Oxidative stress as a component of chromium-induced cytotoxicity in rat calvarial osteoblasts

**Jun Fu · Xing Liang · Yue Chen · Li Tang ·
Qing-hong Zhang · Qiang Dong**

Abstract It has been documented that medical prosthetic alloys release metal ions into surrounding tissues and cause cytotoxicity, but the mechanisms remain undefined. In that regard the cellular oxidative stress may be a common pathway in cellular responses to metal ions. The objective of this study was to approach the hypothesis that oxidative stress mediates chromium-induced cytotoxicity in rat calvarial osteoblasts. Osteoblasts were exposed to different concentrations of Cr^{6+} or Cr^{3+} (5–20 μM) in the presence or absence of the antioxidant *N*-acetyl-cysteine (NAC; 1–5 mM). Cellular viability, differentiation, and intracellular ultrastructural alterations were evaluated by MTT assay, alkaline phosphatase (ALP) activity assay, and transmission electron microscopy. Cellular oxidative stress was evaluated by intracellular reactive oxygen species (ROS) production. ROS production was monitored by the oxidation-sensitive fluorescent probe 2′7′-dichlorofluorescin diacetate (DCFH-DA). A time- and concentration- dependent increased cytotoxicity, time-dependent increased intracellular ROS production were indicated on exposure to Cr^{6+}. Pretreatment of osteoblasts with 1–5 mM NAC afforded dose-dependent cytoprotective effects against Cr^{6+}-induced cytotoxicity in osteoblasts. NAC decreased the level of intracellular ROS induced by Cr^{6+}, too. While Cr^{3+} and NAC did not have any significant effects on osteoblasts (5–20 μM). These results suggest that oxidative stress is involved in Cr^{6+}-induced cytotoxicity in osteoblasts, and NAC can provide protection for osteoblasts against Cr^{6+}-induced oxidative stress. Cr^{3+} (5–20 μM) have no significant cytotoxicity in osteoblasts based on the results of this study.

Keywords Reactive oxygen species (ROS) · MTT · Alkaline phosphatase (ALP) · Antioxidant *N*-acetyl-cysteine (NAC) · Ultrastructure

Introduction

Prosthetic biomaterials have a large impact on replacement surgery such as dental implants, orthopedic implants, and artificial joints. Knowledge of biological processes occurring at the biomaterial–tissue interface is of utmost importance in predicting implant integration and host response. The interactions of cells and prosthetic metals have been the subject of many studies (Garvey et al. 1995; Yang et al. 2003). Some prosthetic alloys release metal ions to surrounding tissues and cause cytotoxicity (Granchi et al. 1996; Wataha and Lockwood 1998; Messer

J. Fu · X. Liang (✉) · Y. Chen · L. Tang ·
Q.-h. Zhang · Q. Dong
Key Laboratory of Oral Biomedical Engineering
of Chinese Ministry of Education,
West China College of Stomatology, Sichuan University,
Chengdu, Sichuan 610041, People's Republic of China
e-mail: Liangfujuninhuaxi@sina.com

et al. 1999; Wang et al. 2002; Schmalz and Garhammer 2002; Dayeh et al. 2005; Reclaru et al. 2005; Okazaki and Nishimura 2001; Okazaki and Gotoh 2005). But the mechanisms remain unclear. It has been long known that chromium exists widely in prosthetic alloys that belong to the first series of transition elements in the periodic system. Previous studies implicated the toxicity of chromium picolinate in renal impairment; skin blisters and pustules; anemia; hemolysis; tissue edema; liver dysfunction; neuronal cell injury, impaired cognitive, perceptual and motor activity; enhanced production of hydroxyl radicals; chromosomal aberration; depletion of anti-oxidant enzymes; and DNA damage (Bagchi et al. 2002). In the biological evaluation of metal ions, an interesting possibility would be to evaluate the cellular oxidative stress, resulting from the imbalance of cellular free radical generation and antioxidative defense, which is a common pathway in cellular responses to metal ions (Stohs and Bagchi 1993; Cuypers et al. 1999; Ercal et al. 2001; Shi et al. 2004; Elbekai and El-Kadi 2005). Oxidative stress is a ubiquitous phenomenon in all cell types, and it is primarily produced in mitochondria, which is essential for multicellular life. Oxidative stress is a host-defense mechanism whose involvement in maintaining homeostasis and/or inducing disease has been widely investigated over the past decade. Oxidative stress targets can be wide ranging and include nucleic acids and a variety of macromolecules.

A free radical may be defined as "any species capable of independent existence that contains one or more unpaired electrons" (Halliwell 1991). In recent years, the term "reactive oxygen species"(ROS) has been adopted to include molecules such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), and singlet oxygen ($^1O_2$), which, while not radicals in nature, are capable of radical formation in the extra- and intracellular environments (Halliwell and Gutteridge 1990). ROS can cause tissue damage by means of the following a variety of different mechanisms, including DNA damage, lipid peroxidation, and damage of protein, oxidation of important enzymes, and stimulation of proinflammatory cytokine released from monocytes and macrophages. "Oxidative stress" has broadly been defined as an unbalance in favor of pro-oxidants and disfavor of antioxidants. It is imposed as a result of one of three factors: (1) an increase in oxidant generation, (2) a

decrease in antioxidant protection, and (3) a failure to repair oxidative damage (Chapple 1996, 1997). Oxidative stress has been implicated in a variety of degenerative processes, diseases, and syndromes. Some of these include atherosclerosis, myocardial infarction, stroke, and ischemia/reperfusion injury; chronic and acute inflammatory conditions such as wound healing; central nervous system disorders such as forms of familial amyotrophic lateral sclerosis and glutathione peroxidase-linked adolescent seizures; Parkinson's disease and Alzheimer's dementia; and a variety of other age-related disorders (Davies and Pryor 2005; Kubo et al. 2006; Mantell 2006). Generally, the human body is protected from free radical-induced oxidative damage by various antioxidants with different functions which constitute a defense system either independently, cooperatively, or even synergistically. Antioxidants are becoming increasingly popular in oxidative stress-related disorders and hold promise as therapeutic agents (Mittoo 2006). Reduced glutathione (GSH), the most abundant low-molecular-weight thiol in animal cells, plays a central defense role in the antioxidant against ROS (Hultberg et al. 2001; Camera and Picardo 2002). Meanwhile, for tissue GSH synthesis, the availability of cysteine is generally the limiting factor, and one of the effective precursors of cysteine is its synthetic derivative, N-acetyl-cysteine (NAC). NAC may also provide SH-groups and scavenge ROS directly (Hsu et al. 2004; Grinberg et al. 2005; Barreiro et al. 2006; MacKenzie et al. 2006).

Although previous investigations have demonstrated that chromium ions induce oxidative damage in murine macrophages and human diploid dermal fibroblasts (Vandana et al. 2006; Rudolf et al. 2005; Rudolf and Cervinka 2006), little is known about the oxidative status of osteoblasts exposed to these metal ions. It is well known that bone remodeling depends on a delicate balance between bone formation and bone resorption, wherein bone-forming osteoblast and bone-resorbing osteoclasts play central roles. The metal ions released from prosthetic alloys probably affect the viability of osteoblasts and result in the resorption of bones and the aseptic loosening of the implants or artificial joints. Therefore, it is necessary to make osteoblasts as a model for evaluating the response to metal ions. The present study was designed to test the hypothesis that oxidative stress mediates chromium-induced cyto-

toxicity in rat calvarial osteoblasts, and antioxidant NAC can provide protection for osteoblasts against chromium-induced oxidative stress through assessing the effects of chromium ions on cell viability, alkaline phosphatase (ALP) activity, cellular ultrastructures, and intracellular levels of ROS in the presence or absence of NAC.

Materials and methods

Osteoblast isolation and culture

First of all, the protocol employed here was reviewed and approved by the Committee for Ethical Use of Experimental Animals at Sichuan University. Every effort was made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques. Rat (male Wistars) calvarial osteoblasts (RCOBS) were isolated from neonatal (<1 day) rat calvarias by a sequential enzymatic digestion method as described previously (Hinoi et al. 2002) and were used between passages 5 and 10. In brief, rat calvarias were gently incubated at 37°C for 10 min with 0.2% (*w/v*) collagenase in F-12 medium (GIBCO, Invitrogen, USA), followed by collection of cells in supernatants thus obtained. This incubation was consecutively repeated five times. Cells obtained from the last three digestion processes were altogether collected in F-12 medium containing 10% fetal bovine serum (Hyclone Labs, Logan, UT, USA) and several antibiotics (100 U/ml penicillin-G and 100 μg/ml streptomycin, Sigma), followed by centrifugation at $250 \times g$ for 5 min. The pellets were suspended in F-12 medium containing 10% fetal bovine serum (FBS). Cells were plated at a density of 1×10^4 cells/cm^2 in appropriate dishes, and then cultured at 37°C for different periods under 5% CO_2 with medium change every 2 days. Throughout experiments, F-12 medium containing 10% FBS, 50 μg/ml ascorbic acid and 5 mM sodium *b*-glycerophosphate was used. The phenotype was identified by immunohistochemical staining using osteocalcin antibodies (Biodesign, Kennebunk, ME, USA).

Preparation of ions solution

Cr^{6+} and Cr^{3+} were obtained from their metal salts (sodium dichromate in distilled, deionized water and

chromium chloride hexahydrate in distilled, deionized water) (Sigma-Aldrich, USA).

Cellular survival testing (MTT assay)

RCOBS seeded in 96-well plates (2×10^4 cells/well) were grown in complete medium for 24 h. The cell cultures were exposed to various concentrations of chromium ions (5, 10, and 20 μM) for 24 or 72 h. Also, to assess the ability of antioxidant NAC (Sigma-Aldrich, A9165, USA) to protect osteoblasts against chromium ion-induced cytotoxicity, osteoblasts were preincubated with NAC (0, 1, 2, and 5 mM) for 1 h. Then, the medium was removed, and cells were washed by cold phosphate-buffered saline (PBS). After that, cells were incubated for further 24 or 72 h with Cr^{6+} or Cr^{3+} (10 μM). In view of possible effects of NAC on the viability of osteoblasts, the NAC-only controls were added, too. At the end of the incubation period, 20 μl MTT solution (sigma, 0.5 mg/ml in PBS) was added into each well. After a 4-h incubation period at 37°C, the MTT solution was removed, and the insoluble fomazan crystals formed were dissolved in 200 μl of dimethyl surfoxide (Sigma, USA). The optical density (OD) was immediately measured at 570 nm using PERKIN ELMER HTS 7000 Plus Bio Assay Reader (PE, USA). The data represented three experiments and were shown as the mean±SD of sextuplicate wells.

Alkaline phosphatase activity assay

Osteoblasts seeded in 24-well plates were treated with chromium ions and NAC as before mentioned in MTT assay. The measurements of ALP activity and protein content were performed as described previously (Kirsch et al. 1997). Cells were lysed by sonication on 0.05% Triton X-100 in PBS for 60 s at room temperature. Total cellular ALP activity in the lysates was measured in 2-amino-2-methyl-1-propanol buffer, pH 10.3, with *p*-nitrophenyl phosphate as a substrate at 37°C (Sigma, USA). Reactions were terminated by the addition of 0.5 M NaOH. Absorbance of the reaction was measured at 405 nm using PERKIN ELMER HTS 7000 Plus Bio Assay Reader (PE, USA). Total protein levels in the lysates were measured according to Bradford (1976) using the bovine serum albumin as a standard. ALP activity was expressed as unit mg^{-1} protein. The data were

from a representative of three experiments shown as the mean±SD of quadruplicate wells.

Measurement of the level of reactive oxygen species

Production of ROS was measured using an oxidation-sensitive fluorescent probe, 2′7′-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich, D6665, USA) methods, based on the ROS-dependent oxidation of DCFH-DA to DCF. The fluorescent intensity was measured with flow cytometic analysis. Osteoblasts seeded in 6-well plates (1×10^6/well) were grown in complete medium for 24 h. The cells were treated with 10 μM Cr^{6+}, 10 μM Cr^{3+}, or 200 μM H_2O_2 (positive control) at 37°C for 15–120 min. To investigate whether NAC has the ability of decreasing the levels of ROS induced by chromium ions, we pretreated osteoblasts with various concentrations of NAC (0–5 mM) for 1 h. Medium was removed and cells were washed by cold PBS. And then, cells were incubated with 10 μM Cr^{6+}, 10 μM Cr^{3+}, or 200 μM H_2O_2 (positive control) for 30 min. After that, cells were detached by 250 mM trypsin/4 mM EDTA, and resuspended in F-12 medium without serum at a density of 2×10^6 cells/ml. DCFH-DA (10μM) was added for 30 min at 37°C in the dark. The cells were washed with F-12 medium without serum and resuspended in F-12 medium without serum. We used a FACScan flow cytometer (Becton Dickinson, USA), to measure ROS generation by the mean fluorescence intensity of 10,000 cells at an excitation wavelength of 495 nm and an emission wavelength of 530 nm. Mean fluorescence intensity was derived by Cell-Quest software.

Transmission electron microscopy

Osteoblasts were treated with different concentrations of Cr^{6+} in the presence or absence of 2 mM NAC as before mentioned in MTT assay. The cell suspension was immediately fixed with 2% glutaraldehyde, rinsed with PBS, and then postfixed in 1% sodium cacodylate buffered osmium tetroxide (OsO_4). The OsO_4-fixed specimens were subsequently dehydrated through a graded series of ethanol and finally embedded in situ by covering with a layer of Spurr epoxy resin (Polysciences, Warrington, PA, USA), which was allowed to polymerize. The intracellular ultrastructures were observed using transmission electron microscopy (TEM; CM-120, Philips, Eindhoven, Netherlands) at 80 kEv.

Statistical analysis

All variables were tested in three independent cultures for each experiment. The results were reported as a mean±SD of replicate wells. Statistical analysis of the data was performed by t-tests or a one-way analysis of variance (ANOVA) followed by a Tukey HSD test for the multiple comparison tests with the use of software SPSS 10.0. A value of $p < 0.05$ was considered statistically significant.

Results

Cell survival rates were assessed by the OD value in MTT assay after exposing osteoblasts to chromium ions (5–20 μM). It showed that incubating osteoblasts with Cr^{6+} induced a significant decrease in cell survival rates in a concentration- and time-dependent manner ($p < 0.05$). On the contrary, incubating osteoblasts in Cr^{3+} (5–20 μM) for 24 or 72 h did not result in significant negative effects on the survival rates of osteoblasts ($p > 0.05$; Fig. 1a). The protective effects of the antioxidant NAC on Cr^{6+}-induced cytotoxicity in cultured osteoblasts were illustrated in Fig. 1b. It was found that NAC afforded dose-dependent reduction to the cytotoxicity induced by Cr^{6+}. And also, 1–5 mM NAC alone had no significant effects on the survival rates of osteoblasts ($p > 0.05$; data were not shown). The concentrations of chromium ions in our study were based on the following reasons. We reviewed some literature about the studies of cytotoxicity induced by chromium ions. Messer (1999) reported the effects of metal ions on human gingival fibroblasts morphology. The concentration they chose was 0.5 ppm sodium dichromate. The concentration of Cr^{6+} was about 3.5 μM. According to their report, the morphology of human gingival fibroblasts was damaged significantly. Vandana (2006) researched the cytotoxicity of chromium ions in murine macrophages. The concentration of Cr^{6+} they chose was 500 ng/ml. It is about 20 μM. In our preliminary studies, we chose a wide range of concentrations of chromium (5–400 μM). We selected proper concentrations by MTT assay. The results showed that when

Fig. 1 a Survival rates of osteoblasts assessed by MTT assay after exposure to different concentrations of Cr^{6+} or Cr^{+3} (0–20 μM) for 24 or 72 h. *Single asterisk p<0.05, double asterisk p< 0.001*; significant difference compared to controls. The results were reported as mean±SD of three independent experiments. **b** Effects of NAC on Cr^{6+}-induced cytotoxicity. Osteoblasts were preincubated with NAC (0, 1, 2, and 5 mM) for 1 h. After that medium was removed, and cells were washed with cold PBS. And then, cells were incubated for further 24 or 72 h with 10 μM Cr^{6+}. In nontreated control groups, cells were grown in normal medium without any treatment. Survival rates of osteoblasts were examined by MTT assay. *Single asterisk p<0.05 compared to column 0 (group treated with 10 μM Cr^{6+}), double asterisk p<0.001 versus column 0. Single pound sign p<0.05 versus column nontreated control (untreated group), double pound sign p<0.001 versus column nontreated control.* The results were reported as mean±SD of three independent experiments
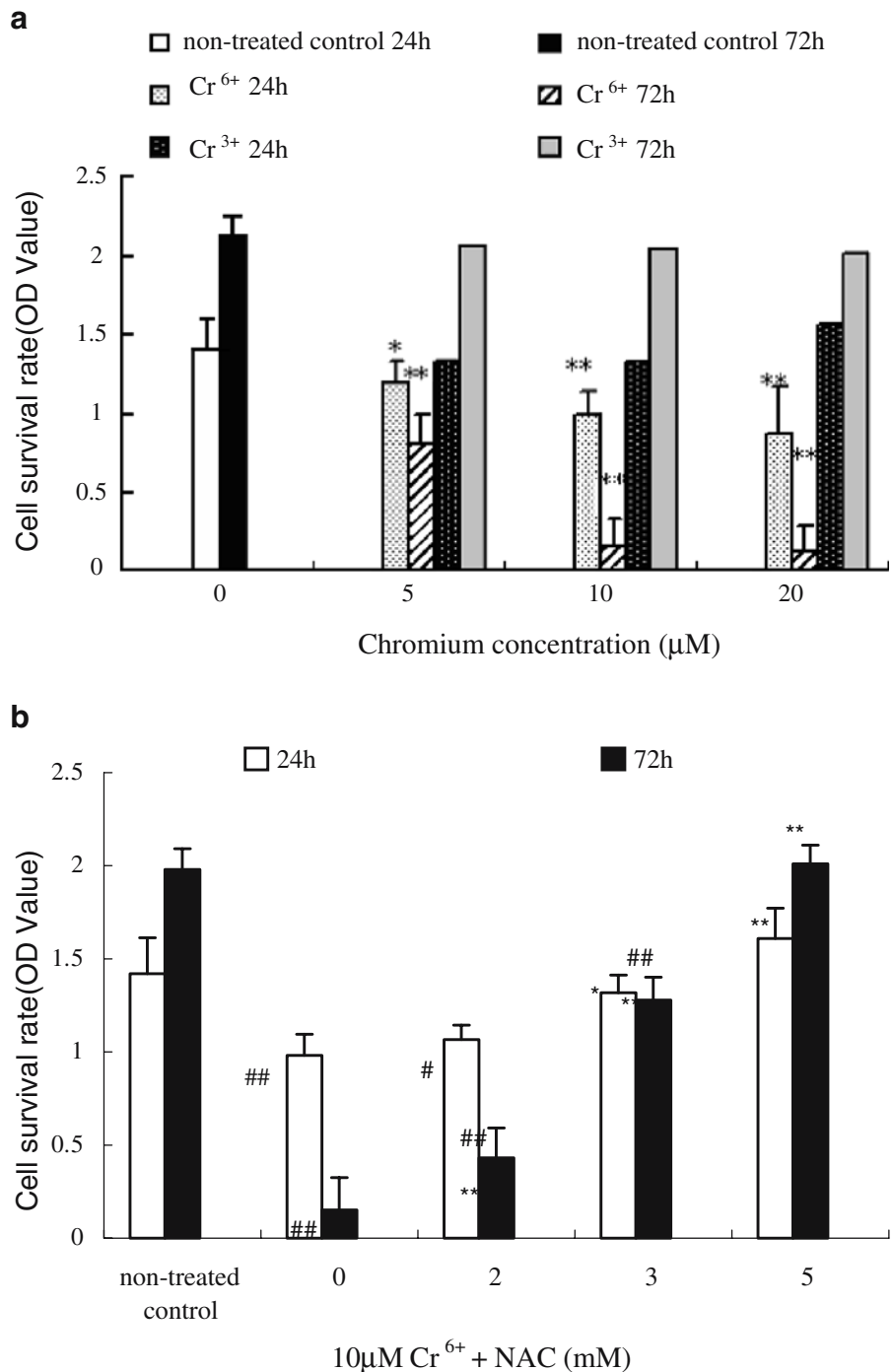

the concentration of Cr^{6+} was 40 μM or higher, after 24 h incubation, the OD value was zero. When the concentrations were in the range of 5–20 μM, the OD value reduced in a dose-dependent manner, and the differences were significant ($p<0.05$). And when the osteoblasts were incubated in 10 μMCr^{6+} for 24 h,

OD value was about half of the control groups. On the other hand, the concentrations of metal ions released from prosthetic alloys were lower than that in in vitro study. The effects of metal ions on human body is multiple and correlative with the microsurroundings and incubation time. So in in vitro study, the

concentrations of metal ions should be higher than in in vivo study. Based on these reasons, we decided the concentrations of chromium ions in our study.

The ALP activity, which indicates osteoblast differentiation directly, was measured as a second marker of the chromium-induced cellular injury of osteoblasts. Osteoblasts firstly were treated in the absence (control) or presence of various concentra-

tions of Cr^{6+} or Cr^{3+} for 24or 72 h. Then, ALP activity was tested. Exposing osteoblasts to Cr^{6+} (5–20 μM Cr^{6+}) inhibited ALP activity dose- and time-dependently with a maximal effect at 20 μM for 72 h ($p<0.001$; Fig. 2a). In contrast, exposure to Cr^{3+}(5–20 μM) for 24 or 72 h had no significant negative effects on the ALP activity in osteoblasts ($p>0.05$; data were not shown). When the cells were pretreated
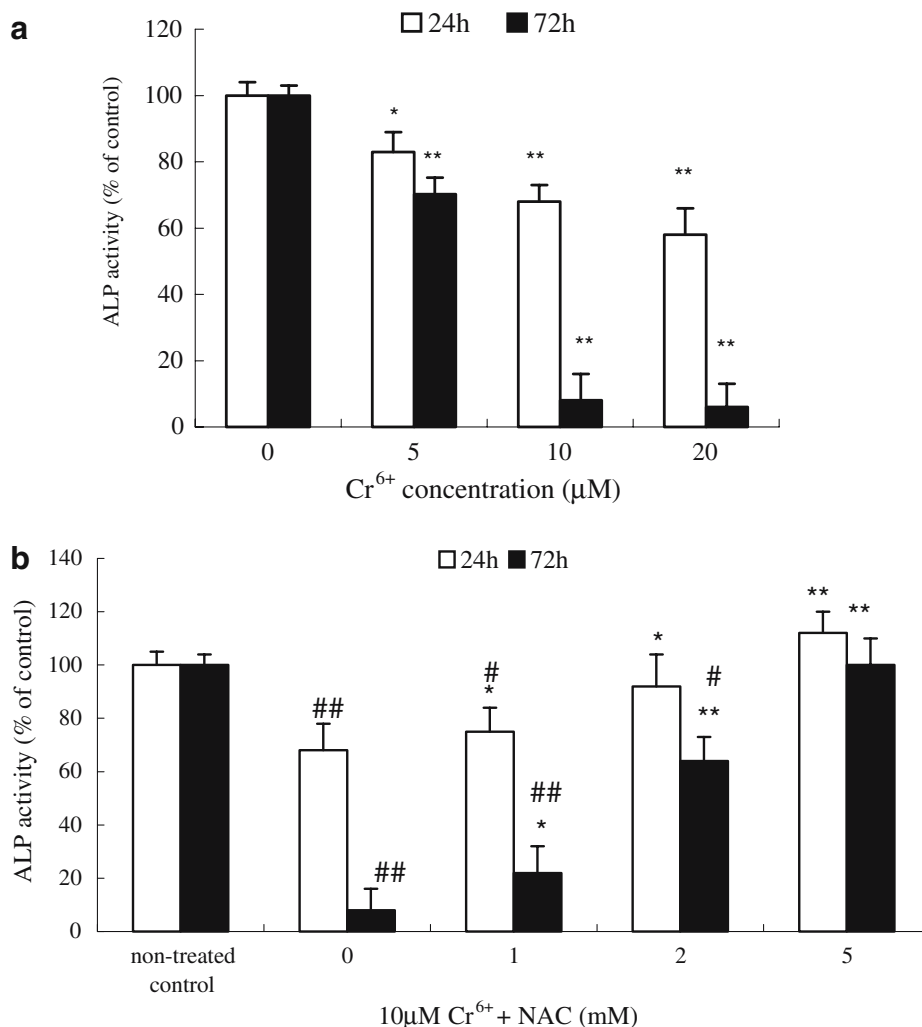
Fig. 2 a Effects of Cr^{6+} on ALP activity in osteoblasts. Cells were treated for 24 or 72 h with the indicated concentrations of Cr^{6+} (μM). ALP activity was measured from whole-cell lysates as described in "Materials and methods". Single asterisk $p<0.05$, double asterisk $p<0.001$; significant difference compared to controls. The results were reported as mean±SD of three independent experiments. b Effect of NAC on the Cr^{6+}-induced reduction of cells' ALP activity. Osteoblasts were preincubated with NAC (0, 1, 2, and 5 mM) for 1 h. After that, medium was removed, and cells were washed with cold PBS. And then, cells were incubated for further 24 or 72 h with 10 μM Cr^{6+}. In nontreated control groups, cells were grown in normal medium without any treatment. ALP activity was measured as mentioned. Dose-dependent reductions to the cytotoxicity induced by Cr^{6+} were observed significantly. Single asterisk $p<0.05$ versus column 0 (group treated with 10 μM Cr^{6+}), double asterisk $p<0.001$ versus column 0. Single pound sign $p<0.05$ versus column nontreated control, double pound sign $p<0.001$ versus column nontreated control. The results were reported as mean±SD of three independent experiments

with 1–5 mM NAC and then exposed to 10 μM Cr^{6+} for 24 or 72 h, ALP activity was measured also. Figure 2b showed the effects of NAC on the ALP activity of cells incubated with Cr^{6+} for 24 or 72 h. It was shown that Cr^{6+}-induced decrease of ALP activity was restrained by NAC in a dose-dependent manner ($p < 0.05$). An amount of 1–5 mM NAC alone did not have significant effects on the ALP activity in osteoblasts ($p > 0.05$; data were not shown).

To investigate if the cytotoxicity of the chromium ions is correlated with the oxidative stress, the intracellular ROS production was monitored. It was found that intracellular amounts of the ROS increased in a time-dependent pattern when cells were exposed to 10 μM Cr^{6+} or 200 μM H_2O_2 (positive control) for 15–120 min ($p < 0.05$). The amounts of ROS in osteoblasts incubated in 10 μM Cr^{6+} increased immediately during the first 30 min (220% of the control), and then the increase became slower. At the end of the 120 min, the amount of ROS was about 310% of the control. The similar trend was observed in the positive control groups. In contrast, exposure to 10 μM Cr^{3+} did not induce a significant ROS increasing in our study ($p > 0.05$; Fig. 3a). NAC decreased the amounts of intracellular ROS induced by 10 μM Cr^{6+} or 200 μM H_2O_2 (positive control) in a dose-dependent manner significantly ($p < 0.05$; Fig. 3b).

Figure 4a–f showed TEM photographs of the 0–20 μM Cr^{6+}-damaged osteoblasts pretreated with or without 2 mM NAC. The nontreated osteoblasts were observed in Fig. 4a, showing such organelles of normal active cells, as a regular nucleus, a large amount of rough endoplasmic reticulum (rER), a well-developed Golgi apparatus, and variously shaped mitochondria. The cells treated with Cr^{6+} had irregular nucleus, swollen mitochondria, and disorganized rER and contained numerous vacuoles in cytoplasm (Fig. 4b–d). These intracellular ultrastructural alterations were noticeably reduced by the NAC pretreatment (Fig. 4e). NAC-alone-treated cells were similar with nontreated control groups (Fig. 4f). The photographs shown in this figure were representative of three independent experiments, showing similar results.

Discussion

Prosthetic alloys release metal ions to surrounding tissues and cause cytotoxicity, but the biological mechanisms are still largely undisclosed (Yang and Pon 2003; Lin and Bumgardner 2004; Lopez-Alias et al. 2006; Cobb and Schmalzreid 2006). Chromium exists widely in prosthetic alloys and belongs to the first series of transition elements in the periodic system. Chromium ions can occur in oxidation states of −2 to +6. The most common valence is +2, +3, and +6. But divalent chromium does not occur in biological or aqueous systems due to its strong reducing power. So in the present study, Cr^{6+} and Cr^{3+} were tested. Cytotoxicity induced by chromium ions was studied by many investigators. Hexavalent chromium compounds induced dose-dependent cytotoxicity and anchorage independence in cultured human diploid fibroblasts (Rudolf et al. 2005; Rudolf and Cervinka 2006). Soluble hexavalent chromium compound calcium chromate (CaCrO4) was shown to induce dose-dependent cytotoxicity in C3H/10T1/2 mouse embryo cells and dose-dependent cytotoxicity and mutagenesis in Chinese hamster ovary cells (Patierno et al. 1988). Metal ions damaged the morphology and ultrastructure of human gingival fibroblasts. Ultrastructural alterations observed included irregular-shaped nuclei and formation of ER-derived vacuoles for cells exposed to hexavalent chromium (Messer et al. 1999). Cellular oxidative stress is a common pathway in cellular responses to metal ions. Vandana et al. (2006) demonstrated that Cr^{6+} induced cytotoxicity and oxidative stress in murine macrophages. Vitamins C and E and beta-carotene inhibited oxidative stress induced by chromium. Wataha et al. (2000) researched the effects of metal ions on GSH levels in THP-1 human monocytes. The results indicated that metal ions induced oxidative stress in monocytes which is manifested by a lower GSH-GSSG ration.

Few studies focused on the cytotoxicity and oxidative stress in osteoblasts. It has been known that the proliferation and biological activity of osteoblasts have important effects on bone remodeling. The integration between bones and biological implants is the key factor to the success of replacement surgery such as dental implants. Any deleterious effects on osteoblasts can result in the loss of the bones and the failure of the implants. We used rat calvarial osteoblasts as a model to research the relation between chromium ion-induced cytotoxicity and oxidative stress. The culture of the rat calvarial osteoblasts is a very classical technique and popularly used in the

a

b
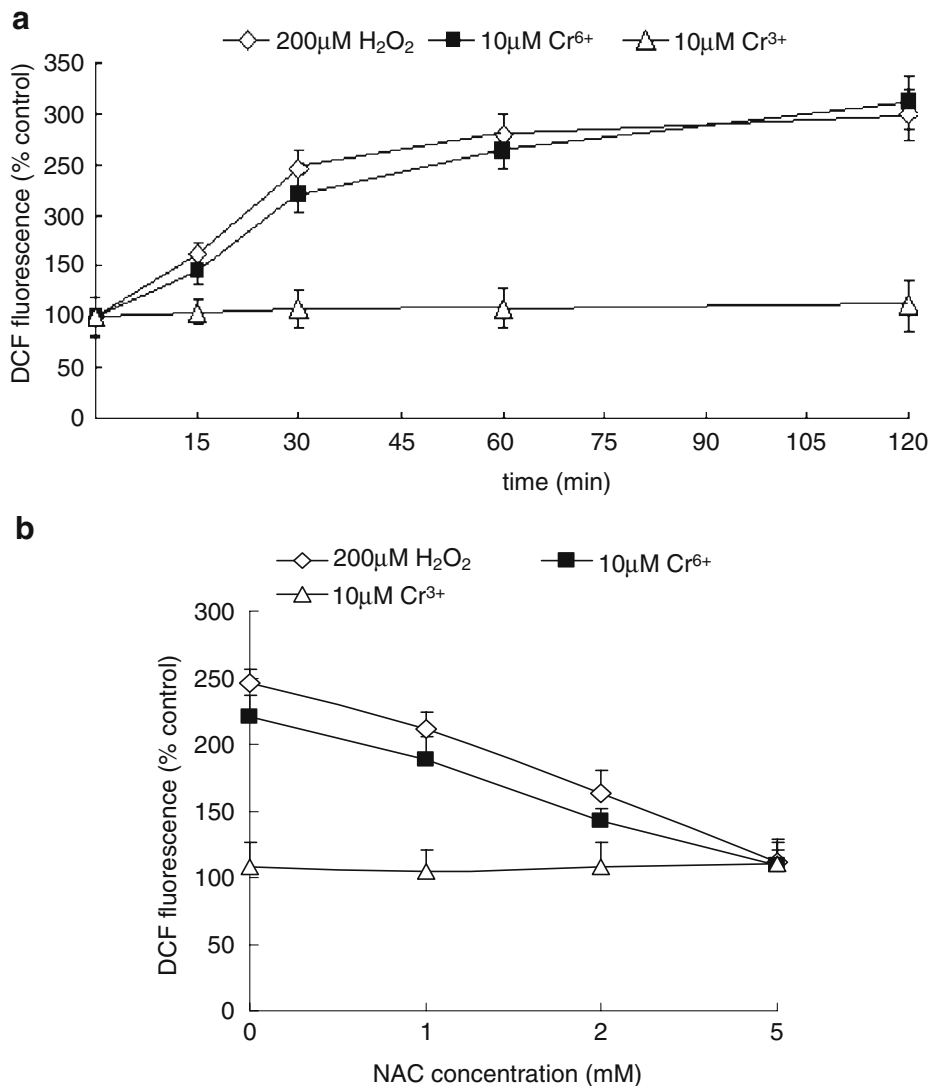
Fig. 3 a Production of ROS in osteoblasts, stimulated by H_2O_2 (positive control), Cr^{6+} or Cr^{3+}. Osteoblasts were treated with 200 μM H_2O_2, 10 μM Cr^{6+}, or 10 μM Cr^{3+} for 15–120 min. Then, cells were loaded with 10 μM DCFH-DA for 30 min. The formation of DCF was monitored by flow cytometer at an excitation wavelength of 495 nm and an emission wavelength of 530 nm. Control (cells without any treatment) re-presents the basal level of intracellular ROS production. The results were reported as mean±SD of three independent experiments. **b** Effects of different concentrations of NAC (1–5 mM) on the production of ROS induced by chromium ions in osteoblasts.

The cells were preincubated with NAC (0, 1, 2, and 5 mM) for 1 h, and then medium was removed, and cells were washed with cold PBS. After that, cells were incubated for further 30 min with 200 μM H_2O_2 (positive control), 10 μM Cr^{6+}, or 10 μM Cr^{3+}. After that, cells were loaded with 10 μM DCFH-DA for 30 min. The formation of DCF was monitored by flow cytometer at an excitation wavelength of 495 nm and an emission wavelength of 530 nm. Control (cells without any treatment) represents the basal level of intracellular ROS production. The results were reported as mean±SD of three independent experiments

research of osteoblasts in vitro (Dee et al. 1996; Geissler et al. 2000; Park et al. 2003; Utting et al. 2006). Moreover, this culture can obtain enough osteoblasts in a high degree of pureness.

In this study, the injurious effects of chromium on both viability and differentiation in osteoblasts were examined using MTT assay and ALP activity assay. MTT assay is known to be used as a means of

Fig 4 **a** TEM of control cells (×10,000). **b** TEM of cells exposed to 5 μM Cr^{6+} for 24 h (×10,000). **c** TEM of cells exposed to 10 μM Cr^{6+} for 24 h (×8,000). **d** TEM of cells exposed to 20 μM Cr^{6+} for 24 h (×8,000). **e** TEM of cells pretreated with 2 mM NAC for 1 h and then exposed to 10 μM Cr^{6+} for 24 h (×8,000). **f** TEM of cells pretreated with 2 mM NAC for 1 h and then incubated in normal medium for 24 h (×6,000)
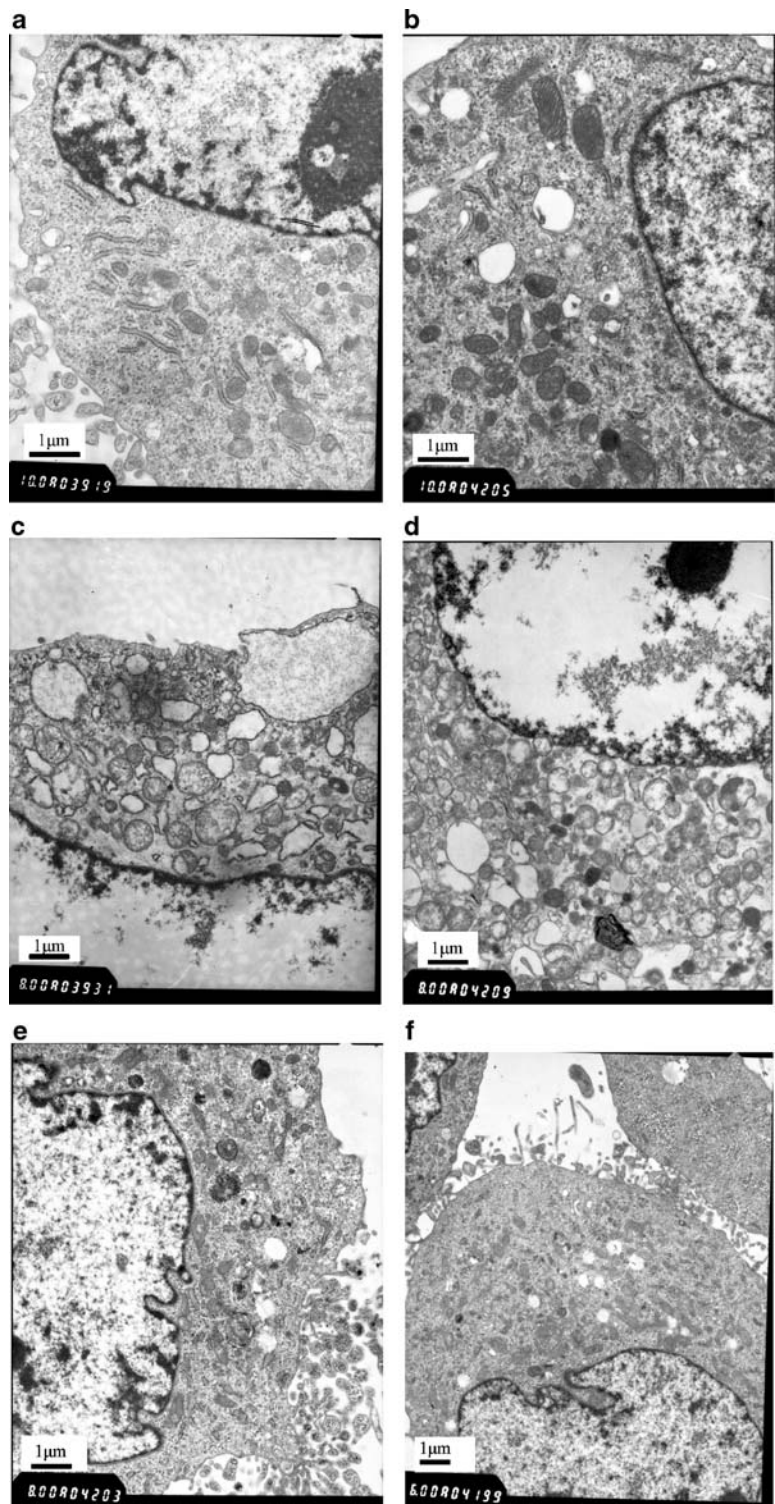
assessing cell viability. ALP is considered a marker enzyme of osteoblast differentiation in in vitro experiments, since ALP can mediate bone mineralization by decomposing phosphate compounds and stimulating the combination of phosphate and calcium in extracellular matrix (Lee et al. 2006). Furthermore, intracellular ultrastructural alterations were observed by TEM when osteoblasts were exposed to 5–20 μM Cr^{6+} for 24 h. It was clear now from the present study that the species of chromium were toxic at different degrees at different stages in osteoblasts. Exposure to Cr^{6+} (5–20 μM) induced a time- and concentration-dependent decreased OD value in MTT assay, and ALP activities were reduced. The intracellular ultra-structures were destroyed, too. It indicated that Cr^{6+} induced cytotoxicity in osteoblasts in a time- and concentration- dependent manner. Cr^{3+} (5–20 μM) did not result in significant effects on the survival rates and ALP activity in osteoblasts.

To verify the hypothesis that oxidative stress mediates chromium-induced cytotoxicity in rat calvarial osteoblasts, the intracellular ROS production was measured by DCFH-DA probe. 2,7-Dichlorofluorescin diacetate is a nonpolar compound that readily diffuses into cells and is cleaved by intracellular esterase to the nonfluorescent derivative DCFH. This is trapped within the cells owing to its polarity. Upon production of ROS (mainly hydrogen peroxide), this compound is oxidized to DCF with emission of fluorescence. The fluorescence intensity is directly proportional to the level of DCF formed intracellularly. The fluorescence values were quantified using flow cytometer (Kampen et al. 2004; Spagnuolo et al. 2004). Time 120 min was chosen as an end point for the assay, as ROS have been produced to a substantial amount at this time, which is also technically convenient. From the results we can see that intracellular ROS production increased significantly when exposed to 10 μM Cr^{6+} at 15–120 min. The trend was similar to H_2O_2 (positive control). About 10 μM Cr^{3+} did not result in a significant increase in the level of ROS. Noticeably, during the first 30 min, the level of ROS increased more quickly when exposed to 10 μM Cr^{6+} or 200 μM H_2O_2, and the increase became slow at 30–120 min. The possible reason was that ROS formation is very transient and declines immediately. With the development of the damage, we were probably not measuring ROS caused by the Cr directly but rather cellular ROS secondarily induced

from damage by the Cr. So the increase of the ROS production became slow. It should be pointed out that DCFH-DA is an oxidation-sensitive fluorescent probe. Formation of fluorescent DCF has been typically considered to be a result of oxidative reaction generated by ROS during the reduction of chromium from hexavalent to trivalent. Quievryn et al. (2001, 2003) made an in-depth study on the mechanism of Cr^{6+}-induced toxicity and mutagenesis. They found that DCF fluorescence probably partially came from DCFH oxidation by intermediate chromium forms such as CrV and CrIV. So some other quantitative methods should be added to measure intracellular ROS production in our study in the future.

The toxic properties of Cr^{6+} originate from the action of this form itself as an oxidizing agent, as well as from the formation of free radicals during the reduction of Cr^{6+} to Cr^{3+} occurring inside the cells. The chromate ion [CrO4]2, the dominant form of Cr^{6+} in neutral aqueous solutions, can readily cross cellular membranes via nonspecific anion carriers, while Cr^{3+} is poorly transported across membranes. The differences in membrane transport explain the differences in the abilities of these two valence states of chromium to induce the formation of ROS and produce oxidative tissue damage. On the other hand, Cr^{3+} is not an oxidizing agent and cannot produce ROS. So it cannot induce cellular oxidative stress. In our study, cells exposed to Cr^{3+} (5–20 μM) exhibited no changes in survival rates and maturation in osteoblasts. Some other studies demonstrated that exposure to high concentrations of Cr^{3+} can also cause toxic effects due to its ability to coordinate various organic compounds resulting in inhibition of some metalloenzyme systems (Messer et al. 2000).

NAC is the pre-acetylized form of the simple amino acid cysteine, a powerful antioxidant, premier antitoxin, and immune support substance, and is found naturally in foods. It is a precursor for GSH, an important antioxidant that protects cells against oxidative stress (Ramudo et al. 2005). NAC is a more stable form of L-cysteine because it has an acetyl group (CH_3CO) attached and has all the properties of L-cysteine but is more water-soluble and said to be more bioavailable than L-cysteine. Mohamadin and Abdel-Naim (1999) researched chloroacetonitrile (CAN)-induced toxicity and oxidative stress in rat gastric epithelial cells (GECs). They found that

pretreatment of GECs with 5 mM NAC plays a critical role against CAN-induced cellular damage and oxidative stress. Suppression of oxidant-mediated toxicity in chromate-treated cells preincubated with 1 mM NAC has already been found in hamster CHO cells (Messer et al. 2006). The concentrations of NAC chosen in our study were based on literature reports and our preliminary studies. From our results, we found that 1–5 mM NAC afforded dose-dependent reduction to the cytotoxicity and the level of cellular ROS induced by Cr^{6+}, which supports a role for NAC as a free radical scavenger (Barreiro et al. 2006; MacKenzie et al. 2006). The results can be attributed to direct interaction of NAC with ROS or enhancement of cellular GSH synthesis. On the other hand, NAC has some side effects, including being pro-oxidant, suppressing respiratory burst, and causing a toxic ammonia accumulation in case of liver problems (Holdiness 1991; Jones et al. 1997). More attention should be paid to the selection of the indication and dose of NAC in clinical application.

From these studies, it was shown that exposing osteoblasts to Cr^{6+} inhibited cell viability and enhanced intracellular ROS production. Cr^{6+}-induced oxidative stress was at least partly responsible for its cytotoxicity. Antioxidant NAC can play a critical role against Cr^{6+}-induced cellular damage.

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