The cytotoxicity of corrosion products of nitinol stent wire on cultured smooth muscle cells

Chun-Che Shih,[1,2] Shing-Jong Lin,[1,3,4] Yuh-Lien Chen,[5] Yea-Yang Su,[6] Shiau-Ting Lai,[2] Gaston J. Wu,[7] Ching-Fai Kwok,[8] Kwok-Hung Chung[9]

[1]*Institute of Clinical Medicine, National Yang-Ming University School of Medicine, Taipei 112, Taiwan*
[2]*Division of Cardiovascular Surgery, Taipei Veterans General Hospital, Taipei 112, Taiwan*
[3]*Division of Cardiology, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-Pai Road, Taipei 112, Taiwan*
[4]*Cardiovascular Research Center, National Yang-Ming University, Taipei 112, Taiwan*
[5]*Institute of Anatomy and Cell Biology, National Yang-Ming University, Taipei 112, Taiwan*
[6]*Amorphous Technologies Inc., 965 Alexis Way, Marietta, Georgia 30066-3860*
[7]*Department of Chemistry, National Taiwan Normal University, Taipei 112, Taiwan*
[8]*Division of Endocrinology and Metabolism, Taipei Veterans General Hospital, Taipei 112, Taiwan*
[9]*Department of Dentistry, Taipei Veterans General Hospital, Taipei 112, Taiwan*

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Abstract: Although nitinol is one of most popular materials of intravascular stents, there are still few confirmative biocompatibility data available, especially in vascular smooth muscle cells. In this report, the nitinol wires were corroded in Dulbecco's modified Eagle's medium with constant electrochemical breakdown voltage and the supernatant and precipitates of corrosion products were prepared as culture media. The dose and time effects of different concentrations of corrosion products on the growth and morphology of smooth muscle cells were evaluated with [^{3}H]-thymidine uptake ratio and cell cycle sorter. Both the supernatant and precipitate of the corrosive products of nitinol wire were toxic to the primary cultured rat aortic smooth muscle cells. The growth inhibition was correlated well with the increased concentrations of the corrosion products. Although small stimulation was found with released nickel concentration of 0.95 ± 0.23 ppm, the growth inhibition became significant when the nickel concentration was above 9 ppm. The corrosion products also altered cell morphology, induced cell necrosis, and decreased cell numbers. The cell replication was inhibited at the G0-G1 to S transition phase. This was the first study to demonstrate the cytotoxicity of corrosion products of current nitinol stent wire on smooth muscle cells, which might affect the postimplantation neointimal hyperplasia and the patency rate of cardiovascular stents. © 2000 John Wiley & Sons, Inc. J Biomed Mater Res, 52, 395–403, 2000.

Key words: corrosion; nitinol; cytotoxicity; nickel; smooth muscle cell

INTRODUCTION

Coronary stenting has been widely used clinically for the treatment of coronary artery disease. The constitution and surface characteristics of the stent determine the nature of host response, and immediate as well as long-term patency rates.[1] Research efforts have been focused on improving their surface properties.[2] However, the electrochemical corrosion effects on the tissue biocompatibility have not been well studied, especially in vascular smooth muscle cells. Most of the current quantitative analyses of metal corrosion toxicity or biocompatibility use a combination method of incubating cultured cells in metal-immersed media.[3–8] Alloy discs,[3] wires,[4] particulates[5] of test metals have been used in cell cultures to explore the dose–effect relationship.[4] The cell growth around pieces of metal in culture dishes was observed and compared with that in control cultures. However, even in the same experimental conditions, the occurrence and severity of corrosion could not be expected and controlled; therefore, variable amounts of corrosion products could be produced. The release of oxides and metal

Correspondence to: S.-J. Lin; e-mail: sjlin@vghtpe.gov.tw
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ions could be detected in the first few days immediately after the stent implantation.[3] However, corrosive breakdown might take place any time, even several years after implantation. At the tissue contact interface, the released ions may be "burst-out" locally, accumulated, and then taken up by the adjacent cells.[6] The cytotoxicity of these corrosion products needed to be evaluated.

High concentrations (15–30 ppm) of either cobalt or nickel have been shown to cause morphological changes and depression in the cell growth rate of cultured fibroblasts.[7,8] The purpose of this study was to evaluate the effects of corrosion products of nitinol wire on the growth and morphology of cultured rat aortic smooth muscle cells. Furthermore, the affected phases of cell replication were analyzed by cell cycle sorter. Both the early leaching-ion effects by low metal dissolution and late breakdown or long-term cumulative effects of metal corrosion by a range of higher metal dissolution were studied.

MATERIALS AND METHODS

Initiation and assurance of corrosion occurrence by using Potentiostatic control

The electrochemical corrosive breakdown of nitinol wires was initiated by applying a potential higher than the predicted breakdown potential. This was conducted by the three electrodes system and recorded by a computer program of Potentiostat (EG & G model 273) as described previously.[9–12]

Collection of corrosive products at different applied potentials

The three electrodes system consisting of a working (nitinol wire), a reference AgCl, and a counter platinum wire were degreased with 70% ethanol in an ultrasonic vibrobath and sterilized by exposure to a dose of 2.5×10^4 Gy-radiation before each experiment. The nitinol wires tested in this study have a polycrystalline oxide surface. The surface oxide of nitinol wires, has been determined by transmission electron microscopy, selected area diffraction, and scanning electron microscopy.[10–12] The chemical composition of the polycrystalline oxide film was determined by the Energy Dispersion X-ray Analysis or Energy Dispersion Spectrum. Five nitinol wires, 0.2 mm in diameter and 15 cm in length, were used in this study.

After filling 50 mL of Dulbecco's modified Eagle's medium (DMEM) into plastic centrifugal tubes, three electrode wires were immersed separately. Different potentials were applied and recorded. The mild leaching medium was prepared with nitinol wire immersed and without any applied potential for 24 h in a 37°C water bath. The severe and cumulative corrosive media were prepared with nitinol wires immersed and with a constantly applied potential at +1.0 V (reference to AgCl electrode) potentiostatic control for 6 h in a 37°C water bath. As described previously,[9–14] the wires of nitinol or stainless steel covered with polycrystalline oxide usually showed corrosion breakdown at the relatively low potentials between +0.2 to +0.6 V versus AgCl reference electrode. The electrochemical corrosive reaction continued until the nitinol wire had been completely dissolved. These media contained both the solid precipitated corrosive products and dissolved ion solutes. After centrifugation, the supernatant and the precipitates were separated and collected, respectively. The corrosive precipitates were resuspended with standard DMEM. The DMEM without wire immersion and any potential application was used as the control assay.

The nickel ion concentration in each conditioned medium was measured by a graphite atomic absorption spectrophotometer. To all of these media were added the same amount of 10% fetal calf serum and 1% penicillin (100 IU/mL) plus streptomycin (100 mg/mL), and pH value was maintained between 7.30 and 7.35 after sterilization.

Surface morphology analysis

Corrosive degradation of the wires after experiment was examined with a scanning electron microscope (SEM) (Hitachi model S-800, San Jose, CA). To prevent the charge problem, samples were sputtered with a thin layer of gold. SEM operated at 15 kV was used to characterize the wire surface and determine the degree of degradation.

Aortic smooth muscle cell cultures

Rat aortic smooth muscle cells were isolated and cultured according to a modified method of Kwok et al.[15] About 10^5 cells, between the 6th and 16th passage, were cultured in 12-well Falcon tissue culture plates (2.9 cm^2/well), and incubated in DMEM in a humidified atmosphere at 37°C in the presence of 5% CO_2. The cell growth and morphology were examined regularly with a phase contrast microscope. The cells were characterized as smooth muscle cells by morphological criteria,[16] and with a murine anti-α-actin antibody to recognize a unique epitope of α-smooth muscle actin.

The influence of specially prepared culture media on the growth of rat aortic smooth muscle cells

After the cells had been growing to occupy above 90% of the wells, serum-free condition was maintained for 12 h. One negative control polystyrene patch immersed DMEM

and three specially prepared culture media (media with wire immersed and without any applied potential, media with supernatant portion of corrosive products after 1.0 V potential applied, and media with precipitate portion of corrosive products after 1.0 V potential applied) were poured into 12-well plates in triplicate.

Twelve hours later, [^{3}H]-methyl thymidine was added to some plates of cultured cells for DNA incorporation test. The other plates were trypsinized down the cells from each well and stored in 10% dimethylsulfoxide with fetal calf serum and DMEM at –70°C for further cell cycle analysis.

[^{3}H]-thymidine uptake test

The uptake of radiolabeled [^{3}H]-thymidine was used as an index of cell proliferation. DNA synthesis was quantified by measuring [^{3}H]-thymidine uptake as described previously.[15] Wells added with standard DMEM were used as controls. The negative-control media, mild leaching media, and different dilution media of severe corrosion were prepared and tested simultaneously. The smooth muscle cells, grown in 12-well plates, were incubated with the addition of 1 mCi/mL [^{3}H]-thymidine at 37°C for 2 h. The exposure to [^{3}H]-thymidine was terminated by washing the cells twice, replacing the medium with ice-cold phosphate buffer saline (pH 7.2 without Ca^{2+} and Mg^{2+}). After solubilizing the cells in 0.1% sodium dodecyl sulphate for 30 min, aliquots were taken for protein assay and liquid scintillation counting. The radioactivity of aliquots was measured in a Beckman, LS-6500 liquid scintillation counter after DNA was precipitated with ice cold 10% trichloroacetic acid and filtered with GF/C glass microfibre filters (Whatman 24-mm ϕ circle). Protein content of the solubilized cells in each well was also determined by the method of Kwok et al.[15] with some modifications. Briefly, 0.1 mL of aliquots of solubilized cells was mixed and reacted with 0.8 mL of working solution A (2% Na_2CO_3 in 0.1*N* NaOH/1% $CuSO_4$/2% NaK = 100:1:1) for 10 min. Then, after adding 0.1 mL of solution B (Folin-phenol/H_2O = 1:1) and standing for 30 min, the resulting solutions were checked for absorbance at 562 nm using a Beckmann DU-64 spectrophotometer. Standards were mixed using bovine serum albumin from 0 to 0.6 mg/mL.

Cell cycle analysis

DNA ploidy and content were analyzed by flow cytometry (Epics Elite, Coulter, Miami, FL) following the method of McGuire[17] with some modifications. The frozen cells from each well, which stored at –70°C, were defrosted to 37°C immediately, centrifuged for 30 s, and the supernatant solution was discarded. A 0.5-mL staining solution A [50 μg/mL propidium iodide (No. p-4170; Sigma Chemical Co., St. Louis, MO), 3% polyethyleneglycol 6000 (ART 807491; Merck, Darmstadt, Germany), 0.1% Triton x-100, 180 U/mL RNAase (No. R-5503; Sigma), 4 m*M* citrate buffer, pH 7.2] was added, incubated at 37°C for 20 min, followed by the addition of 0.5 mL staining solution B [50 μg/mL propidium iodide, 3% polyethyleneglycol 6000, 0.1% Triton X-100, 0.4*M* NaCl, pH 7.2]. The samples were then stored at 4°C for 1 h and filtered before being analyzed using a flow cytometer.

The time-courses and dose-response studies of [^{3}H]-thymidine uptake inhibition by severe corrosive media

Five culture media with the supernatant of severe corrosion of test nitinol wires were mixed together and the final nickel concentration was detected. Then this culture medium was diluted further and separated into 1×, ½×, ¼×, and 1/16× dilution groups for time-course and dose-response studies. During these studies, the incubation period for each different dilution of corrosion medium was 12 h. At time intervals of 0, 6, 8, 10, and 11 h, culture media of near confluent smooth muscle cells in each 12-well plate were replaced with standard DMEM, negative control polystyrene media, or severe corrosion media of different dilutions simultaneously and sequentially with duplicate individual samples.

The detection of nickel dissolution

The supernatant of corrosion media was analyzed by a graphite atomic absorption spectrophotometer, GFAAS (Hitachi Z 8100), with deuterium and polarized Zeeman-effect background correction system and autosampler. Medium samples were analyzed according to the thermal programs described in Table I with reference setting at 232.0-nm wavelength, 10-mA lamp current, 0.4-nm slit, and 20-μL sample volume.

STATISTICS

The data of nickel concentration were expressed as ppm and data of [^{3}H]-thymidine uptake were calculated as absolute value in c.p.m. per milligram of protein. The effects of negative control and corrosion

TABLE I
The Thermal Programs for Nickel Ion Detection in Culture Media by Graphite Atomic Absorption Spectrophotometry

Step	Stage	Temperature (°C) Start	Temperature (°C) End	Time (s)	Carrier Gas (mL/min)
1	Dry	80	120	30	200
2	Ash	1200	1200	30	200
3	Atom	2850	2850	10	30
4	Clean	3000	3000	4	200

products of different treatments were expressed as the ratio of [^{3}H]-thymidine uptake as compared with control uptake of vascular smooth muscle cells cultured with standard DMEM. Six experiments were performed on triplicate individual samples.

In the time-course and dose-response curves, the [^{3}H]-thymidine uptake experiment of different dilutions of corrosion media at different time intervals was expressed as individual points. Each point was the mean of six measurements on duplicate individual samples and expressed as mean ± standard deviation of the percentage of uptake in the control group. The cell cycle phase analysis was performed on duplicate individual samples with a total of six experiments.

The difference of means among and within groups was compared by analysis of variance, using a Scheffe test for *post hoc* comparisons. A $p < 0.05$ was considered statistically significant.

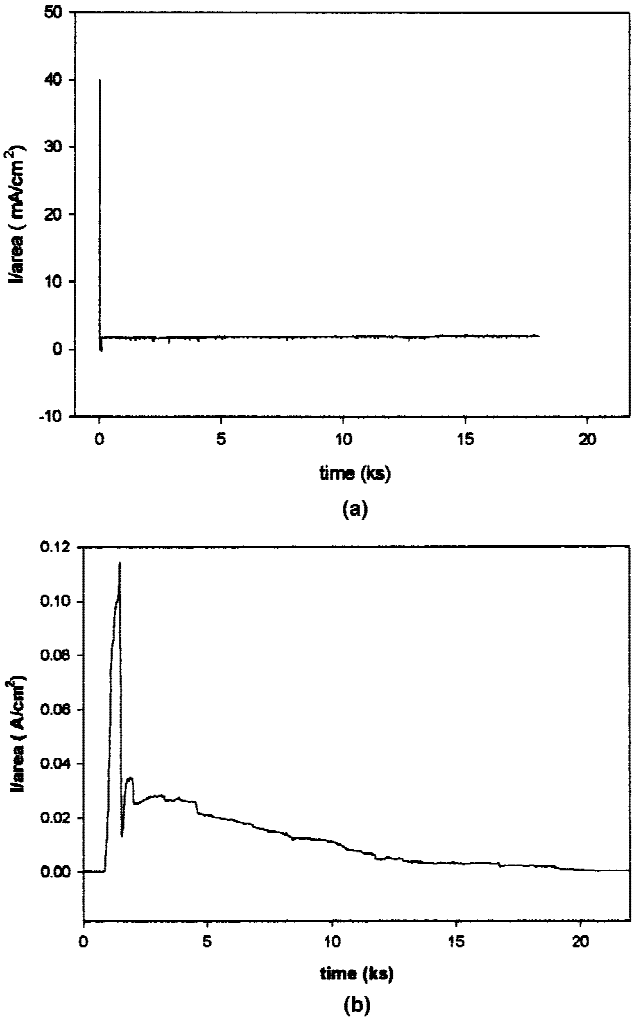

Figure 1. The initiation and occurrence of nitinol wire corrosion recorded for 4–6 h by Potentiostat. (a) Flat linear curve without any applied potential voltage and nickel dissolution in media with concentration of 0.97 ppm. (b) Curve of severe corrosion with conduction of 1.0 V voltage and nickel dissolution with a concentration of 208 ppm.

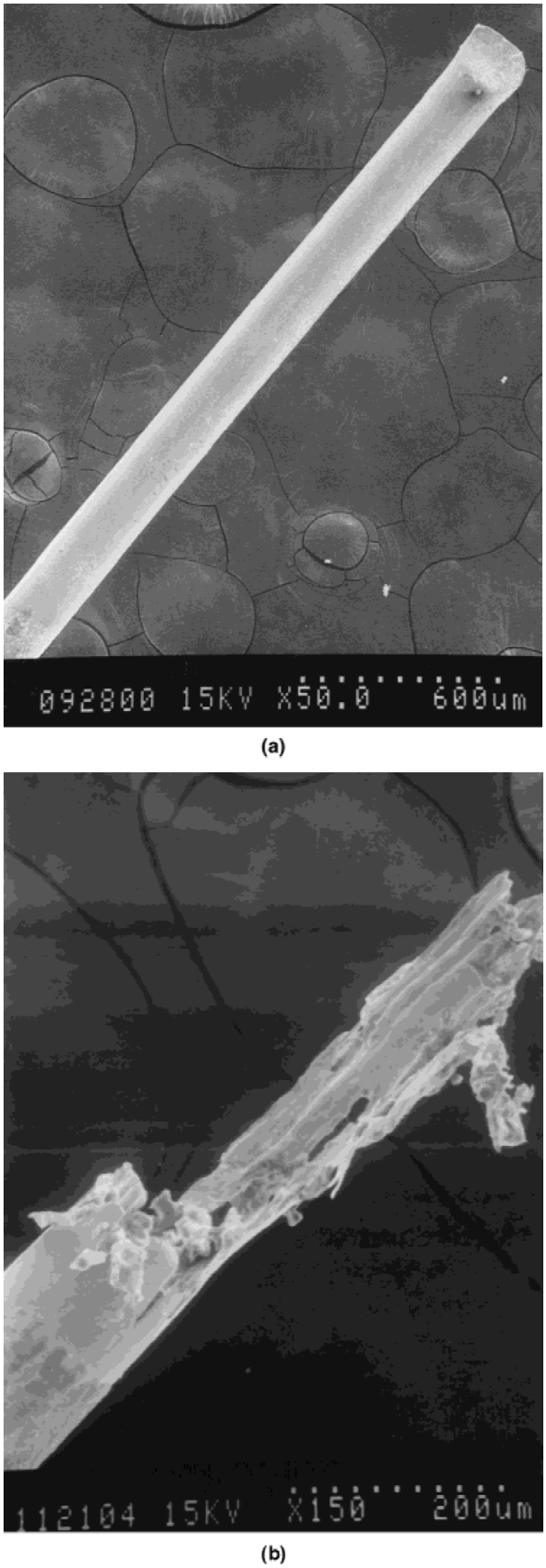

Figure 2. Surface morphology of degradation of the wires examined under scanning electron microscopy. (a) Sample of Figure 1(a) with smooth surface and no pitting or crevice corrosion. (b) Sample of Figure 1(b) with severe corrosion of wire.

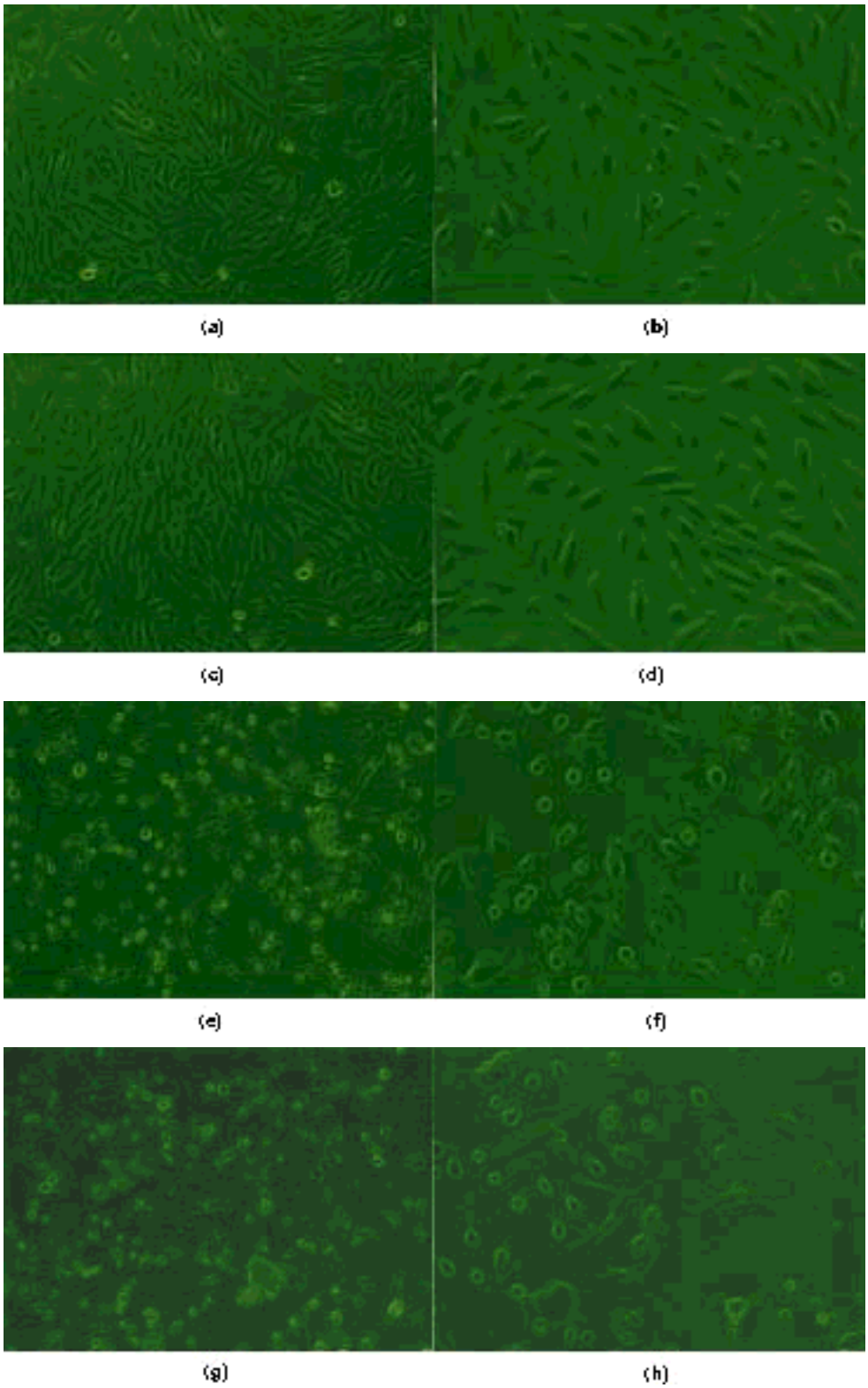

Figure 3. Rat aortic smooth muscle cells observed under phase contrast microscope after incubation for 12 h with different corrosion media. (a) and (b): Confluence culture of smooth muscle cells incubated with negative control polystyrene patch-immersed medium. Original magnification: (a) 100×, (b) 200×. (c) and (d): Confluence culture of smooth muscle cells incubated with nitinol wire immersed media without any applied potential (nickel: 0.97 ppm). Original magnification: (c) 100×, (d) 200×. (e) and (f): Large numbers of dead smooth muscle cells appeared after incubation with the supernatant portion of severe corrosive products (nickel: 208 ppm). Original magnification: (e) 100×, (f) 200×. (g) and (h): Large numbers of dead smooth muscle cells appeared after incubation with the resuspended precipitate portion of severe corrosive products (nickel: 267 ppm). Original magnification: (g) 100×, (h) 200×.

RESULTS

Severe corrosion of nitinol wires was initiated and conducted by applying 1.0 V of potentiostatic control and recording for 4–6 h by Potentiostat as shown in Figures 1 and 2. Nickel concentrations of different test media were: 0.95 ± 0.23 ppm for the wire-immersed mild leaching media, 208 ± 12 ppm for the supernatant portion of severe corrosion, and 286 ± 23 ppm for the resuspended precipitate portion of severe corrosion, respectively. The final concentration of nickel in the supernatant of mixed media for time-course and dose-response studies was 149 ppm.

The corrosive products of nitinol wire were authenticatedly toxic for the primary culture of rat aortic vascular smooth muscle cells. Morphologically, under a phase contrast microscope (Fig. 3), cellular death appeared after incubation with the corrosion media and the affected cells were increased along with the concentration increase and time exposure.

The wire-immersed mild leaching media with less than 1 ppm of nickel ion had a small but insignificant stimulation effect on [^{3}H]-thymidine uptake by cultured vascular smooth muscle cells. However, corrosive products of severe corrosion displayed significant inhibition of replication, as shown in Figure 4. Both the supernatant and the precipitated products have similar inhibition effects on the cell replication.

The time-course and dose-response curves of [^{3}H]-thymidine uptake ratio of cultured vascular smooth muscle cells are shown in Figure 5. The replication inhibition initiated immediately after changing media and reached the maximal effect 4–6 h later. This inhibition effect could be observed even in the 1/16× diluted media with a nickel concentration of 9 ppm.

The cell numbers in severe corrosion media were significantly less than those in wire-immersed mild leaching media, which showed a small though statistically insignificant increase as compared with standard DMEM and negative-control media (Table II).

Media of both the supernatant and resuspended precipitates of severe corrosion had similar cytotoxic effects. The cell growth was inhibited during the transition of G0-G1 phase to S1 phase by cell cycle analysis (Table II).

DISCUSSION

Nickel–titanium alloy (nitinol) is a metallic biomaterial that has unique thermal shape memory, superelasticity, and high damping properties. At present,

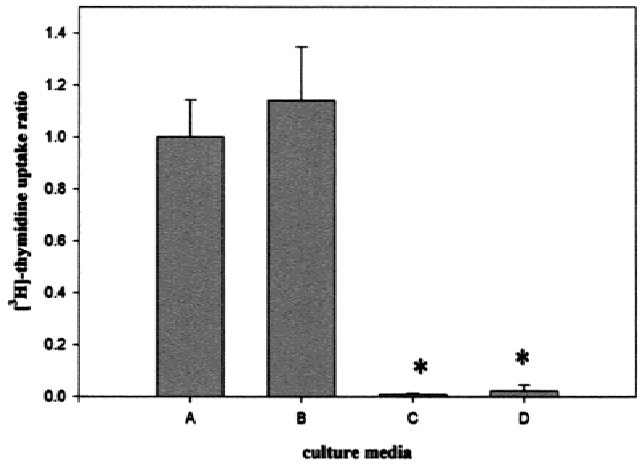

Figure 4. [^{3}H]-thymidine uptake ratio of rat aortic smooth muscle cells incubated with different culture media. (a) Polystyrene patch in DMEM for 24 h. (b) nitinol wire with polycrystalline oxide (PO) surface in DMEM without applied potential for 24 h (nickel: 0.97 ppm). (c) Nitinol wire with PO surface in DMEM with applied potential of 1.0 V (the supernatant of corrosive products) (nickel: 208 ppm). (d) Nitinol wire with PO surface in DMEM with applied potential of 1.0 V (the resuspension of precipitated corrosive products with DMEM) (nickel: 267 ppm). Cells were incubated with media a, b, c, or d for 12 h, then thymidine uptake was measured. Results were expressed as mean ± standard deviation of [^{3}H]-thymidine uptake ratio as compared with control uptake in standard DMEM. Each bar represents the average of six determinations performed on triplicate individual samples (*$p < 0.01$).

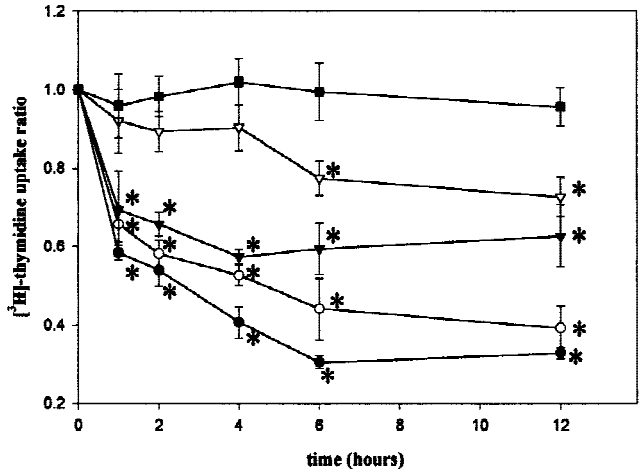

Figure 5. The time course and dose response of [^{3}H]-thymidine uptake ratio in rat aortic smooth muscle cells after incubation with different dilutions of corrosion medium of nitinol. Cells were incubated with media containing different concentrations of corrosive products [nickel: 1× = 149 ppm (closed circle), ½× = 74 ppm (open circle), ¼× = 37 ppm (closed triangle), 1/16× = 9 ppm (open triangle), negative control media (closed square)] for up to 12 h. Then thymidine uptake was measured and data were expressed as mean ± standard deviation of [^{3}H]-thymidine uptake ratio as compared with control uptake at start point. Each point represents the average of six determinations performed on duplicate individual samples. *$P < 0.05$ as compared with that of each corresponding time point of negative control polystyrene patch-immersed media.

TABLE II
Cell Numbers and Cell Cycle Analysis of Rat Aortic Smooth Muscle Cells Incubated with Different Corrosion Media

	Total Cell Counts	G0–G1 Phase (%)	S Phase (%)	G2 + M Phase (%)
Standard DMEM	129573 ± 26118	75.9 ± 7.3	18.9 ± 6.3	5.3 ± 2.8
Negative control polystyrene media	120312 ± 15962	77.5 ± 5.9	15.3 ± 4.7	7.2 ± 2.5
Mild leaching media (0 V)	134000 ± 14880	75.1 ± 8.3	19.6 ± 6.5	5.5 ± 2.7
Media of supernatant of severe corrosion (1.0 V)	90634 ± 18141*	89.6 ± 2.3*	6.0 ± 1.6*	4.4 ± 2.2
Media of resuspended precipitates of severe corrosion (1.0 V)	91452 ± 17654*	86.3 ± 6.2	8.2 ± 3.3*	5.5 ± 4.3

Six experiment were done on duplicate individual samples.
*$p < 0.05$ as compared with standard DMEM group.

there is not much confirmative biocompatibility data available on nitinol, especially for vascular smooth muscle cells. *In vivo* histological studies[18,19] have shown that there is always some ionization and solubilization of metal or alloy when in contact with tissue after implantation, regardless of alleged corrosion resistance. The constituent elements of alloys can be detected in the local tissues and the severity of tissue reaction around an implanted prosthetic alloy is related to the degree of concentration of metal ions released into the tissues.

In the present study, the *in vitro* cell cultivation with corrosive products of nitinol has proven its cytotoxicity to the primary culture of rat aortic smooth muscle cells, and the degree of toxicity has also been quantified.

Mechanism of nitinol corrosion

When metals are exposed to reactive circulatory milieu after implantation, corrosion, mostly electrochemical in nature, occurs on the surface. The rates of chemical and electrochemical reactions are strongly dependent on the properties of the passive layer on the surface of these metals or alloys. This passive layer is made of metallic oxides, the constitution of which is a function of the metallic corrosion and the passivation process. The formation of two types of metallic oxide, polycrystalline oxide and amorphous oxide, depends on the compositions of the metal or alloy material and industrial processes used in surface finishing. The current commercial intravascular stents of stainless steel or nitinol with polycrystalline oxide have been proven to have poorer corrosion resistance.[9–14] Degradation such as pitting often occurs if the polycrystalline oxide passive layer is locally ruined by the high chloride content of body fluid,[20] and metal ions are released from the degenerative metallic stent.

The nitinol is a simple equiatomic alloy of nickel and titanium (Ni/Ti = 1:1). Reaction on the metal surface at the anode includes the dissolution of metal ions or oxide formation. Nickel is the major released ion in the supernatant, and titanium oxide as well as nickel oxide are the major solid precipitates in corrosion media. The released nickel ions may act as cofactors or inhibitors in enzymatic processes involved in protein synthesis and cell replication,[21] disrupt intracellular organelles, alter cell morphology, and decrease cell numbers.[22–26]

Several studies have found nickel ion to be toxic to cultured cells, as well as carcinogenic.[8,27–30] By quantifying the effects of increased concentrations (7.5–30 μg/mL) of nickel (as $NiCl_2 . 6 H_2O$) on the growth and morphology of cultured 3T6 embryo mouse fibroblasts, Bearden and Cooke[7] found that lower concentrations of nickel may have produced some stimulation of cell growth; however, depressions in the cell growth rate with morphological change was noted in high concentrations (15–30 μg/mL) of nickel. Similar *in vitro* effects of several metal cations including nickel ion on Balb/c 3T3 fibroblast metabolism have been observed.[8] In the present study, a small stimulation effect on cell growth of smooth muscle cells was also found in mild leaching media at a lower concentration of less than 1 ppm of nickel (Fig. 4). Instead, all concentrations of nickel higher than 9 ppm (1/16× dilution) in corrosion products of nitinol displayed growth inhibition of cultured smooth muscle cells, which were demonstrated by [^{3}H]-thymidine uptake study and cell cycle analysis.

Therefore, as one of the major constituents of nitinol matrix and lack of nickel in the polycrystalline oxide surface coating (Table III), nickel ion dissolution from nitinol has been regarded as hazardous to vascular smooth muscle cells.

The dose-response and time-course studies (Fig. 5) have clearly demonstrated that the degrees of cytotoxicity of nitinol are increased along with the exposure time and dose increment. The growth inhibition

TABLE III
The Compositions (Weight Percent) of Nitinol[10–12]

Elements	Polycrystalline Oxide	Matrix
Titanium	83.99	50
Nickel	Nil	50
Oxygen	16.01	Nil

achieved maximal effect (more than 50% of inhibition) 4 to 6 h after reaction initiated.

The inhibition on transition of G0-G1 phase to S phase

From the [^{3}H]-thymidine uptake test, the replication of rat aortic smooth muscle cells was significantly inhibited by the corrosion products of nitinol. Cell cycle analysis by flow cytometry had further displayed that the inhibition process occurred during the transition of G0-G1 phase to S phase, which caused the diminution of cell numbers (Table II).

A number of growth factors and cytokines may be involved during this transition period and affect the cell cycle manifestation, the cell viability, and proliferation. To avoid potential nickel cytotoxicity, enhancement of corrosion resistance of nitinol by surface treatment with either preliminary oxidation, melt-spraying with biocompatible material, or surface morphological modification during the final manufacturing process of cardiovascular stent is very important and needs further investigation.

CONCLUSION

This was the first study to demonstrate that both the supernatant and the precipitated corrosive products of current nitinol stent wire were potentially toxic to vascular smooth muscle cells, especially when the released nickel concentration was higher than 9 ppm. The growth of cultured smooth muscle cells was inhibited during the transition of G0-G1 phase to S phase. The cell number diminution with morphology alteration, or even cell death was more prominent along with the time exposure and dose increment of corrosion media.

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