Cytotoxicity and its test methodology for a bioabsorbable nitrided iron stent

Wenjiao Lin,1 Gui Zhang,1 Ping Cao,2 Deyuan Zhang,1 Yufeng Zheng,3 Rangxiu Wu,1 Li Qin,1 Geqi Wang,1 Taoyuan Wen1
1R&D Center, Lifetech Scientific (Shenzhen) Co. Ltd., Shenzhen 518057, China
2Shenzhen Testing Center of Medical Devices, Shenzhen 518057, China
3Department of Materials Science and Engineering, College of Engineering, Peking University, Beijing 100871, China

Published online 26 July 2014 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.33246

Received 23 September 2013; revised 30 April 2014; accepted 20 June 2014

Abstract: Comprehensive assessments of the cytotoxicity of nitrided iron, a promising bioabsorbable metallic material, were conducted using in vitro methods. Extracting and standing experiments were conducted to determine the factors influencing the precipitation of the extract during extraction and incubation. The MTT method, fluorescent staining, and direct contact method were used to explore the in vitro cytotoxicity of nitrided iron stent extracts, nitrided iron foils, and their bulk corrosion products. The extracting and standing experiments confirmed that the extraction medium and available oxygen are crucial for precipitation during the extraction and incubation processes. In the MTT test, the extract of nitrided iron stents with a high iron ion concentration (124.11 ± 7.55 µg/mL) was not cytotoxic to L929 fibroblasts. Thus, the in vitro cytotoxicity of nitrided iron stents was actually caused by the size effect of corrosion particles and not the material itself. Test methodology for in vitro cytotoxicity of biodegradable iron-based materials was analyzed, and the results demonstrate that multiple methods should be combined for comprehensive evaluation of the cytocompatibility of bioabsorbable iron-based materials to get an impartial conclusion.

Key Words: bioabsorbable, nitrided iron stent, cytotoxicity, size effect, test methodology


INTRODUCTION

For decades, high corrosion resistance has been important for metallic biomaterials.1–4 Corrosion of these materials has been recognized to cause inflammation in local tissues and to decrease the mechanical integrity, thus decreasing the effectiveness of the devices.5 However, recently, biodegradable metallic biomaterials have also been proposed for specific applications, including pediatric, orthopedic, and cardiovascular applications.6–9 One of these applications is bioabsorbable stents, which are expected to provide the fourth revolution in cardiovascular interventions following Percutaneous Transluminal Coronary Angiography (PTCA), Bare Metal Stent (BMS), and Drug Eluting Stent (DES) because they have numerous advantages over permanent stents.10–13 The stent is a foreign object within the blood vessel, and its permanent presence is associated with the potential for long-term inflammatory reactions, progressive neointima development, endothelial damage, and associated late thrombosis risk.14

Iron-based and magnesium-based alloys have been under research and development as biodegradable metallic materials for application in cardiovascular intervention.15–19 In vivo studies have demonstrated that iron stents can be safely implanted without significant obstruction of the stented vessel as a result of inflammation, neointimal proliferation, or thrombotic events, but a faster degradation rate is desirable.20–22 However, many previous studies also reported in vitro cytotoxicity of iron for endothelial cells (ECs), smooth muscle cells, and L929 fibroblasts.23–27 These previous studies show inconsistency in the in vivo and in vitro cytocompatibility of iron-based materials.

Iron-based materials are biodegradable and thus different from traditional corrosion-resistant biomaterials. Because the human in vivo environment is complicated, simple in vitro evaluations of cytocompatibility may produce biased or false conclusions.28,29 Investigators worldwide have shown that results from in vitro testing often predict...
failure of biodegradable metals in a biological environment, although animal models may show an excellent host response. Future in vitro studies need to carefully consider the use of multiple test methods because each in vitro method provides different information.\textsuperscript{30,31}

Given these considerations, the current study determined the in vitro cytotoxicity of nitrided iron as a biodegradable metallic material using multiple test methods and compared it with the pure iron. Composition, microstructure, and mechanical properties were characterized for both pure iron stent and nitrided iron stent for coronary artery application. Extracting and standing experiments were conducted to determine the factors influencing precipitation during extraction and incubation. The MTT method was used to explore the in vitro cytotoxicity of extracts of nitrided iron stents and pure iron stents. The direct contact method and fluorescent staining were used to analyze the cytotoxicity of nitrided iron foils and the bulk corrosion products of nitrided iron stents. The real purpose of this study was to conduct test methodology analysis for in vitro cytotoxicity of iron-based materials, which was meaningful for impartial assessment of the potential iron-based materials for medical applications.

**MATERIALS AND METHODS**

**Materials**

Pure iron stents (Ø 4.0 mm × 18 mm) and pure iron foils (Ø 10 mm × 10 mm) were laser-cut from the as-drawn pure iron tubings (Ø 1.6 mm × wall 0.11 mm) manufactured by Liftech Scientific (Shenzhen, China). Vacuum plasma nitriding for 2 h at 500°C with 50 Pa pressure was applied to obtain the nitrided iron stents and nitrided iron foils using an in-house-designed vacuum nitriding furnace. All the stents (Ø 4.0 mm × 18 mm) were electrochemically polished to the designated size ( strut 95 μm, wall 75 μm, weighing ~18 mg), and the foils were also polished to remove the oxide layer. The finished stents and foils after sterilization were used for the following mechanical, extracting, and cell culture experiments.

**Composition and microstructure**

Ultimate analysis of the pure iron stents and nitrided iron stents was conducted. Both stents were embedded in resin, grinded, mechanically polished, and then eroded for cross-section microstructure observation using JEOL 6510 scanning electron microscope (SEM; Japan).

**Mechanical properties**

**Vicker’s hardness.** A HXD-1000TMC digital microhardness tester was used to measure the Vickers hardness of the stents with and without nitriding with a load of 10 g and a dwelling time of 15 s. After embedding and mechanically polishing the samples, at least two areas on each of the randomized four struts were tested to obtain the average microhardness.

**Radial strength and stiffness.** Balloon catheter and inflation device were used to expand the pure iron stents and nitrided iron stents to the nominal size (Ø 4.0 mm). Radial strength and stiffness were measured with a compression rate of 0.1 mm/s using a RX550-100 type radial strength tester (Machine Solution). We defined the stent radial strength (kPa) as the strength at 90% of the nominal diameter and stent stiffness (N/mm) as the force needed to produce unit change in the stent diameter during the radial compression process. At least five samples were tested in each stent group.

**Extracting and standing experiments**

Extraction of the nitrided iron stents and pure iron stents (with a total surface area of 1.15 cm\(^2\), weighing ~18 mg) was conducted according to ISO-10993-12:2007 (Ref. 32) by using an extraction ratio of 0.2 g/mL (equivalent to 12.6 cm\(^2\)/mL) in a 37°C water bath for 72 h under 5% CO\(_2\). A blood collection tube was used as the extraction container because of its good sealing performance. Extracts (2 mL) were prepared in the blood collection tube with a 5.5-mL air column left above the samples: extract A was obtained in 0.9% NaCl with good tube sealing, and extracts B and C were obtained in DMEM culture medium containing 10% fetal bovine serum with poor sealing and good sealing, respectively, as presented in Table I.

Iron ion concentration in extract C was tested using an atomic absorption spectrometer (SpectraAA240; Agilent Technologies) for both pure iron stent and nitrided iron stent, with DMEM culture medium containing 10% fetal bovine serum as blank (samples were filtered using membrane filter with 0.22 μm pore size before iron concentration testing).

The filtered nitrided iron stent extract C was also incubated in the wells of a 96-well cell culture plate in a humidified atmosphere under 5% CO\(_2\) at 37°C for 48 h to observe its precipitation behavior by using an inverted biological microscope (XDS-200; Caikon Optical Instrument, China).

**Cytotoxicity assessment by the MTT method**

Extracts B and C of pure iron stents and nitrided iron stents were prepared as mentioned in the "Composition and microstructure" section for cytotoxicity assessment. The L929 fibroblast cell line was used in this test. After the cells were incubated with the extract in a humidified atmosphere under 5% CO\(_2\) at 37°C for 24 h, the 96-well cell culture plates were observed under an optical microscope to determine the cell morphology. Then, 100 μL of culture medium and 50 μL of MTT were added to each well. The cells were incubated with MTT under a 5% CO\(_2\) atmosphere at 37°C for 2 h, and then, the medium was discarded, and 100 μL of isopropanol was added to each well, followed by shaking of the culture plates. The absorbance of the samples was measured by a microplate reader (RT-6000) at 570 nm, and a reference wavelength of 630 nm was used for cell viability calculation. During this process, four sample groups were identified as follows (presented in Table II): B2: extract B was used for incubation without change of medium during incubation, but the wells were rinsed with DMEM before MTT was added; C1: extract C was incubated without
change of medium or rinse; C2: extract C was incubated without change of medium but with a rinse; and C3: extract C was incubated with change of medium every 6 h, and the wells were rinsed with DMEM before MTT was added. DMEM containing 10% fetal bovine serum was used as negative control, and DMSO was used as a positive control. Staining of dead cells was also conducted in sample groups C2 and C3 of nitrided iron stent as well as the negative and positive control groups.

Cytotoxicity assessment for nitrided iron foil by direct contact
Nitrided iron foils (10 mm × 10 mm) were half-embedded in paraffin and corroded in the 0.9% NaCl solution for 24 h; the paraffin was then removed for epoxylanethane sterilization. After culture in 12-well cell culture plates with 2 mL of smooth muscle cells (SMCs) or ECs (1 × 10^5 cells per milliliter) under 5% CO_2 at 37°C for 72 h, the iron plates were removed, rinsed with PBS, fixed with glutaraldehyde, dehydrated with ethanol, and naturally dried for SEM observation of cell morphology.

Cytotoxicity assessment for bulk corrosion products by direct contact
The bulk corrosion products were obtained from extract A of nitrided iron stent with size ranging from 100 to 2000 μm. Ultrasonic cleaning of these bulk corrosion products was applied to ensure that the small corrosion particles (<100 μm) would not adhere to surfaces. The bulk corrosion particles derived from nitrided iron stents were cultured with 2 mL of L929 fibroblasts (1 × 10^5 cells per milliliter) in 12-well cell culture plates under 5% CO_2 at 37°C for 24 h. The bulk corrosion products were removed for fixation and analysis by using SEM and energy dispersive spectroscopy (EDS). In addition, fluorescent staining (nucleic acid dye for live cells) was also applied to evaluate cell viability (Leica DM2500).

Statistical analysis
The results were expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) was conducted using SPSS11.5 to evaluate the cytotoxicity of extracts of nitrided iron stents. Statistical significance was defined as p ≤ 0.05. Student-Newman-Keuls tests (q tests) were performed between the experimental and control groups when ANOVA showed statistical significance.

RESULTS
Composition and microstructure
The elemental composition of the pure iron and nitrided iron is shown in Table II. It could be seen that the purity of Fe in the pure iron stent is over 99.5 wt %. The nitrogen content in the nitrided iron stent is only 0.074 wt %, whereas in the pure iron, the base line for nitrogen is 0.011 wt %. Nitrogen in the nitrided iron stent is definitely in an extremely low content.

Figure 1 shows the microstructure of the pure iron stent and nitrided iron stent. It could be seen from Figure 1(a) that the pure iron has very fine grains (several

<table>
<thead>
<tr>
<th>Extract Groups</th>
<th>When Prepared</th>
<th>Extraction Medium</th>
<th>Oxygen Control</th>
<th>Medium Change</th>
<th>When Used for Cytotoxicity Evaluation</th>
<th>Rinse Before Adding MTT</th>
<th>Change</th>
<th>Rinse Before Adding MTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Good Sealing</td>
<td>0.9% NaCl</td>
<td>Bad Sealing</td>
<td>No</td>
<td>Good Sealing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>Good Sealing</td>
<td>DMEM + 10% Fetal Bovine Serum</td>
<td>Good Sealing</td>
<td>Every 6 h</td>
<td>Good Sealing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Good Sealing</td>
<td>DMEM + 10% Fetal Bovine Serum</td>
<td>Good Sealing</td>
<td>No</td>
<td>Good Sealing</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
micrometers) in the transection and from Figure 1(c) that the grains are stretched significantly along the longitudinal axis of the pure iron tubings, resulting from the severe plastic deformation during cold drawing of tubings. After nitriding, dispersion layer is formed with second-phase particles (possibly Fe₄N or Fe₁₆N₂) evenly scattered in the pure iron matrix as shown in Figure 1(b,d). The size of the iron nitride particles is below 1 μm.

Mechanical properties
Figure 2 presents the typical radial compression curves of stents, and the linear area emphasized with gray dash line indicates recoverable deformation. The calculated radial strength and stiffness together with Vickers hardness are shown in Table III. It is obvious that the nitrided iron stent shows significantly elevated microhardness increased from HV175.92 ± 6.35 to HV305.25 ± 9.91 (p < 0.01), radial strength increased from 80.52 ± 5.36 kPa to 120.80 ± 6.25 kPa (p < 0.01), and stiffness increased from 52.98 ± 3.97 N/mm to 97.86 ± 3.48 N/mm (p < 0.01) because of the uniform dispersion of the second-phase particles in the pure iron matrix after nitriding.

Extracting and standing experiments
According to ISO-10993-12:2007, an acceptable extraction condition is 37°C for 72 h with an extraction ratio of 0.2 g/mL based on an appropriate consideration of product use. For nitrided iron stents, Figure 3(a) shows that in a polar extraction medium (physiological saline), dark black turbid liquid is obtained after 72 h of extraction, that is, extract A has numerous black corrosion particles. Comparatively, the use of DMEM with 10% fetal bovine serum as the extraction vehicle reduced the formation of corrosion products from the nitrided iron stent. A few dark yellow corrosion product particles were observed in the light yellow 72-h extract with bad sealing (i.e., extract B) as shown in Figure 3(b), whereas the 72-h extract with good sealing (i.e., extract C) was still clear on macroscopic examination as shown in Figure 3(c).

For cytotoxicity evaluation using the MTT method, DMEM with serum is preferred for extraction because of its ability to support cellular growth and to extract both polar and nonpolar substances according to ISO-10993-5:2009. As shown in Table IV, the pH of pure iron stent extract C and nitrided iron stent extract C is 7.44 ± 0.39 and 7.46 ± 0.48, respectively, and that of the blank is 7.39 ± 0.45. The iron ion concentration in pure iron stent extract C and nitrided iron stent extract C is 82.9 ± 5.86 and 124.11 ± 7.55 μg/mL, respectively, whereas that in the blank is 2.89 ± 0.72 μg/mL.

As shown in Figure 4, precipitation occurred in nitrided iron stent extract C when it was incubated in the cell culture environment for 48 h (i.e., at 37°C in an atmosphere of
5% CO2) and the particle number increased. In Figure 4(a), extract C is clear under microscopic examination at the zero time, whereas Figure 4(b) shows the absence of precipitated particles after incubation for 6 h. However, a small quantity of precipitated particles was observed at the bottom after 24 h of incubation presented in Figure 4(c), and many precipitated particles overspread the entire well bottom after 48 h of standing as shown in Figure 2(d). The precipitated particles had a size of approximately 2–5 μm, which is much smaller than L929 fibroblasts.

**Cytotoxicity assessment for the extracts of nitrided iron stent**

Figure 5 illustrates the morphology of L929 fibroblasts after culture for 24 h in nitrided iron stent extracts. The cells in the negative group showed normal growth and morphology in Figure 5(a), whereas reduced number and abnormal morphology was observed for the positive control group as shown in Figure 5(b). It is worth noting that in the negative group, no apparent precipitation particles were formed after 24 h of incubation with fibroblasts. Apparent apoptosis was observed in group B2 as shown in Figure 5(c). For both groups B2 and C1, a thick layer of precipitation particles was observed only after 24 h of culture, at which time the fibroblasts were embedded. The precipitation of extract C incubated with cells was much faster than that of extract C incubated alone in the cell culture environment. Rinsing caused fibroblasts to be exposed in group C2 in Figure 5(e); the fibroblasts were less in number and may have had low cell activity because they maintained a normal morphology. This prestudy validation thus demonstrated nearly no precipitation particles in extract C after 6 h of incubation with cells; hence, for group C3, a medium change every 6 h was chosen to prevent the precipitation of particles during the 24 h of culture. Cells in group C3 maintained normal morphology and growth as shown in Figure 5(f).

It is difficult to determine only from morphology observation whether cells maintaining a normal morphology are still alive or have high metabolic activity. Trypan Blue works well in this context as it stains the dead cells. As shown in Figure 5(a,b), none of the fibroblasts in the negative group were stained, thus indicating high cell activity, whereas nearly all of the cells in the positive group were stained light blue, which indicated that the cells had extremely low activity or were dead. Most cells in group C2 were stained, although they maintained normal morphology as indicated by Figure 5(c). In Figure 6(d), only a few cells were stained.

The MTT method was used to evaluate cytotoxicity after 24-h culture with 72-h extracts of pure iron stents and nitrided iron stents. As shown in Figure 7, the viability of cultured cells in groups B2 and C2 of nitrided iron stent was significantly decreased ($p < 0.05$) relative to the negative control, which indicates obvious cytotoxicity. There was no significant difference among group C3 for both pure iron stent and nitrided iron

| TABLE III. Mechanical Performance of Pure Iron Stent and Nitrided Iron Stent |
|---------------------------------|----------------|----------------|----------------|
| **Φ 4.0 × 18 mm**               | **Microhardness (HV0.05)** | **Radial Strength (kPa)** | **Stent Stiffness (N/mm)** |
| Pure iron stents               | 175.92 ± 6.35 | 80.52 ± 5.36 | 52.98 ± 3.97 |
| Nitrided iron stents           | 305.25 ± 9.91 | 120.80 ± 6.25 | 69.89 ± 4.38 |
| $p$                             | <0.01          | <0.01          | <0.01          |

| TABLE IV. Iron Ion Concentration |
|----------------------------------|-----------------|-----------------|
| **Sample Description**           | **pH Value**    | **Iron Ion Concentration (μg/mL)** |
| Blank                            | 7.39 ± 0.45     | 2.89 ± 0.72     |
| Pure iron stent extract C        | 7.44 ± 0.39     | 82.9 ± 5.86     |
| Nitrided iron stent extract C    | 7.46 ± 0.48     | 124.11 ± 7.55   |
stent and the negative group ($p > 0.05$), which demonstrates nontoxicity. These findings are in agreement with the results of cell morphology and Trypan Blue staining. However, the significant increase ($p < 0.05$) of the viability of cultured cells in group C1 of nitrided iron stent was unexpected because it contradicts the results of cell morphology and Trypan Blue staining. It should be noted that the only difference between groups C1 and C2 of nitrided iron stent is that in group C1, the wells were not rinsed prior to the addition of MTT.

As shown in Figure 8(a), the insoluble formazan formed by negative control fibroblasts on treatment with MTT manifested as a purple floccule, which was transformed from MTT by enzymes in live cells. As shown in Figure 8(b), when positive group was treated with MTT, there was no formazan formed. Figure 8(c) illustrated the subgroup C1 treated with MTT. Although no floccule formazan formed because of few live cells, the aggregated dark purple particles formed probably from the reaction between MTT and the precipitates formed during incubation. For a demonstration that the MTT would react with the precipitates, light yellow MTT solution was added to the extract C on standing for 48 h in 96-well cell culture plate, which had numerous precipitates formed as shown in Figure 4(d). After 2-h
incubation of the 48-h standing extract C with MTT, a dark purple substance was found on the bottom of the well presented in Figure 8(d). Subsequently, the supernatant was discarded, and colorless transparent isopropyl alcohol was added. Figure 8(e) demonstrates that this dark purple reaction product was dissolved by colorless isopropyl alcohol to form a purple solution, which added to the absorbance and resulted in a false conclusion of greatly increased cell viability.

**Cytotoxicity assessment for nitrided iron foil**

The cytotoxicity of the nitrided iron foil was evaluated using the direct contact method according to ISO-10993-5:2009. It is clear from Figure 9 that both HA-vascular SMCs (VSMCs) and HUVECs in the noncorroded area maintained normal morphology and metabolism as shown in Figure 9(a,c). In the severely corroded area with many corrosion products (the mean particle size was much smaller than the diameter of the cultured cells), toxicity in HA-VSMCs and HUVECs is obvious in Figure 9(b,d), as indicated by the large area containing dead and abnormal cells.

**Cytotoxicity assessment for bulk corrosion products of nitrided iron stents**

The EDS linear analysis in Figure 10(a,b) shows that for the cell-shaped substance, carbon content increased with sharp reduction in iron and oxygen content, whereas for the matrix, both iron and oxygen content increased greatly with decrease in carbon content. This finding demonstrates that dark spindle-shaped spots are L929 cells and that the matrix is a compound of iron and oxygen (i.e., it is an iron corrosion product). The submicron iron nitride particles should also be mixed in the bulk corrosion products; however, because of the low particle amount and extremely low nitrogen content, it is not easy to identify by EDS or SEM. The L929 fibroblasts, which maintained their normal morphology, covered the entire surface of the bulk corrosion product. The white small particles on the surface are not corrosion products; rather, they represent crystallization or precipitation from the residual saline solution during dehydration.

Figure 11(a) shows that after 24-h incubation with the bulk corrosion products (the yellow material) of nitrided iron stent, the L929 fibroblasts took on a typical long spindle shape with abundant cytoplasm and visible nucleus; this morphology was not different from that of normally cultured cells. Although the L929 fibroblasts maintained a virtually normal morphology, further experiments are necessary to confirm that they are still alive after 24 h of culture on the surface of the bulk corrosion products. Figure 11(b) shows fluorescent staining using a nucleic acid dye to identify live cells. Every fluorescent spot in Figure 11(b) represents a nucleus, thus indicating a cell. The cell density on the bulk corrosion products was comparable with the background cell density (i.e., cells growing at the bottom of the well). Because the cells growing on the bulk corrosion products and well bottom were not on the same focal plane, the boundary of the bulk corrosion products could be easily identified [the corrosion product in Figure 11(b) had a diameter of ∼700 μm].

**DISCUSSION**

**Composition, microstructure, and mechanical performance**

In the current work, it has been demonstrated that extremely low nitrogen alloying into the pure iron stent could lead to significantly increased mechanical performance because of the solution strengthening of nitrogen and dispersion strengthening resulting from the evenly dispersed fine iron nitride particles. Stiffness is used to characterize the capability of the components against elastic deformation. There is pronounced stiffness enhancement of the nitrided stent, meaning less deformation to ensure the same radial support. Accordingly, a smaller degree of overdilatation could be applied to the nitrided stent to reduce...
the trauma to the vessel wall so as to lower the in-stent restenosis risk. With high radial strength and hardness, nitrided iron stent can have smaller strut dimensions (e.g., strut width and thickness) to minimize profile of the stent system so as to improve its flexibility and crossability in the tortuous and stenosis blood vessel. More importantly, smaller strut dimensions can shorten the stent degradation period. In our previous work, nitriding was also demonstrated to strengthen pure iron by solid solution strengthening and dispersion strengthening, more importantly, to accelerate corrosion by introducing microgalvanic corrosion\textsuperscript{33} for peripheral application.

![Figure 8](image1.png)

**FIGURE 8.** (a) Formazan formed on treatment of the negative control group with MTT; (b) observation on treatment of the positive group with MTT; (c) observation on treatment of sample group C1 of nitrided iron stent with MTT; (d) observation after 2-h incubation of the light yellow MTT solution with the 48-h standing nitrided iron stent extract C and thereafter discard the supernatant for (e) observation after the addition of colorless transparent isopropyl alcohol to dissolve the dark purple reaction product on the well bottom. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

![Figure 9](image2.png)

**FIGURE 9.** Morphology of HA-VSMCs (a and b) and HUVECs (c and d) after culture on the surface of nitrided iron foils for 72 h; (a) and (c) non-corroded area, and (b) and (d) corroded area.
Cytotoxicity assessment for bioabsorbable nitrided iron stents

The toxicity of metallic materials is governed mainly by the toxicity of the component elements and the corrosion products. Three different methods for in vitro cytotoxicity evaluation are presented in ISO-10993-5:2009, including testing of extracts (MTT method), the direct contact method, and the indirect contact method (agar diffusion assay). Most studies on the cytotoxicity of iron-based materials are based on MTT data.

Many previous studies indicated that the iron-based materials had cytotoxicity. The response of human ECs to various concentrations of ferrous ions was investigated by Huang et al. using the WST-8 assay, who found that lower iron concentrations (<10 μg/mL) may produce a favorable effect on the metabolic activity of ECs. Conversely, very high iron ion concentrations (>50 μg/mL) may be cytotoxic to ECs. Mueller et al. found that ferrous ion concentrations from 5 to 50 μg/mL had similar effects and arrested smooth muscle cell growth. Zheng and coworkers reported that nanocrystalline pure iron stimulates the proliferation of fibroblast cells and promotes endothelialization, while effectively decreasing the viability of VSMCs. This decrease in VSMC proliferation caused by iron ions was also reported by Moravej et al. and Liu et al. In addition, MTT results obtained by Zhang et al. revealed that extracts of pure iron typically were toxic to mouse bone marrow stem cells and that the toxicity increased with increases in the iron ion concentration and incubation time.

However, in the current study, the MTT assay demonstrated that the corrosion particles (2–5 μm) precipitating during extraction and incubation processes are significantly toxic to L929 fibroblasts. When such precipitation is prevented or minimized, extracts with an iron ion concentration up to 124 μg/mL show no cytotoxicity to L929 fibroblasts. With lower corrosion rate, iron ion concentration in the extract C of pure iron stent is also lower than that of the nitrided iron stent; however, there is no significant difference of cell viabilities between the two stent extracts when used for cell culture, both showing nontoxicity. Further experiments were conducted in this study to identify the reason for the cytotoxicity of precipitated fibroblast cells and promotes endothelialization, while effectively decreasing the viability of VSMCs. This decrease in VSMC proliferation caused by iron ions was also reported by Moravej et al. and Liu et al. In addition, MTT results obtained by Zhang et al. revealed that extracts of pure iron typically were toxic to mouse bone marrow stem cells and that the toxicity increased with increases in the iron ion concentration and incubation time.

However, in the current study, the MTT assay demonstrated that the corrosion particles (2–5 μm) precipitating during extraction and incubation processes are significantly toxic to L929 fibroblasts. When such precipitation is prevented or minimized, extracts with an iron ion concentration up to 124 μg/mL show no cytotoxicity to L929 fibroblasts. With lower corrosion rate, iron ion concentration in the extract C of pure iron stent is also lower than that of the nitrided iron stent; however, there is no significant difference of cell viabilities between the two stent extracts when used for cell culture, both showing nontoxicity. Further experiments were conducted in this study to identify the reason for the cytotoxicity of precipitated...
corrosion particles. Direct contact and fluorescent staining revealed that L929 cells cultured on the surface of bulk corrosion products (100–2000 μm, i.e., much larger than L929 cells) from nitrided iron stent maintained normal morphology and grew healthily. Bulk corrosion products of nitrided iron stent shall contain both the corrosion products of pure iron matrix and the remained submicron iron nitride particles, however, showed no toxicity to the L-929 fibroblasts.

Consequently, this study confirmed that the in vitro cytotoxicity of nitrided iron stents is derived from the size effect of their corrosion products (the corrosion particles of pure iron matrix mixed with the remained iron nitride particles) and not from the stent material itself. The corrosion particles generated from nitrided iron stents during extraction and incubation were highly toxic to L929 cells, probably because they precipitated and covered the cells during the incubation and consequently inhibit the nutrient supply to the cells or because they were smaller than the cells. Many other studies have also reported that the cytotoxicity of particles is mainly caused by a particle size effect and not by the particle material itself. Kumazawa et al. and Tamura et al. found that fine Ti particles (1–3 μm) that were smaller than neutrophils (approximately 5–10 μm) were phagocytized by the neutrophils, with similar results observed in vivo. These previous studies showed that the cytotoxic effect of Ti particles is size dependent and that the particles must be smaller than the cells to cause pronounced cytotoxicity.35,36 Ying and Hwang37 used the A3 human T lymphocyte as an in vitro model to investigate the dependence of cytotoxicity on particle size and the surface coating of iron oxide nanoparticles; they found that size and surface coating affect nanotoxicity in biological systems as well and that the interpretation of the cytotoxicity of nanoparticles can vary with the mass concentration, total number of particles per well, and total surface area of particles per well. It has, therefore, been confirmed in previous studies that particles that are small enough to be phagocytosed by macrophages or lymphocytes are more toxic than large agglomerates.

Cytotoxicity testing methodology for bioabsorbable iron-based materials

Nearly all studies on the in vitro cytotoxicity of iron-based materials were based on the MTT method and concluded that iron-based materials show obvious cytotoxicity to ECs, SMCs, and L929 fibroblasts. Therefore, it is necessary to more carefully analyze this testing method and its applicability to the cytotoxicity assessment of bioabsorbable iron-based materials.

Extraction medium and available oxygen. For the MTT method, the particles that precipitated during the extraction and incubation process were small enough (2–5 μm) to produce in vitro cytotoxicity. Hence, it is important to determine how process factors, that is, the extraction medium and available oxygen, affect precipitation during extraction and incubation to minimize precipitation.

Chlorine ions have been found to accelerate the corrosion of iron materials.38 Therefore, under the same extraction conditions, extract A contained numerous corrosion products, whereas extract C was still clear, that is, precipitation was determined by the medium applied during extraction. However, DMEM with serum is preferred for extraction because of its ability to support cellular growth and extract both polar and nonpolar substances.39 Previous studies have shown that culture medium with serum provides a more sensitive evaluation of cytotoxicity.40 The results illustrated in Figure 1 also confirm that control of sealing exerts an effect by modulating the amount of available oxygen, that is, precipitation is determined by the amount of oxygen available during extraction. The in vitro cytotoxicity of the corrosion products of biodegradable materials is becoming increasingly recognized, and it has been suggested that the supernatant and degradation products in the extract should be assessed separately.30 However, by choosing an appropriate extraction medium and sealing to control the amount of available oxygen, precipitation can be inhibited during the extraction process. Furthermore, even if a precipitate forms, filtration can be used to remove it, as filtration is already often performed when the extracts of biodegradable materials are used in biological evaluations, probably to reduce the risk of microbial contamination.27,34,41 However, precipitation may still occur during incubation, and such precipitation is typically neglected.

The available oxygen plays an important role in precipitation during incubation because of the two unique characteristics of iron, that is, the significant difference of solubility product between Fe2+ and Fe3+ [e.g., at 25°C, Ksp = 4.87 × 10−17 for Fe(OH)2 and 2.79 × 10−39 for Fe(OH)3; Ref. 42] and the strong tendency for Fe2+ to be transformed to Fe3+ in the presence of sufficient available oxygen because of the reducibility of Fe2+. The concentration of Fe2+ and Fe3+ is determined only by extract pH, as indicated in formulae (1) and (2) based on the definition of the solubility product. A higher pH is associated with a lower concentration. For a solution with an extremely high concentration of iron ions, the pH should be rather low, for example, 3.2 for FeCl2 and 1.7 for FeCl3.41 If the extract has a fixed pH, there is a saturation concentration for Fe2+ and Fe3+ ions, and once this concentration is exceeded, precipitation occurs. For example, in a 25°C extract (pH = 7.4) in 0.9% NaCl, the saturated concentration for Fe2+ and Fe3+ ions is 43.22 mg/L and 9.86 × 10−15 mg/L, respectively, which means that once the Fe2+ ions are oxidized to Fe3+ ions in the presence of sufficient oxygen, precipitation occurs because of the extremely low saturation concentration of Fe3+ ions. DMEM with 10% serum is a buffer solution (pH maintained around 7.4) with a complicated composition. The protein in this solution will combine some iron ions, and this might be why the total iron ions detected in this solution is higher than the saturation concentration Fe2+ in the saline solution with pH value around 7.4. However, the current study demonstrates that a saturation tendency similar to that described above also occurs in this medium at 37°C, and the precipitation particles are likely a compound of iron, oxygen, and other possible elements. Because cell growth requires a humidified 5% CO2
atmosphere at 37°C, it is very difficult to control the amount of oxygen available during incubation. However, it needs time for Fe^{2+} ions to be transformed to Fe^{3+} ions (reaction rate), consequently, precipitation might be reduced to minimum by extract change at a reasonable short interval before sufficient Fe^{2+} ions transformation.

\[
\begin{align*}
\lg [C(Fe^{2+})] & = \lg Ksp + 28 - 2pH \quad (1) \\
\lg [C(Fe^{3+})] & = \lg Ksp + 42 - 3pH. \quad (2)
\end{align*}
\]

Furthermore, precipitation during incubation corresponds to precipitation of Fe^{2+} (saturated Fe^{2+} in an oxygen-deficient environment) or precipitation of Fe^{3+} (saturated Fe^{3+} in an oxygen-abundant environment). When the former occurs, Fe^{2+} ions in the solution after filtration can still be detected by devices (e.g., ICP-OES) because of their high saturation concentration, whereas when the latter occurs, oxygen will oxidize Fe^{2+} to Fe^{3+}, and the extremely low saturation concentration of Fe^{3+} is below the detection limit of the device (usually at the ppm or ppb grade). The inability of Schinhammer et al.\textsuperscript{41} to detect iron ions in a filtered eluate might have been because of lack of oxygen control. Hence, oxygen control is critical during both extraction and cell incubation for the evaluation of cytotoxicity of iron ions.

**Reaction between MTT and precipitation particles.** MTT is reduced to insoluble formazan (a substance with a purple color) by enzymes in live cells. The formazan is then dissolved by colorless isopropyl alcohol to form a purple solution whose absorbance can be measured. More live cells (higher viability) produce greater formation of formazan, which results in a darker purple solution and higher absorbance. The current study demonstrated that particles precipitated during the incubation react with MTT to form a dark purple particle substance that is also dissolved by isopropyl alcohol to form a purple solution, which then contributes to the absorbance and produces a false conclusion of greatly increased cell viability. Similar findings were also reported by Fischer et al.,\textsuperscript{43} who found that tetrAzolium salt-based assays, which are widely used in practice, are influenced by the corrosion products of Mg-based alloys, that is, corroded Mg converts tetrAzolium salts to formazan, which leads to increased background and inflated results for cell viability.

Based on the above analysis, when the MTT method is used for assessing the cytotoxicity of biodegradable materials, the obtained in vitro cytotoxicity findings may get affected by the following conditions: (1) if the extraction conditions are not strictly controlled and filtration is not performed, small particles precipitated during extraction may produce in vitro cytotoxicity; (2) if the precipitation during incubation is not effectively minimized, in vitro cytotoxicity may occur; and (3) if rinsing is not performed prior to the addition of MTT or if inappropriate rinsing is performed, the reaction between MTT and the precipitated particles would artificially increase the observed cell viability. Therefore, analyses of test method details should be conducted to avoid biased or false conclusions. Moreover, multiple tests should be combined for a comprehensive cytocompatibility assessment, especially when considering the size effect of the corrosion particles.

**Correlation between in vitro and in vivo findings**

With regard to in vivo experiments, our previous study\textsuperscript{33,44} demonstrated that at 1 month postoperatively, a nearly intact layer of ECs formed on the vessel in the stented area. Thus, the nitrided iron stent presented excellent cytocompatibility, and consequently, the thrombosis risk was reduced. Moreover, nearly all other in vivo studies have demonstrated that iron materials show excellent cytocompatibility after implantation.\textsuperscript{20–22}

Various studies have demonstrated the complex nature of the in vivo environment, which is not easily simulated in vitro. Previous studies have indicated that the in vivo degradation rate of pure iron is much slower than the in vitro corrosion rate in simulated blood-like fluids.\textsuperscript{20} Another study further demonstrated that the in vivo degradation rate of iron materials in contact with blood is much slower than that of iron materials in contact with vessel wall tissue.\textsuperscript{35}

Although the small corrosion products obtained in in vitro tests present cytotoxicity because of their size effect, the size distribution of particles in in vitro tests may not be the same as that in vivo, which may be why in vivo experiments typically indicate excellent cytocompatibility of biodegradable iron-based materials. Additionally, other in vivo factors, such as macrophages, will help in mitigating or eliminating negative effects of particles on cells and tissue.

**CONCLUSIONS**

This study evaluated the cytocompatibility of nitrided iron stents from different perspectives and explored different test methods. Following are the conclusions drawn:

1. Iron ions in high concentrations (up to 124 μg/mL) show no in vitro cytotoxicity to L929 fibroblasts at a pH of ~7.4. Nitrided iron foils and bulk corrosion products of nitrided iron stents were also not toxic to L929 fibroblasts. The in vitro cytotoxicity of bioabsorbable nitrided iron stents is therefore caused by the size effect of the corrosion products and not by the material itself. Corrosion product particles that are smaller than the cultured cells significantly decrease cell viability. Cytotoxicity of the extract of the nitrided iron stent shows no significant difference when compared with that of the pure iron stent.

2. When the MTT method is used for cytotoxicity assessment of bioabsorbable iron-based materials, precipitation during extraction and incubation processes and the resulting reaction of the precipitate with MTT should be prevented or minimized. Multiple tests should be combined for an overall cytocompatibility assessment for bioabsorbable iron-based materials.
In vitro cytotoxicity of bioabsorbable iron-based materials is derived from the size effect of corrosion product particles; however, the corrosion situation and particle size distribution in vitro may not be the same as that in vivo. Additionally, in vivo factors such as macrophages may eliminate negative effects of particles. Therefore, a comprehensive analysis should be conducted to confirm the in vivo cytocompatibility of bioabsorbable iron materials and avoid biased or incorrect conclusions.

REFERENCES


37. Ying EB, Hwang HM. In vitro evaluation of the cytotoxicity of ion oxide nanoparticles with different coating and different sizes in A3 human T lymphocytes. Sci Total Environ 2010;408:4475–4481.