行政院國家科學委員會專題研究計畫成果報告

計畫名稱：Menadione-induced Cytotoxicity On Rat Osteoblasts Cell

(英、中文) Menadione **對骨母細胞之細胞毒性研究**

計畫類別：☒ **個別型計畫** □ **整合型計畫**

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個別型計畫：計畫主持人：韓毅雄

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整合型計畫：總計畫主持人：

子計畫主持人：

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中 華 民 國 八 十 九 年 十 月 十 三 日

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中文摘要：

關鍵詞：酮類化合物、骨母細胞、氧化自由基

氧化自由基導致細胞破壞為可能原因之一。退化性骨關節炎為一種與老化有關之疾病，在此種關節老化過程程中常併發有發炎反應。過去經驗看來 Non-Steroid Anti-Inflammaory Drug **所能提供之療效抑不過是止痛效果而已。氧化自由基可能與退化性骨關節炎有某種程度之相關，只是尚未確立。稍後亦有學者指出軟骨細胞產生之氧化自由基與其基質退化具有相關。本研究將探討** menadione **引發之氧化壓力與退化性骨關節炎以及骨母細胞之關係。**

以 200 μM Menadione **成份將與懸浮之小鼠骨母細胞共同培養並測量其微量激光反應。再以** methylthiazol tetrazolium (MTT)**及脂質過氧化**(MDA)**釋放法測量** 200 μM Menadione **對細胞造成傷害程度。各種抗氧化合物如**: superoxide dismutase (SOD), catalase, or dimethylsulfoxide (DMSO)**將加入培養以測定其對** Menadione **所引發細胞毒性之解毒效果。並以組織免疫螢光法偵測細胞磷酯質變性及骨母細胞之自然凋亡。**

經由本研究，氧化壓力對於骨母細胞之作用機轉將可以得到釐清，氧化自由基對於退化性骨關節炎病程中所佔角色也可以提供一可能之解答。

英文摘要：

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 the osteoblasts. The osteoblasts were mixed in vitro culture with 200 μM menadione. The cytotoxic effect by menadione-induced oxidative stress was monitored by the lucigenin- or luminol-amplified chemiluminescence, MTT assay, and immunocytochmical study. The result showed that the adding of menadione can induce an oxidative stress on the osteoblasts by superoxide and hydrogen peroxide production, which can be eradicated by superoxide dismutase (SOD) and catalase in a dose dependent mode. The catalase has a protective effect on the cytotoxicity induced by menadion, while the SOD does not. The menadione treated osteoblasts had a strong affinity for annexin V and the nucleus are strongly stained with the TUNEL (TdT-mediated dUTP nick end labeling) reaction mixture. From the result, we suggest that menadione-triggered reactive oxygen species production might lead to the apoptosis of the osteoblasts.

KeyWords: menadione, oxidative stress, osteoblasts.

INTRODUCTION

Hundreds of naturally occurred quinones have been isolated from biological tissue. Some quinones (eg. ubiquinones) have important roles in the biochemistry of energy production and serve as vital link in electron transport. Other quionones have been attributed to a defense role as a result of their effectiveness at inhibiting the growth of bacteria, fungi, and parasites [1]. Quinones (eg. 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. Thus the industrial solvent and gasoline component benzene is thought to be carcinogenic by being metabolized 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]. This includes a decrease in cell redox capacity as observed in connection with cell aging and certain genetic diseases [4-6]. Oxidative stress has also been suggested to alter membrane 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, which forms superoxide anion radicals that induce a progressive impairment of several cellular processes [4].

Osteoarthritis are age-related diseases, in which degenerative changes (arthrosis) and superimposed inflammatory reactions (arthritis) lead to progressive destruction of the joints. Current pharmacotherapy for osteoarthritis (OA) 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 the modes of action is not well established. Later Rathakrishnan [8] 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 studying oxidative process against the osteoblasts. As shown, menadione-induced oxidative stress has a cytotoxic effect on the osteoblasts. The cytotoxic 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 by 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 pre-warmed (37^{o}C) solution containing 137 mM NaCl, 2.7 mM KCl, 3 mM NaH_2PO_4, pH 7.2 (solution A), for 10 minutes three times, the fragments were sequential digested with collagenase (180 U/ml, Sigma Co.) in solution A with EDTA. The sequential digestion consisted of four times' treatments for 5 minutes followed by two times' treatments for 10 minutes. 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, UK), penicillin G sodium 100 units /ml and streptomycin 100 mg /ml (Gibco, UK) and then incubated in 5 % CO_2 at 37^{o}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 types [10]. In this study, 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 PBS at 1 x 10^5 cells per ml for chemiluminescence examination or cultured in 10% FBS-DMEM with 9.4 cm^2 culture dishes (Corning, NY, USA), or 96-well flat-bottomed plates at the density of 1.0 x 10^4 cells /cm^2. The osteoblast suspension was used to measure their chemiluminescence within 4 hours after trypsinization. The culture dishes were incubated in 5% CO_2 at 37^{o}C for 2 days and chemiluminescence was measured.

Menadione treatment

Fourty-eight hours after seeding, the culture medium was replaced with a phosphate buffered saline (PBS) supplemented with 1 mM $CaCl_2$ and $MgCl_2$, pH 7.3). Osteoblasts were treated with various concentration of menadione [0, 10 μ M, 20 μ M, 100 μ M, 200 M, 2 mM, 20 mM, and 200mM menadione (2-methyl-1,4-naphthoquinone, Sigma) diluted in dimethyl sulphoxide (DMSO) by adding 200X stock solution] for 24 hours. 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 using MTT assay [15]. MTT assay results showed that menadione at concentration more than 200 μM showed toxicity to osteoblasts (Table 1). Therefore menadione level of 200 μM was tested in this experiment for various periods of incubation (0.5, 1.0, 3.0, 6.0 hours). To determine the effect of cytotoxicity on menadione-induced injury, assays were carried out as described below.

Colorimetric MTT (Tetrazolium) assay for cell viability [15]

The mitochondrial activity of the osteoblasts after exposure to menadione was determined by colorometric assay which detects the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma catalog no. M2128). For the assay, 2.5 x 10^4 cells per well were incubated (5% CO_2, 37^{o}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^{o}C for 4 h to allow the formation of formazan crystal. Again the supernatant was removed and acid-isopropanol (100 µl of 0.04 N HCl in 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 Microelisa reader (Emax Science Corp, USA), using a test wavelength of 570 nm against a reference wavelength of 690 nm. Plates were normally read within 1 h after adding the isopropanol.

Determination of free radical production by luminol- and lucigenin-dependent chemiluminescence:

The measurement of luminol- and lucigenin-dependent chemiluminescence was similar to that described previously [16]. Briefly, 6.25 x 10^4 osteoblasts were resuspended in 3.0 ml 10% FBS-DMEM and cultured with 9.4 cm^2 culture dishes (Corning, NY, USA) for 48 hours. After washing with PBS solution, osteoblasts were incubated with menadione (200 µM). The chemiluminescence was then measured in an absolutely dark chamber of the Chemiluminescence Analyzing System (Tohoku Electronic Industrial Co., Sendai, Japan) as described above. At 100 second time point, 1.0 ml of 0.01 mM lucigenin (bis-N-methylacridinium nitrate, Sigma Co., U.S.A.) or 10^{-4} M luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma Co., U.S.A.) in PBS (pH = 7.4) was injected into the cell. The chemiluminescence in the sample was continuously measured for a total 1000 seconds' time period. The total amount of chemiluminescence 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 chemiluminescence counts/ 10 sec.

Lipid peroxidation

After the cells were exposed to menadione for 0.5, 1, 3 and 6h, the supernatant in 6-well plates was collected and stored at -80^{o}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 was 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^{o}C in a water bath. After cooling with tape water, 4.0 ml of n-butanol was added and the mixture was shaken vigorously for 30 seconds. After centrifugation at 2,000 rpm for 10 min, the n-butanol layer was used for fluorometric measurement at 553 nm with 515 nm excitation. The value of fluorescence was calculated by comparing with standard curve prepared from tetraethoxypropane.

Statistical analysis

The data were evaluated by using analysis of variance (one way ANOVA) and student 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 x 10^5 cells/ ml in 9.4 cm^2 culture dish were incubated (5% CO_2, 37^{o}C) in the presence of menadione. After various time intervals the supernatants were removed, cell samples were

washed with cold PBS twice and then resuspended in HEPES buffered saline solution supplemented with 25 mM $CaCl_2$. The fluorescein-conjugated annexin V and propidium iodide regent (R&D systems, MN, USA) was added, vortexed and then incubated for 15 min at room temperature in the dark. Samples were then analyzed under fluorescence microscope.

Immunohistochemical detection of apoptosis

Detection of apoptosis in osteoblasts after menadione treatment were performed by the immunohistochemical methods described by Sgonc et al. [19]. For the assay, 1.0 x 10^5 cells / ml in 9.4 cm^2 culture dish were incubated (5% CO_2, 37^oC) in the presence of menadione. After various time intervals the supernatants were removed, cell samples were fixed with paraformaldehyde solution (4% in PBS, pH 7.4) for 30 min at room temperature. The 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^oC). The DNA strand breaks in osteoblasts were then fluorescence-labeled by 100 µl TUNEL (TdT-mediated dUTP nick end labeling) reaction mixture (Boehringer Mannheim, Mannheim, Germany) and incubated for 60 min. Anti-fluorescence antibody conjugated with POD (Peroxidase, Boehringer Mannheim, Mannheim, Germany) was added and then incubated with samples for 30 min. DAB-substrate solution was added and incubated for 10 min. Samples were then analyzed under light microscope.

RESULTS

MTT is a pale yellow substrate that produces a dark blue formazan product when incubated with living cells. MTT ring is cleaved in active mitochondria, and the reaction occurs only in living cells [15]. When cultured with menadione for 24 hours, even low concentration of menadione (10 μM) showed toxicity to the osteoblasts; when the concentration of the osteoblasts increased to 100 μM, the viability of osteoblasts nearly totally lost. In this study, we selected the concentration of 200 μM to produce the oxidative streses on the osteoblasts. In the presence of 200 μM menadione, the viability of the osteoblasts significantly decreased even after 0.5 hours′ culture; the viability of the osteoblasts decreased significantly and reached to its maximal effect at 6 hours′ culture.

Luminol- and lucigenin-dependent chemiluminescence (CL):

The oxidative stress induced by menadione was shown by measurement of the chemiluminescence amplified by the lucigenin and luminol. In the control osteoblasts without menadione treatment, the lucigenin or luminol-amplified chemiluminescence was quite low and near the backgrond level. The CL levels of control osteoblasts without menadione treatment were 122 counts/10 sec.(S.D.: 18.0 counts/10 sec.). After adding the menadione, the lucigenin or luminol-amplified chemiluminescence were significantly increased. The amplified chemiluminescence can be reduced by the pretreatment of the SOD and catalase in a dose-response pattern. After treatment with 200 μM menadione, the lucigenin-amplified CL levels increased significantly. The CL can be reduced significantly by SOD pretreatment. There is a statistically significant difference existed in these measurements ($P<0.00001$ by ANOVA test). After treatment with 200 μM menadione, the luminol-amplified CL levels also increased significantly The luminol-amplified CL can be reduced significantly by catalase pretreatment ($P<0.0001$). The catalase had a scavenger effect on the osteoblasts when the osteoblasts is under the oxidative stress of menadione.

MTT assay for cell viability

After treatment with 200 μM menadione for 0.5 hr, the optic density (O.D.) of the MTT test in osteoblasts can be decreased significantly. The difference existed between the experimental osteoblasts with or without SOD pretreatment was not statistically significant ($P>0.05$). When osteoblasts treated with 200 μM menadione for 3 hr, the vaibility increased significantly when osteoblasts pretreated with moderate concentration of SOD (100 U/ml in this study). After treatment with 200 μM menadione for 0.5 hr, there is statistically significant difference existed between the experimental osteoblasts with or without catalase pretreatment ($P< 0.005$). When catalase-pretreated osteoblasts were treated with 200 μM menadione for 3 hr, the increase in the osteoblast viability was even more significant. In this experimental condition, the catalase had a positive effect on the viability of the osteoblasts, even in the oxidative stress of the menadione.

Lipid peroxidation

After the cells were exposed to menadione for 0.5, 1, 3 and 6h, the extent of lipid peroxidation was measured by malondialdehyde (MDA) content. There was no significant difference existed between these measurement.

Changes in cell membrane phospholipid asymmetry

The changes in cell membrane phospholipid asymmetry after menadione treatment were quite obvious. After 0.5 hr′s oxidative stress of the menadione

treatment on osteoblasts, many osteoblasts showed increased annexin V-FITC adherence in the cell membrane, while the stain of the nucleus was not quite obvious. The annexin V binding in the cell membrane was even much more obvious under 3 hrs′ oxidative stress of menadione treatment; the propidium iodide stain on the nucleus was evident at this time.

In situ detection of apoptosis of osteoblasts after menadione treatment

The osteoblasts undergoing apoptosis after menadione treatment was quite obvious with the in situ immunohistochemical stain for apoptosis. After 0.5 hr′s oxidative stress of the menadione treatment on osteoblasts, the cellularity of osteoblasts decreased significantly and apoptosis of the osteoblasts was detected in some of the osteoblasts by showing dense stain in the samples. The decrease in the cellularity and apoptosis of osteoblasts were even more significant after 3 hrs′ treatment of menadione.

DISCUSSION

Reactive oxygen species can be generated either during the physiological metabolism of the cell (i.e., by the mitochondrial and endoplasmic reticular electron transport chains) or following the bio-transformation of several drugs and chemicals. Among these, quinones have been extensively investigated [20,21]. The one-electron reduction of quinones, by a number of flavoenzymes, results in the formation of the semiquinone radical, which can rapidly reduce molecular oxygen forming superoxide anion free radical and regenerating the parent quinone; such as a ″redox cycling″ pathway [22]. Dismutation of O_2^- to hydrogen peroxide and the production of other highly reactive species quickly lead to a condition of oxidative stress as redox cycling of the quinone continues. This is followed by a progressive impairment of several cellular processes eventually leading to cytotoxicity [23]. Menadione is a redox-cycling quinone which has been shown to lead to a condition of oxidative stress [1,24,25] followed by a progressive impairment of several cellular processes which eventually lead to cytotoxicity [20,26,27]. The purpose of this study is to study the cellular mechanism of the menadione-induced oxidative stress against the osteoblasts.

Chemiluminescence is defined as the light produced from chemical reactions. Allen et al first described cellular chemiluminescence as the light emitted by phagocytic cells following phagocytosis [28]. Cells that exhibit respiratory burst, such as phagocytes, produce chemiluminescnce [29]. The emitted light is very weak and needs the introduction of chemiluminigenic probes to increase the efficiency of light detection [30]. Luminol-dependent chemiluminescence is thought to reflect the production of hydrogen peroxide (H_2O_2) and singlet oxygen [30]. Lucigenin, on the other hand, was found to be insensitive to hypochlorite and hydroxyl radicals and to specifically measure superoxide radicals [30]. The chemiluminescent techniques have been used to study a number of clinical conditions, such as chronic granulomatous disease [31,32]. Chemiluminescence has also been used to study the effects of biologic response modifiers, cytokines, and pharmacologic agents on oxygen radical production [29]. In this study, the oxidative stress induced by menadione was shown by the measurement of the chemiluminescence amplified by the lucigenin and luminol. After adding the menadione, the lucigenin- or luminol-amplified chemiluminescence was significantly increased. The amplified chemiluminescence can be reduced by the pretreatment of the SOD and catalase in a dose-response pattern. The significant inhibition of lucigenin-derived chemiluminescence by superoxide dismutase indicates that superoxide has been involved in this assay system [28,33]. This was similar to that reported by Rembish and Trush that the lucigenin-induced CL was mainly used to quantitatively assess the modulation of mitochondrial superoxide generation of the mononuclear cells [34]. The significant inhibition of luminol derived chemiluminescence by catalase indicates that H_2O_2 has been 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 demonstrated 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 mitochondria of living cells [15,36]. Menadione can cause significant cellular damage in osteoblasts, as evidence by the decreased cell vaibility measured with MTT assay. Since MTT is cleaved only by active

mitochondria [15], menadione-induced decrease of cell viability measured with MTT assay suggests that menadione may have damaged the mitochondria of osteoblasts. As shown above, both SOD and catalase have a scavenger effect on the superoxide and H_2O_2 produced by menadione reaction. We performed MTT test to evaluate the scavenger effect of SOD and catalase on the viability of menadione treated osteoblasts. The result also showed that the scavenger effect of SOD on the osteoblasts viability was not so obvious. Superoxide dismutase can remove the superoxide in the tissue, and the product of this reaction is H_2O_2. The increased concentration of H_2O_2 can inactivate CuZnSOD and FeSOD by removal of histidine [35]. The scavenger effect of SOD was then partially inhibited. Preatment of osteoblasts with catalase increased the amount of formazan formation in MTT test, suggesting a protective effect of catalase on mitochondria of osteoblasts. Our data indicated that preincubation of osteoblasts with catalase protect osteoblasts from the cytotoxic effect induced by menadione. The catalase can scavenge the end products of the reactive oxygen intermediates H_2O_2, while the end product of this reaction are H_2O and O_2. Increased O_2 concentration will activate pentose phosphate pathway [37], then increase the production of ribose-5-phosphate production and thus increased DNA synthesis and cell proliferation. This can explain the increased cellularity of the osteoblasts after catalase treatment.

Phospholipids are the principal structural component of cell membranes. The fatty acyl composition of phospholipids modulate the function of membrane receptors, enzymes, and ion channels [38], and is therefore 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 phospholipid synthesis [41]. The resulting changes in membrane phospholipids may lead to increased calcium permeability, integral protein failure, and cell death. Free radicals are very reactive and directly attack lipids [42] and proteins [43] in the biological membranes at local sites of generation, causing their dysfunction [44,45]. Malondialdehyde produced by lipid peroxidation can cause cross-linking and polymerization of membrane components. Under extreme conditions, peroxized membranes can lose their integrity and detrimental to cell viability [46]. In the present study, incubation of menadione with osteoblasts did not result in a significant increase of lipid peroxidation. This seemed to contradictory to our hypothesis that the cause of cell injury manifested by loss of mitochondrial functions indicated by MTT assay.

Annexin V, a member 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 high affinity for phosphotidylserine [18]. Normally phosphotidylserine is only found on the inner side of cell membranes. 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 after menadione treatment, the phospholipid of the cell membrane showed significant change in integrety. After 0.5 hr′s oxidative stress of the menadione treatment on osteoblasts, many osteoblasts showed increased annexin V binding in the cell membrane, while the stain of the nucleus was not quite obvious. The annexin V binding in the cell membrane was much more obvious

under 3 hrs′ oxidative stress of menadione treatment; the propidium iodide stain on the nucleus was also evident. This fact indicated that there is expression of the phospotidylserine on the outer leaflet of cell membrane with compromised cell membrane. We therefore assume that one triggering event for menadione-mediated osteoblast destruction is the impairment of function of cell membrane thereby destroying the integrety of cell membrane. This will lead to the disintegration of the cells which is finally detectable by the plasma membrane damage.

Two distinct modes of cell death, apoptosis and necrosis, can be distinguished based on differences in 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 undegoing apoptosis display a characteristic pattern of structural changes in nucleus and cytoplasm, including rapid blebbing of plasma membrane and nuclear disintegration. The 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]. In the TUNEL technique, DNA ploymerase as well as terminal deoxynucleotidyl transferase (TdT) have been used for the incorporation of lebeled nucleotides to DNA strand breaks in situ [19,54-56]. The osteoblasts undergoing apoptosis after menadione treatment was quite obvious with the in situ TUNEL stain for apoptosis. After 0.5 hr′s oxidative stress of the menadione treatment on osteoblasts, the cellularity of osteoblasts decreased significantly and apotosis stain was detected in some of the osteoblasts. The decrease in the cellularity and apoptosis stain of osteoblasts were even more significant after 3 hrs′ treatment of menadione. These findings suggest that oxidative stress induced by menadione will damage many different constitutents of the osteoblasts, including nuclear DNA which may also contribute to osteoblast death.

Menadione can induce cytotoxic effect on the osteoblasts. From our study, it is quite obvious that reactive oxygen species, including superoxide, H_2O_2, and others, were produced immediately after the adding of menadione. These reactive oxygen species induce damage in cell membrane integrity, even DNA fragmentation, and lead to the apoptosis of the osteoblasts. The pathogenetic role of mitochondrial and extra mitochondrial damage in menadione-induced oxidative stress on osteoblasts is now under investigation.

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