity to rat osteoblasts

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Abstract. Oxygen-derived free radical injury has been associated with several cytopathic conditions. Oxygen radicals produced by chondrocytes is an important mechanism by which chondrocytes induce matrix degradation. In the present study, we extend these observations by studying oxidative processes against osteoblasts. Osteoblasts were mixed in in vitro culture with 200 μM menadione. The cytotoxic effect of menadione-induced oxidative stress was monitored by lucigenin- or luminol-amplified chemiluminescence,tetrazolium assay and immunocytochemical study. Results showed that adding menadione induces an oxidative stress on osteoblasts, via superoxide and hydrogen peroxide production, that can be eradicated by superoxide dismutase (SOD) and catalase in a dose-dependent manner. Catalase and the appropriate concentration of dimethyl sulfoxide have a protective effect on cytotoxicity induced by menadione, whereas SOD does not. Menadione-treated osteoblasts have a strong affinity for annexin V, and the nuclei are strongly stained by TUNEL (TdT-mediated dUTP nick-end labelling). The results suggest that menadione-triggered production of reactive oxygen species leads to apoptosis of osteoblasts.

Key words. Menadione; oxidative stress; osteoblasts; superoxide dismutase (SOD); catalase.

Hundreds of naturally occurred quinones have been isolated from biological tissue. Some quinones (e.g. ubiquinones) have important roles in the biochemistry of energy production and serve as vital links in electron transport. Other quinones have been attributed a defence role because they inhibit growth of bacteria, fungi and parasites [1]. Quinones (e.g. phenanthrenequinone) and aromatic hydrocarbons are also prevalent as ubiquitous environmental pollutants formed by various combustion processes, e.g. cigarette smoke, automobile exhaust, diesel exhaust and urban air particulates. Metabolism of environmental aromatic hydrocarbons has been suggested to contribute to their carcinogenicity. The molecular mechanisms involved in quinone or hydroquinone cytotoxicity are still largely unknown. So far it is clear that quinones can alkylate essential proteins or inactivate enzymes either directly or following reduction. However, the most prominent characteristic of quinones is their ability to undergo reversible oxidation-reduction. Thus, the industrial solvent and gasoline component benzene is thought to be carcinogenic due to its metabolism to phenol and hydroquinone in the liver. The hydroquinone may then undergo activation by oxidation to benzoquinone by prostaglandin synthetase and/or myeloperoxidase in the bone marrow [2].

Oxygen-derived free radical injury has been associated with several cytopathic conditions [3]. These include a decrease in cell redox capacity as observed in connec-

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tion with cell ageing and certain genetic diseases [4–6]. Oxidative stress has also been suggested to alter amembrane integrity, including both lipid peroxidation and modifications of membrane fluidity [1]. In particular, the naphthoquinone menadione was demonstrated to be reduced to a semiquinone radical that forms superoxide anion radicals, which in turn induce a progressive impairment of several cellular processes [4–6].

Osteoarthritis (OA) is an age-related disease, in which degenerative changes (arthrosis) and superimposed inflammatory reactions (arthritis) lead to progressive destruction of the joints. Current pharmacotherapy for osteoarthritis is aimed at relief of pain and functional disability. Although an inflammatory component may be found in some cases, there is little evidence that anti-inflammatory drugs commonly used in the treatment of OA provide more relief than simple analgesics. A growing body of knowledge about the pathophysiology of OA now offers opportunities to develop interventions aimed at retarding the progressive degeneration of articular cartilage. Active oxygen species derived from various sources play a role in this process [7], but their mode of action is not well established. Rathakrishnan [8] has suggested that oxygen radicals produced by chondrocytes may also be an important mechanism by which chondrocytes induce matrix degradation. In the present study we extend these observations by examining the effect of oxidative processes on osteoblasts. Our results show that menadione-induced oxidative stress has a cytotoxic effect on osteoblasts. This effect was mainly exhibited by the loss of cell membrane integrity, DNA fragmentation and apoptosis of osteoblasts.

Materials and methods

Culture of osteoblasts. Sequential digestion of newborn Wistar-rat calvaria was performed using a modification of the methods described by Wong and Cohn [9]. Briefly, after pretreatment of the dissected calvaria with 4 mM Na_2-EDTA in a prewarmed (37 °C) solution containing 137 mM NaCl, 2.7 mM KCl, 3 mM NaH_2PO_4, pH 7.2 (solution A), for 10 min $\times 3$, the fragments were sequentially digested with collagenase (180 U/ml; Sigma, St. Louis, MO, USA) in solution A with EDTA. The sequential digestion consisted of $\times 4$ treatments for 5 min followed by $\times 2$ treatments for 10 min. The cells released after each treatment were immediately harvested by centrifugation and resuspended in culture medium containing 10% FBS-DMEM (Dulbecco's modified Eagles' medium supplemented with 10% fetal calf serum; Gibco, Grand Island, New York, USA), penicillin G sodium 100 U/ml and streptomycin 100 mg/ml (Gibo, Grand Island, New York, USA) and then incubated in 5% CO_2 at 37 °C. Unambiguous identification of cell populations as osteoblasts is complex, since none of the parameters used for defining osteoblast-like cells are unique to this cell type [10]. In this tudy, the presence of alkaline phosphatase, an early marker of osteoblasts [11], is used to assess the osteoblastic character of the isolated cells [9, 12–14].

After the cells grew 80% confluence, osteoblasts were resuspended in phenol-red free phosphate-buffered saline at 1×10^5 cells/ml for chemiluminescence (CL) examination or cultured in 9.4 cm^2 culture dishes in 10% FBS-DMEM (Corning; NY, USA), or 96-well flat-bottomed plates at a density of $1.0 \cdot 10^4$ cells/cm^2. The suspension was used to measure CL of osteoblasts within 4 h of trypsinization. The culture dishes were incubated in 5% CO_2 at 37 °C for 2 days, and CL was measured.

Menadione treatment. Forty-eight hours after seeding, the culture medium was replaced with phosphate-buffered saline supplemented with 1 mM $CaCl_2$ and $MgCl_2$, pH 7.3. Osteoblasts were treated with various concentrations of menadione (0, 10, 20, 100, 200, 2, 20 and 200 mM) (2-methyl-1,4-naphthoquinone; Sigma) diluted in dimethyl sulfoxide (DMSO) by adding 200 $\times$ stock solution for 24 h. Cells treated with equal amounts of vehicle alone were considered as controls.

In the pilot study, the minimal toxic dosage levels of menadione were first established by MTT (tetrazolium) assay [15]. Results showed that menadione at a concentration greater than 200 μM was toxic to osteoblasts (fig. 1A). Thus a menadione level of 200 μM was tested in this experiment for various periods of incubation (0.5, 1.0, 3.0 and 6.0 h). To determine the effect of cytotoxicity on menadione-induced injury, assays were carried out as described below.

Pretreatment with antioxidant. For testing the scavenger effects of various antioxidants, superoxide dismutase (SOD; Sigma: 50, 100, 500 and 1000 U/ml), catalase (Sigma: 600, 3000, 6000, 12,000 and 24,000 U/ml) or DMSO (Sigma: 1.5×10^{-7} M, 1.5×10^{-8} M, 1.5×10^{-9} M and 1.5×10^{-10} M) was added 30 min before menadione treatment. The cytotoxic effects of menadione-induced injury were then determined.

Colorimetric MTT (tetrazolium) assay for cell viability [15]. The mitochondrial activity of osteoblasts after exposure to menadione was determined by colorimetric assay, which detects the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma catalogue no. M2128). For the assay, 2.5×10^4 cells per well were incubated (5% CO_2, 37 °C) in the presence of menadione. After various time intervals the supernatant was removed, 100 μl per well of an MTT solution (1 mg/ml in test medium) was added and the wells were incubated at 37 °C for 4 h to allow the formation of formazan crystal. The supernatant was removed again, and acid-isopropanol (100 μl of 0.04 N HCl is isopropanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After a few minutes at

room temperature to ensure that all crystals were dissolved, the plates were read on a Microelisa reader (Emax Science, Sunnyvale, CA, USA), using a test wavelength of 570 nm against a reference wavelength of 690 nm. Plates were normally read within 14 h of adding the isopropanol.

Determination of free-radical production by luminol- and lucigenin-dependent CL. Measurement of luminol- and lucigenin-dependent CL was similar to that described previously [16]. Briefly, 6.25×10^4 osteoblasts were resuspended in 3.0 ml of 10% FBS-DMEM and cultured in 0.4 cm^2 culture dishes (Corning) for 48 h. After washing with phosphate-buffered saline solution, osteoblasts were incubated with menadione (200 μM). CL was then measured in an absolutely dark chamber of the Chemiluminescence Analyzing System (Tohoku Electronic Industrial Co.; Sendai, Japan) as described above. At 100 s, 1.0 ml of 0.012 mM lucigenin (bis-*N*-methylacridinium nitrate; Sigma) or 10^{-4} M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) in phosphate-buffered saline (pH = 7.4) was injected into the cell. The CL in the sample was continuously measured for a total 1000 s. The total amount of CL was calculated by integrating the area under the curve and subtracting it from the background level, which was equivalent to the dark average. The assay was performed in duplicate for each sample and was expressed as CL counts/10 s.

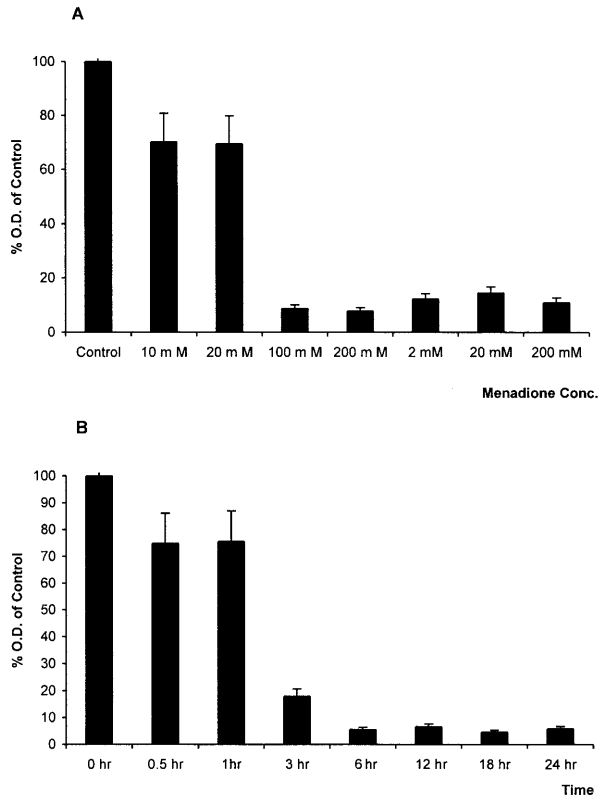

Figure 1. Viability of osteoblasts after treatment with menadione. (*A*) Viability of osteoblasts after treatment with various concentrations of menadione for 24 h (n = 10). Even low concentrations of menadione (10 μM) showed toxicity to osteoblasts when cultured for 24 h. As the concentration of menadione increased to 100 μM, the viability of osteoblasts was nearly totally lost (error bars = SD). (*B*) Viability of osteoblasts after treatment with 200 μM menadione for various time period (n = 10). Viability of osteoblasts significantly decreased after 0.54h of treatment was minimal after 6 h (error bar = SD).

Lipid peroxidation. After the cells were exposed to menadione for 0.5, 1, 3 and 6 h, the supernatant in six-well plates was collected and stored at −80 °C. The extent of lipid peroxidation was determined by measuring malondialdehyde (MDA) according to the method of Yagi [17]. Two ml of 0.083 N sulfuric acid (H_2SO_4) and 0.3 ml of 10% phosphotungstic acid were added to the same sample. After standing at room temperature for 10 min, 1.0 ml of TBA reagent (a mixture of equal volumes of 0.67% thio-barbituric acid aqueous solution and glacial acetic acid) was added. The reaction mixture was heated for 60 min at 95 °C in a water bath. After cooling with tap water, 4.0 ml of *n*-butanol was added, and the mixture was shaken vigorously for 30 s. After centrifugation at 2000 rpm for 10 min, the 1-butanol layer was used for fluorometric measurement at 553 nm with 515 nm excitation. The fluorescence value was calculated by comparison with a standard curve prepared from tetraethoxypropane.

Statistical analysis. The data were evaluated using analysis of variance (one-way ANOVA) and Dunnett's *t*-test. Differences were considered significant if $P < 0.05$.

Immunohistochemical detection of cell-membrane phospholipid asymmetry. Detection of cell-membrane phospholipid asymmetry in osteoblasts after menadione treatment was performed by the immunohistochemical methods described by Andree et al. [18]. For the assay, 1.0×10^5 cells/ml in a 9.4-cm^2 culture dish were incubated (5% CO_2, 37 °C) in the presence of menadione. After various time intervals the supernatants were removed, and cell samples were washed twice with cold phosphate-buffered saline and then resuspended in Hepes-buffered saline solution supplemented with 25 mM $CaCl_2$. The fluorescein-conjugated annexin V and propidium iodine reagent (R&D Systems, Minneapolis, MN, USA) was added, vortexed and incubated for 15 min at room temperature in the dark. Samples were analysed under a fluorescence microscope.

Immunohistochemical detection of apoptosis. Detection of apoptosis in osteoblasts after menadione treatment as performed by the immunohistochemical methods described by Sgonc et al. [19]. For the assay, 1.0×10^5 cells/ml in a 9.4-cm^2 culture dish were incubated (5% CO_2, 37 °C) in the presence of menadione. After various time intervals the supernatants were removed, and cell samples were fixed with paraformaldehyde solution

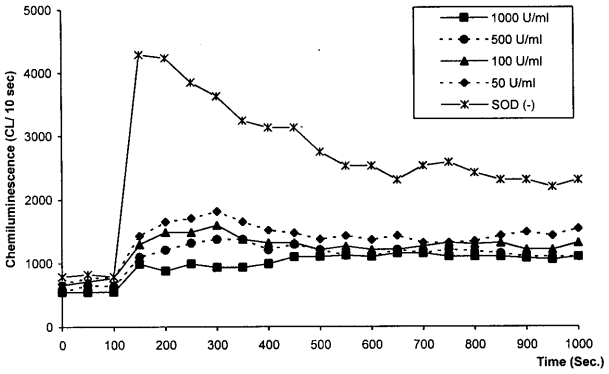

Figure 2. Representative graph of the lucigenin-amplified CL of menadione-treated osteoblasts. After addition of menadione, lucigenin-amplified CL is significantly increased. Amplified CL is reduced by adding SOD in a dose-response pattern.

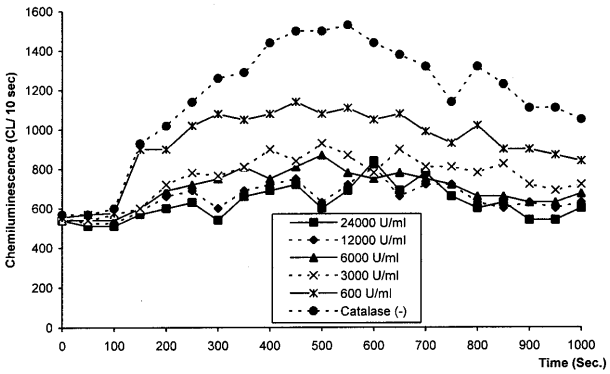

Figure 3. Representative graph of the luminol-amplified CL of menadione-treated osteoblasts. Luminol-amplified CL is significantly increased after addition of menadione. Amplified CL is reduced by additing catalase in a dose-response pattern.

(4% in phosphate-buffered saline, pH 7.4) for 30 min at room temperature. Endogenous peroxidase was blocked by 0.3% H_2O_2, then permeated with 0.1% Triton X-100 in 0.1% sodium citrate (Boehringer Mannheim; Mannheim, Germany) for 2 min on ice (4 °C). The DNA strand breaks in the osteoblasts were then fluorescence-labelled with a 100-µl TUNEL (TdT-mediated dUTP nick-end labelling) reaction mixture (Boehringer Mannheim) and incubated for 60 min. Antifluorescence antibody conjugated with POD (peroxidase; Boehringer Mannheim) was added and then incubated with samples for 30 min. DAB (3,3′-diaminobenzidine tetrahydrochloride)-substrate solution was added and incubated for 10 min. Samples were then analysed under a light microscope.

Results

MTT is a pale yellow substrate that produces a dark blue formazan product when incubated with living cells. MTT rings are cleaved in active mitochondria, and the reaction occurs only in living cells [15]. Figure 1 shows the effect of menadione on osteoblast viability as measured by MTT assay. In 24-h cultures, even low concentrations of menadione (10 µM) were toxic to osteoblasts; when the concentration of menadione was increased to 100 µM, the viability of the osteoblasts was nearly totally lost (fig. 1A). In this study, we selected a concentration of 200 µM to produce oxidative stress on the osteoblasts. In the presence of 200 µM menadione, the viability of the osteoblasts significantly decreased even after 0.5 h; the viability of the osteoblasts decreased significantly and was minimal at 6 h (fig. 1B).

Luminol- and lucigenin-dependent CL. The oxidative stress induced by menadione was shown by measurement of CL amplified by lucigenin and luminol. In the control osteoblasts not treated with menadione, lucigenin- and luminol-amplified CL were quite low and near the background level. The CL levels of control osteoblasts not treated with menadione were 122 counts/10 s (SD: 18 counts/10 s). After addition of menadione, lucigenin- and luminol-amplified CL increased significantly. Amplified CL was reduced by pretreatment of SOD and catalase in a dose-response pattern (figs. 2 and 3). After treatment with 200 µM menadione, lucigenin-amplified CL levels increased significantly; the levels were reduced significantly by SOD pretreatment (table 1). The difference in these measurements was statistically significant ($P < 0.00001$ by ANOVA). After treatment with 200 µM menadione, the luminol-amplified CL levels also increased significantly; these levels were significantly reduced by catalase pretreatment ($P < 0.0001$). The catalase had a scavenger effect on osteoblasts under menadione-induced oxidative stress (table 1).

MTT assay for cell viability. After treatment with 200 µM menadione for 0.5 h, the optic density (OD) of the MTT assay in osteoblasts decreased significantly. The difference between experimental osteoblasts with or without SOD pretreatment was not statistically significant ($P > 0.05$). When osteoblasts treated for 3 h with 200 µM menadione were pretreated with a moderate concentration of SOD (100 U/ml in this study) their viability increased significantly (table 2). The difference between experimental osteoblasts with and without catalase prtreatment treated with 200 µM menadione for 0.5 h was statistically significant ($P < 0.005$). When catalase-pretreated osteoblasts were treated with 200 µM menadione for 3 h, the increase in the osteoblast viability was even greater (table 3). After pretreatment with 1.5×10^{-9} M DMSO, the viability of the mena-

Table 1. Scavenger effect of SOD and catalase on menadione-induced (200 μM menadione) oxidative stress on osteoblasts.

Scavenger	Osteoblast suspension Chemiluminescence (CL/s) Mean (±SD)	Attached osteoblasts Chemiluminescence (CL/s) Mean (±SD)
-	4115.76 (±328.36)	616.36 (±189.20)
SOD		
50 U/ml	585.91 (±119.05)	85.66 (±4.23)
100 U/ml	465.15 (±110.27)	
500 U/ml	454.82 (±74.17)	
1000 U/ml	379.03 (±65.62)	
-	1394.73 (±248.74)	819.82 (±78.07)
Catalase		
600 U/ml	1145.13 (±220.07)	548.14 (±107.44)
3000 U/ml	1009.25 (±56.56)	315.64 (±93.09)
6000 U/ml	757.48 (±33.45)	247.73 (±55.38)
12000 U/ml	585.86 (±39.48)	224.61 (±17.72)
24000 U/ml	443.94 (±79.57)	155.33 (±29.14)

Notes: Differences between measurements were all statistically significant ($P < 0.0005$ by ANOVA).
Differences between measurements with SOD pretreatment (50 U/ml, 100 U/ml, 500 U/ml and 1000 U/ml) and without SOD pretreatment (menadione 200 μM only) were all statistically significant ($P < 0.005$ by Dunnett's t test).
In the osteoblast suspension, differences between measurement with 600 U/ml catalase pretreatment and without catalase pretreatment (menadione 200 μM only) were not statistically significant ($P < 0.070$ by Dunnett's t test); but differences between measurements with 3000 ($P = 0.007$), 6000 ($P = 0.0002$), 12,000 ($P < 0.0005$) and 24,000 U/ml ($P < 0.0005$) catalase pretreatment and without catalase pretreatment (menadione 200 μM only) were all statistically significant (by Dunnett's t test).
In attached osteoblasts, differences between measurements with and without catalase pretreatment (menadione 200 μM only) were all statistically significant ($P < 0.001$ by Dunnett's t test).

Table 2. Scavenger effect of SOD on menadione-induced (200 μM menadione) cell death of osteoblasts.

SOD	Optic density by assay MTT mean (±SD) 0.5 h	Optic density by assay MTT mean (±SD) 3 h
Control		
menadione: −	0.0553 (±0.0145)	0.0631 (±0.0086)
menadione: +	0.0275 (±0.0086)	0.0085 (±0.0042)
50 (U/ml)	0.0369 (±0.0140)	0.0112 (±0.0069)
100 (U/ml)	0.0332 (±0.0094)	0.0130 (±0.0081)
500 (U/ml)	0.0363 (±0.0118)	0.0080 (±0.0050)
1000 (U/ml)	0.0311 (±0.0107)	0.0067 (±0.0038)

Notes: After 0.5 h of menadione treatment, differences between measurements were statistically significant ($P < 0.0005$ by ANOVA). Differences were greatest between the groups with and without menadione treatment. Following menadione treatment, differences between measurements with SOD pretreatment (50, 100, 500 and 100 U/ml) and without SOD pretreatment (menadione 200 μM only) are not statistically significant ($P = 0.13$ by ANOVA).
After 3 h of menadione treatment, differences between measurements were statistically significant ($P < 0.0005$ by ANOVA). Differences were greatest between the groups with and without menadione treatment. Following menadione treatment, only the difference between the measurement with 100 U/ml SOD pretreatment and without SOD pretreatment (menadione 200 μM only) was statistically significant ($P = 0.02$ by Dunnett's t test); differences between the groups with higher or lower concentration of SOD pretreatment and without SOD pretreatment (menadione 200 μM only) were not statistically significant ($P > 0.06$ by Dunnett's t test).

dione-treated osteoblasts was significantly improved ($P < 0.01$ and $P < 0.001$ for 0.5 h and 3.0 h respectively). Changes in DMSO concentration did not improve the viability of osteoblasts (table 4). Under these experimental conditions, catalase and 1.5×10^{-9} M DMSO had a positive effect of the viability of osteoblsts, even under the oxidative stress of menadione.

Lipid peroxidation. After cells were expose to menadione for 0.5, 1, 3 and 6 h, the extent of lipid peroxidation was measured by malondialdehyde (MDA) content. These measurements did not differ significantly.

Changes in cell-membrane phospholipid asymmetry. The changes in cell-membrane phospholipid asymmetry after menadione treatment were quite obvious. After 0.5 h of menadion-induced oxidative stress, many osteoblasts showed increased annexin V-FITC (fluorescein isothiocyanate) adherence in their cell membranes, while staining of the nuclei was not quite as obvious. Annexin V binding in the cell membranes was even more marked after 3 h of menadione-induced oxidative stress; propidium iodide staining of the nuclei was evident at this time (fig. 4).

In situ detection of osteoblast apoptosis after menadione treatment. Ostelblasts undergoing apoptosis after menadione treatment were clearly visible following in situ immunohistochemical staining for apoptosis. After 0.4 h of menadione-induced oxidative stress the cellularity of osteoblasts decreased significantly, and apoptosis of some osteoblasts was evidenced by dense staining. The decrease in the cellularity, and apoptosis of osteoblasts, was more marked after 3 h of treatment with menadione (fig. 5).

Discussion

Reactive oxygen species can be generated either during the physiological metabolism of the cell (i.e. via the mitochondrial and endoplasmic reticular electron transport chains) or following the biotransformation of specific drugs and chemicals. Among these, quinones have been extensively investigated [20, 21]. The single electron reduction of quinones by a number of flavoenzymes results in formation of semiquinone radicals, which can rapidly reduce molecular oxygen, forming superoxide anion free radicals and regenerating the parent quinone in a 'redoc cycling' pathway [22]. Dismutation of O_2^- to hydrogen peroxide and the production of

Table 3. Scavenger effect of catalase on menadione-induced (200 μM menadione) cell death of osteoblasts.

Catalase	Optic density by assay MTT mean (±SD) 0.5 h	Optic density by assay MTT mean (±SD) 3h
Control		
menadione: −	0.0870 (±0.0299)	0.0783 (±0.0076)
menadione: +	0.0537 (±0.0072)	0.0130 (±0.0129)
600 (U/ml)	0.0817 (±0.0199)	0.0184 (±0.0125)
3000 (U/ml)	0.1038 (±0.0256)	0.0411 (±0.0257)
6000 (U/ml)	0.1327 (±0.0290)	0.1038 (±0.0431)
12000 (U/ml)	0.1675 (±0.0171)	0.1738 (±0.0565)
24000 (U/ml)	0.1571 (±0.0239)	0.2096 (±0.622)

Notes: After 0.5 h of menadione treatment, differences between measurements were statistically significant ($P < 0.0005$ by ANOVA). Compared with the positive control (menadione 200 μM without catalase pretreatment), the ODs of catalase-pretreated osteoblasts were all significantly higher ($P < 0.005$ by Dunnett's t test); compared with negative control (without menadione 200 μM treatment), the ODs of 6000, 12000 and 24000 U/ml catalase-pretreated osteoblasts (also with menadione 200 μM treatment) were significantly higher ($P < 0.005$ by Dunnett's t test).
After 3 h of menadione treatment, differences between measurements were statistically significant ($P < 0.0005$ by ANOVA). Compared with the positive control (menadione 200 μM without catalase pretreatment), the OD of 600 U/ml catalase-pretreated osteoblasts was not statistically different ($P = 0.13$ by Dunnett's t test), whereas the ODs of higher concentrations of catalase pretreated (i.e. 3000, 6000, 12000 and 24000 U/ml) osteoblasts were all significantly higher ($P < 0.005$ by Dunnett's t test). Compared with the negative control (without menadione 200 μM treatment), the ODs of 600 and 3000 U/ml catalase-pretreated osteoblasts (also with menadione 200 μM treatment) were significantly lower ($P < 0.03$ by Dunnett's t test), whereas the ODs of 6000, 12000 and 24000 U/ml catalase-pretreated osteoblasts were significantly higher ($P < 0.0005$ by Dunnett's t test).

Table 4. Scavenger effect of DMSO on menadione-induced (200 μM menadione) cell death of osteoblasts.

DMSO	Optic density by assay MTT mean (±SD) 0.5 h	Optic density by assay MTT mean (±SD) 3 h
Control		
menadione: −	0.0890 (±0.0368)	0.0763 (±0.0045)
menadione: +	0.0659 (±0.0050)	0.0315 (±0.0089)
DMSO		
1.5×10^{-7} M	0.0627 (±0.0037)	0.0077 (±0.0046)
1.5×10^{-8} M	0.0632 (±0.0050)	0.0086 (±0.0033)
1.5×10^{-9} M	0.0847 (±0.0142)	0.0687 (±0.0355)
1.5×10^{-10} M	0.0692 (±0.0080)	0.0284 (±0.0066)

Notes: After 0.5 h of menadione treatment, differences between measurements were statistically significant ($P < 0.0001$ by ANOVA). Compared with the positive control (menadione 200 μM without DMSO pretreatment), the OD 1.5×10^{-9} M DNSO-pretreated osteoblasts was all higher ($P < 0.001$). Compared with the negative control (without 200 μM menadione treatment), all the ODs of the tested sample except that pretreated by 1.5×10^{-9} M DMSO were lower ($P < 0.001$).
After 3 h of menadione treatment, differences between measurements were statistically significant ($P < 0.0001$ by ANOVA). Compared with the positive control (menadione 200 μM without DMSO pretreatment), the OD of 1.5×10^{-9} M DMSO-pretreated osteoblasts was higher ($P = 0.01$), while those of 1.5×10^{-7} M and 1.5×10^{-8} M DMSO-pretreated osteoblasts were lower ($P < 0.0001$). Compared with the negative control (without 200 μM menadione treatment), all the ODs of the test sample were significantly lower ($P < 0.001$).

other highly reactive species quickly lead to a condition of oxidative stress as redox cycling of the quinone continues. This is followed by progressive impairment of several cellular processes, eventually leading to cytotoxicity [23]. Menadione is a redox-cycling quinone that has been shown to induce oxidative stress [1, 24, 25] followed by the progressive impairment of several cellular processes, which eventually leads to cytoxicity [20, 26, 27]. The purpose of this study is to study the cellular mechanism of menadione-induce oxidative stress on osteoblasts.

Chemiluminescence is defined as the light produced from chemical reactions. All et al. first described cellular CL as the light emitted by phagocytic cells following phagocytosis [28, 29]. The emitted light is very weak, and chemiluminigenic probes are needed to increase the efficiency of light detection [30]. Luminol-dependent is thought to reflect the production of hydrogen peroxide (H_2O_2) and singlet oxygen [30]. Lucigenin was used to specifically measure superoxide radicals [30]. Chemiluminescent techniques have been used to study a number of clinical conditions, such as chronic granulomatous disease [31, 32], and also been used to study the effects of biological response modifiers, cytokines and pharmacological agents on oxygen radical production [29]. In this study, the oxidative stress induced by menadione was shown by measuring CL amplified by lucigenin and luminol. After addition of menadione, lucigenin- or luminol-amplified CL increased significantly. Amplifed CL can be reduced by pretreatment of SOD and catalase in a dose-response pattern (figs 2 and 3). The significant inhibition of lucigenin-derived CL by SOD indicates that superoxide was involved in this assay system [28, 33]. This result was similar to that reported by Rembish and Trush that lucigenin-induced CL was mainly used to quantitatively assess the modulation of mitochondrial superoxide generation of mononuclear cells [34]. The significant inhibition of luminol-derived CL by catalase indicates that H_2O_2 was involved in this assay system [35]. In our experiments, we added menadione to osteoblasts in the culture medium to mimic an environment of oxidative stress in vivo. Our data demonstrate that both superoxide and H_2O_2 were produced in this assay system.

The conversion of the tetrazolium salt MTT into its formazan product can be cleaved in active mitochon-

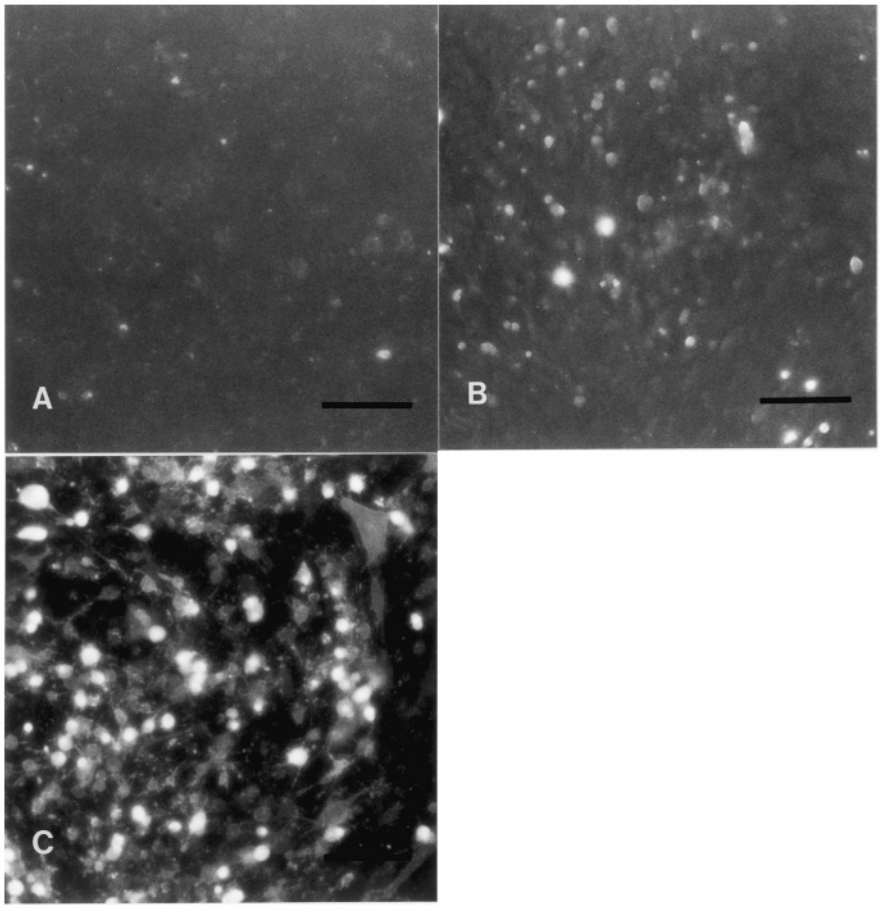

Figure 4. Changes in cell-membrane phospholipid integrity after tretment with menadione. (*A*) Control osteoblast cell structure without menadione treatment. (*B*) After 0.5 h of menadione-induced oxidative stress, many osteoblasts showed increased annexin V content in the cell membrane, while staining of the nucleus was not obvious. (*C*) After 3 h of oxidative stress, the annexin V content in the cell membrane was much more evident; propidium iodide staining of the nucleus was also pronounced (bar = 100 μm).

dria of living cells [15, 36]. Menadione causes significant cellular damage in osteoblasts, as evidenced by the decreased cell viability measured by MTT assay (fig. 1). Since MTT is cleaved only be active mitochondria [15], menadione-induced decreased of cell viability measured by MTT assay suggests that menadione may damage the mitochondria of osteoblasts. As shown above, both SOD and catalase have a scavenger effect of the superoxide and H_2O_2 produced by reaction to menadione (table 1). We used the MTT assay to evaluate the scavenger effect of SOD, DMSO and catalase on the viability of menadion-treated osteoblasts. Our results also showed that the scavenger effect of SOD on the osteoblast viability was not clearly obvious (table 2). SOD removes superoxide in tissue, and the product of this reaction is H_2O_2. The increased concentration of H_2O_2 inactivates CuznSOD and FeSOD by removal of histidine [35], which then partially inhibits the scavenger effect of SoD. Pretreatment of osteoblasts with catalase increased the amount of formazan formation in MTT assay (table 6), suggesting that catalase has a protective effect on osteoblast mitochondria. Our data indicate that preincubation of osteoblasts with catalase protects cells from the cytotoxic effects induced by menadion (table 3). Catalase scavenges the end products of the reactive oxygen intermediate H_2O_2—H_2O and O_2. Increased O_2 concentration activates the pentose phosphate pathway [37], which increases production of ribose-5-phosphate and thus DNA synthesis and cell proliferation. This explains the increased cellularity of the osteoblasts after catalase treatment (table 3), whereas only the appropriate concentration of DMSO (i.e. 1.5×10^{-9} M) has a similar scavenger effect on the viability of the osteoblast culture in the presence of menadione (table 4).

Phospholipids are the principal structural components of cell membranes. The fatty acid composition of phospholipids modulates the function of membrane recep-

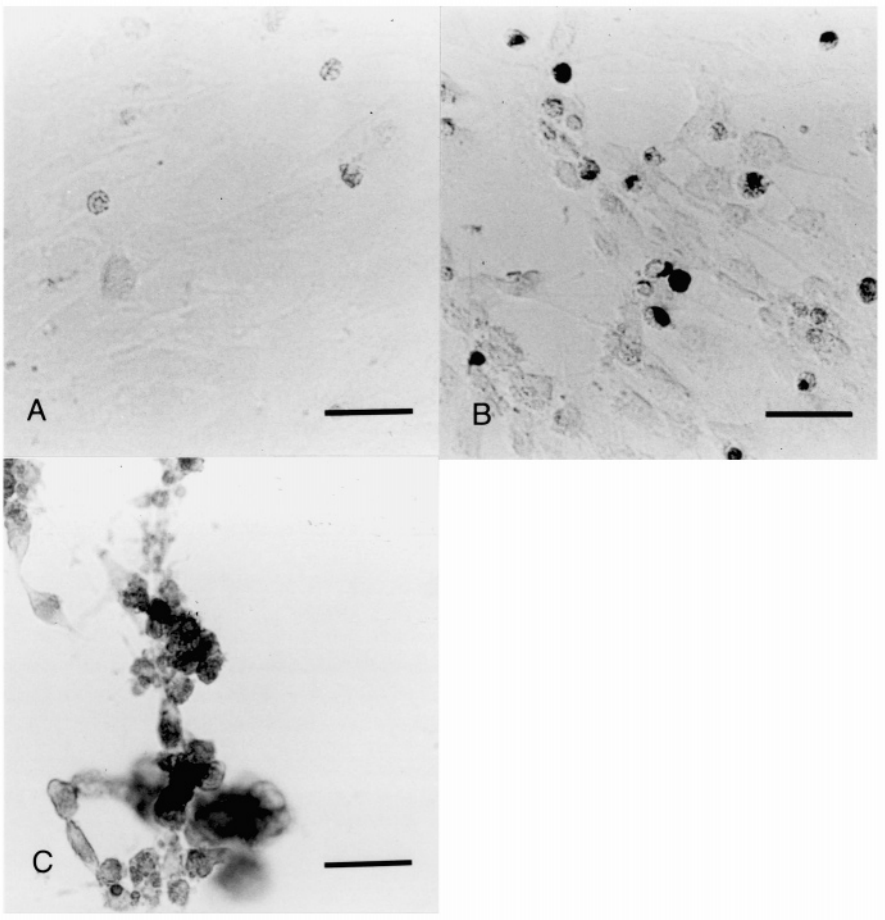

Figure 5. Apoptosis of osteoblasts after treatment with menadione. (*A*) Control osteoblasts without menadione treatment. (*B*) In situ apoptosis assay of osteoblasts after 0.5 h of menadione treatment. There was a decrease in cellularity, with numerous osteoblasts clearly labelled by 100 µl TUNEL reaction stain at the nucleus. (*C*) In situ apoptostic assay of osteoblasts after 3.0 h of menadione treatment. The decrease in cellularity and apoptotic changes in osteoblasts were even more evident (bar = 200 µm).

tors, enzymes and ion channels [38], and is a critical determinant of cell viability. The content and composition of phospholipid fatty acids may be altered by oxygen-free radical-mediated lipid peroxidation [39], activation of phospholipase [40], and decreased activity of reacylation enzymes and de novo phospholoipid synthesis [41]. The resulting changes in membrane phospholipids may lead to increase calcium permeability, integral protein failure and cell death. Free radicals are very reactive and directly attack lipids [42] and proteins [43] in biological membranes at local sites of generation, causing their dysfunction [44, 45]. Malondialdehyde produced by lipid peroxidation causes cross-linking and polymerization of membrane components. Under extreme conditions, peroxized membranes lose their integrity, which is detrimental to cell viability [46]. In the present study, incubation of menadione with osteoblasts did not result in a significant increase in lipid peroxidation. This seemed to contradictory to our hypothesis that menadione can induce cell injury by loss of mitochondrial function as manifested by MTT assay.

Annexin V, a member of a family of calcium and phospholipid binding proteins, is largely found on the cytosolic face of plasma membranes [47]. In the presence of physiological concentrations of calcium, annexin V has a high affinity for phosphotidylserine [18]. Normally, phosphotidylserine is found only on the inner side of cell membranes, but during apoptosis, cells may expose phosphotidylserine to the outer membrane [48]. The differential staining of cells with annexin V has been demonstrated to be useful in identifying cells undergoing apoptosis [49–51]. In this study, we demonstrated that following treatment with menadione, cell membrane phospholipids showned significant changes in integrity. After 0.5 h of menadione-induced oxidative stress many osteoblasts showed increased annexin V

binding in their cell membranes, whereas staining of nuclei was not as evident. Annexin V binding in cell membranes was more pronounced after 3 h of menadione-induced oxidative stress; propidium iodine staining of nuclei was also evident (fig. 4), suggesting expression of phosphotidylserine on the outer leaflet of compromised cell membranes. Thus we conclude that one triggering event for menadione-mediated osteoblast destruction is impairment of the function of the cell membrane, which destroys its integrity. This leads to disintegration of the cells, detectable by the damage to the plasma membrane.

Two distinct modes of cell death, apoptosis and necrosis, can be distinguished based on differences in the morphological, biochemical and molecular changes of dying cells. Programmed cell death, or apoptosis, is the most common form of eukaryotic cell death [52]. In general, cells undergoing apoptosis display a characteristic pattern of structural changes in the nucleus and cytoplasm, including rapid blebbing of the plasma membrane and nuclear disintegration. Nuclear collapse is associated with extensive damage to chromatin and DNA cleavage into oligonucleosomal-length DNA fragments after activation of a calcium-dependent endogenous endonuclease [53]. The TUNEL technique uses DNA polymerase as well as terminal deoxynucleotidyl transferase (TdT) to incorporate labelled nucleotides to DNA strand breaks in situ [19, 54–56]. Osteoblasts undergoing apoptosis after treatment with menadione were clearly obvious with in situ TUNEL staining. After 0.5 h of menadione-induced oxidative stress, the cellularity of osteoblasts decreased significantly, and apototic staining was detected in some of them. These reactions were even more pronounced after 3 h of menadione treatment (fig. 5). These finindgs suggest that oxidative stress induced by menadione damages many different constituents of osteoblasts, including nuclear DNA, and may also contribute to osteoblast death.

Menadione has a cytotoxic effect on osteoblasts. As our study clearly shows reactive oxygen species, including superoxide H_2O_2 and others, are produced immediately after addition of menadione. These reactive oxygen species induce damage in cell membrane integrity, even DNA fragmentation, and lead to apoptosis of osteoblasts. The pathogenic role of mitochondrial and extra mitochondrial damage in menadione-induced oxidative stress on osteoblasts is currently being investigated.

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