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Quantitative biocompatibility evaluation of nickel-free high-nitrogen stainless steel *in vitro/in vivo*

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Received 24 January 2013; revised 14 April 2013; accepted 5 May 2013
Published online 13 July 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/jbm.b.32982

Abstract: Coronary stents must not provoke an inflammatory response; however, some kinds of ions that are released from biometals induce biological reaction. In the present study, we quantitatively evaluated biological reaction of nickel-free high-nitrogen stainless steel (HNS) by endothelial cell culture, and a bioimaging system using NF-κB/luciferase transgenic mice to confirm the potential of HNS for the application of coronary stent. Endothelialization was greater with HNS than with commercial stainless steel (SUS316L). *In vivo* inflammatory response of HNS was lower than that of SUS316L. These differences may be related to the amounts of nickel ion eluted from the stents, as HNS did not elute nickel ion. These data suggest that HNS may be useful as a material for coronary artery stents. © 2013 The Authors. Journal of Biomedical Materials Research Part B: Applied Biomaterials Published by Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater, 102B: 68–72, 2014.

Key Words: stainless steel, endothelialization, stents, *in vivo*, inflammation

How to cite this article: Inoue M, Sasaki M, Katada Y, Taguchi T. 2014. Quantitative biocompatibility evaluation of nickel-free high-nitrogen stainless steel *in vitro/in vivo*. J Biomed Mater Res Part B 2014:102B:68–72.

INTRODUCTION

Stents are important medical devices for coronary intervention therapy.[1,2] Coronary artery stents can be classified into bare metal stents (BMSs) and drug-eluting stents.[3] Both types of stents are composed of metals[4] that possess excellent mechanical properties when compared with other materials. Favorable features of metals for use in coronary artery stents include biocompatibility, corrosion resistance, nonmagnetic, x-ray impermeability, and endothelialization properties after implantation. Common metals used in coronary artery stents that possess these features include low carbon austenitic stainless steel (SUS316L),[5,6] cobalt–chromium (Co-Cr) alloy,[7] and platinum–chromium steel (Pt-enhanced alloy).[8] Once these biometallic stents are implanted into coronary arteries, metal ions are released from the surface of these materials.

Nickel (Ni) is impregnated within the metals that compose coronary artery stents to stabilize austenite, nonmagnetic face-centered cubic structure, and to improve corrosion resistance and mechanical strength. Several enzymes that play an important role in gas exchange utilized Ni ion as cofactors.[9] However, Ni ions can also be associated with adverse effects, including inflammatory, allergic reaction, and DNA mutation.[10–12] Indeed, Ni ions can significantly increase production of inflammatory cytokines, including interleukin-1β (IL-1β), IL-6, and tumor necrosis factor (TNF).[13,14] A recent study reported that Ni ions trigger an inflammatory response by directly activating human Toll-like receptor 4 (TLR4).[15] European regulations limit the amount of Ni ion that can be released from approved Ni-containing products that contact the skin to $\leq 0.5\ \mu g/cm^2/wk$.[16] In Japan, the use of coronary artery stents made of SUS316L is contraindicated in patients with Ni allergies.[17] Because several millions people worldwide have Ni allergies,[18] use of Ni-free coronary artery stents would be of benefit. Indeed, allergic reactions to Ni ion released from coronary artery stents may trigger in-stent restenosis.[19]

The properties of Ni-reduced stainless steels have been investigated.[20–25] For example, the *in vitro* cytocompatibility of Fe-24Cr-2Mo-N, a nickel-free high-nitrogen stainless steel (HNS), was qualitatively higher than that of SUS316L.[26] Furthermore, the *in vitro* blood compatibility of Fe-17Cr-14Mn-2Mo-0.45N (BIOSSN4) and BIOSSN4 with various nitrogen contents was higher than that of SUS316L, and this compatibility increased as the nitrogen content increased.[27,28] Thus, HNS may be a useful material for coronary artery stents. However, the *in vitro* endothelialization property and *in vivo* compatibility of HNS has not yet been investigated.

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Contract grant sponsor: Japan Society for the Promotion of Science (JSPS)
Contract grant sponsor: Funding Program for World-Leading Innovation R&D on Science and Technology (FIRST Program)
Contract grant sponsor: World Premier International Research Center (WPI) Initiative on Materials Nanoarchitectonics

TABLE I. Chemical Composition of HNS and SUS316L (mass %)

	C	Si	Mn	P	S	Ni	Cr	Mo	N	O	Fe
HNS	0.029	0.13	0.06	—	—	<0.005	22.65	1.02	1.03	—	Bal.
SUS316L	0.025	0.48	1.5	0.027	0.016	11.87	16.72	2.01	0.021	0.0042	Bal.

C: carbon; Si: silicon; Mn: manganese; P: phosphate; S: sulfur; Ni: nickel; Cr: chromium; Mo: molybdenum; N: nitrogen; O: oxygen; Fe: iron; —: not analyzed; Bal.: balancing.

Therefore, this study investigated the utility of HNS for use in a BMS and specifically evaluated the endothelialization and inflammatory response associated with this type of stent when compared with a SUS316L stent. SUS316L was used as a control biometal because it is classified in the same as Fe-Cr-based stainless steel.

MATERIALS AND METHODS

Materials

HNS ingot was prepared by the nitrogen gas–pressurized electroslag remelting (P-ESR) process, resulting in Fe–23Cr–1Mo–1N (mass%) biometal. Then, disks (10 mm in diameter, 1 mm in thickness for cell culture and ion release experiments; 5 mm in diameter, 0.5 mm in thickness for animal experiments) were cut from the HNS ingot. For the experiment, the mirror-polished disks were sonicated for 15 min in acetone three times to remove any extraneous matter. The disk was then irradiated using an ultraviolet (UV) light source lamp (8 W, Miyata Elevam, Tokyo, Japan) at 185 and 254 nm for 60 min, followed by sterilization with ethylene oxide gas. After ethylene oxide sterilization, alloys were in vacuo for 2 weeks to remove ethylene oxide. The chemical compositions of HNS and SUS316L are summarized in Table I.

Culture of human umbilical vein endothelial cells on metals

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (Walkersville, MD) and were cultured in basal endothelial medium-2, which was changed several times a week, at 37°C and 5% carbon dioxide (CO_2) until the cells reached confluence. Then, the HUVECs were trypsinized and resuspended in fresh media. Substrate was placed into a 48-well plate (Thermo Fisher Scientific, USA), and 500 μL of HUVECs suspension (5×10^4 cells/mL) was subsequently seeded on each metals and incubated at 37°C with 5% CO_2. After incubation for 1, 3, or 7 days, the viability of HUVECs was determined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

Quantification of adsorbed fibronectin

Each substrate was incubated with human fibronectin (FN) in 10 m*M* phosphate-buffered saline (PBS; pH 7.4) at 37°C for 3 h. The amount of human FN adsorbed on the surface was calculated from the amount of human FN in PBS using an enzyme-linked immunosorbent assay (ELISA) kit containing a biotinylated anti-human FN antibody (Human FN ELISA Kit; Boster Biological Technology Co., USA). The result was shown as relative value to the amount of the FN adsorbed on SUS316L.

Determination of Ni ion elution from metals

Each substrate was immersed in saline (Otsuka Pharmaceutical Factory, Japan) for 1, 3, or 7 days at 37°C. Then, 500 μL of the supernatant was added to 500 μL of saturated trisodium citrate solution for 2 min, followed by mixing with 500 μL of 0.03*M* dimethylglyoxime (DMG) and 500 μL of 2*M* sodium hydroxide solution for 2 min. The mixture was allowed to incubate for 10 min at room temperature, and the complex of Ni ion and DMG was detected by its absorbance at 540 nm using a micro-plate reader.

Quantitative analysis of inflammation of metals

Disks were implanted subcutaneously into the backs of 10- to 15-week-old BALB/c-transgenic (nuclear factor-kappa B (NF-κB)-responsive element (RE)-luciferase (luc))-Xen mice (Xenogen, CA). For *in vivo* imaging, the mice were anesthetized with isoflurane and injected with 150 mg of luciferin per kilogram of body weight. After injection, the mice were placed in a chamber, and luminescence from tissues was analyzed quantitatively using an imaging system (IVIS Lumina II, Summit Pharmaceuticals International Corporation, Tokyo, Japan) at 15 min. Photons emitted from the tissues were quantified using living images software. The signal intensity was quantified as the sum of all detected photon counts per second within the region of interest after subtracting background luminescence and is presented as photons per second. Animal experiments were approved by the Institutional Animal Experiment Committee of the National Institute for Material Science of Japan.

RESULTS AND DISCUSSION

Endothelialization property

Endothelialization on the surface of coronary artery stents helps prevent in-stent thrombosis.[29] Endothelialization property of the HNS and SUS316L was investigated by assessing proliferation of HUVECs. Figure 1 shows HUVEC cultures on HNS and SUS316L after 1, 3, and 7 days. There were no significant differences ($p > 0.05$) in the number of HUVECs at the 1-day time point when comparing HNS and SUS316L. However, the number of HUVECs at 3 days was 1.6-fold higher on HNS than on SUS316L ($p < 0.05$). Furthermore, the number of HUVECs on HNS was 2.8-fold higher at 3 days than at the 1-day time point. Similarly, the number of HUVECs at 7 days was 1.3-fold higher on HNS than on SUS316L, and the number of HUVECs on HNS was fourfold higher at 7 days than at the 1-day time point.

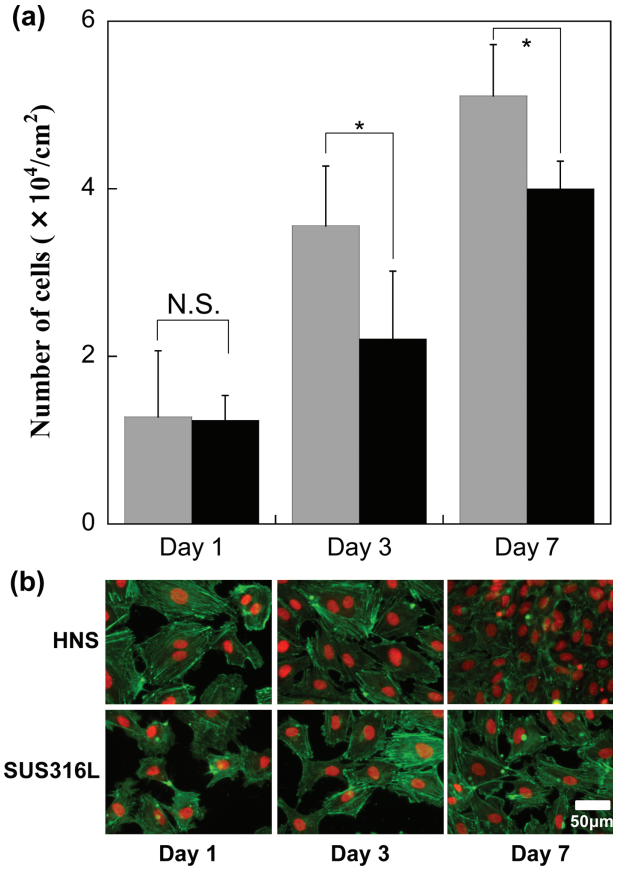

FIGURE 1. Endothelialization property of HNS and SUS316L. (a) Number of HUVECs on HNS (gray bar) and SUS316L (black bar) after cultured for 1, 3, and 7 days. Data are the average ± standard deviations (SD) of five samples, with (*) indicating $p < 0.05$. N.S. means no significant difference. (b) Distribution and morphology of HUVECs on HNS and SUS316L after cultured for 1, 3, and 7 days. Actin filaments (green) and cell nuclei (red) are shown.

The distribution and morphology of HUVECs on HNS and SUS316L after culture for 1, 3, and 7 days are shown in Figure 1(b). After culture for 1 day, there were no differences in HUVEC morphologies when comparing HNS or SUS316L, and cell distribution correlated with the number of HUVECs. After culture for 3 and 7 days, the surfaces of HNS were covered with stretched HUVECs.

Surface morphology of biomaterials and protein adsorption on biomaterials affect cell adhesion and proliferation. However, in our experiment, the surface morphology of metals was not supposed to affect cell adhesion and proliferation, because mirror-polished metals were used. To compare the cell adhesion properties, we incubated each metal in human FN-containing PBS and assessed the level of FN adsorbed, as FN is a common cell adhesion molecule that is present in the extracellular matrix and in the serum.[30] Furthermore, the adsorption of FN is a key mediator of initial cell adhesion. Figure 2 shows the amount of FN adsorbed on the surface. The amount of FN was 1.09-fold higher for HNS than for SUS316L, but this difference did not reach the level of statistical significance. This suggests that factors other than surface morphology and adsorption of cell adhesion protein resulted in the decreased number of HUVECs on SUS316L when compared with HNS.

Elution of Ni ion from metals

After culture for 1 day, there were no significant differences in the number of HUVECs when comparing HNS and SUS316L. This means that the adhesiveness of HUVECs was similar regardless of the types of metal. However, the number of HUVECs after culture for 3 or 7 days was significantly higher on HNS than on SUS316L. To investigate the different proliferation behavior of HUVECs on HNS and SUS316L, we assessed Ni ion elution from these metals. Figure 3 shows the amount of Ni ions released from HNS or SUS316L disks after immersion in PBS for various periods. A total of 37.2 ± 7.7 μg/g/well of Ni ion was eluted from SUS316L after 1 day. The amount of Ni ion eluted from SUS316L gradually increased with time and reached 88.2 ± 19.7 μg/g/well after incubation for 7 days. In contrast, the amount of Ni ions eluted from HNS was below the detection limit (limit of detection: 1.2 ppb)[31] even after immersion in PBS for 7 days. European regulations limit the amount of Ni ion that can be released from approved Ni-containing products that contact the skin to $\leq 0.5\,\mu g/cm^2$/wk.[16] In Japan, the use of coronary artery stents made of SUS316L is contraindicated in patients with Ni allergies.[17] Thus, HNS may be more useful than SUS316L on the basis of Ni ion elution.

In vivo biocompatibility test

It was expected that the inflammatory response of HNS to body would be much lower than that of SUS316L, because *in vitro* experiments revealed that HNS does not elute the Ni ion. Furthermore, studies have reported that NF-κB-related gene expression is upregulated by Ni ions[32,33] and

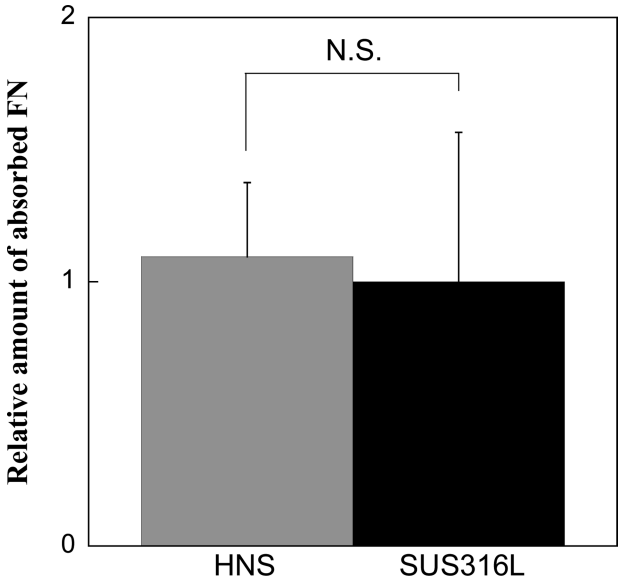

FIGURE 2. Relative value of adsorbed FN on the surface of HNS (gray bar) and SUS316L (black bar) assuming that SUS316L is 1. Data are the average ± SD of five samples. N.S. means no significant difference.

that expression of IL-1β, IL6, and TNF are regulated by NF-κB.[34] To investigate NF-κB expression after implantation of HNS *in vivo,* we evaluated the bioluminescence of the HNS- or SUS316L-implanted area in NF-κB-RE-luc-Xen mice. Figure 4(a) shows the bioluminescent signal of the on or around implanted specimen after implantation of HNS or SUS316L. The metal embedding parts are surrounded by a dotted line. The emission intensity of the HNS was significantly lower than that of the SUS316L. Figure 4(b) shows the intensity of the bioluminescent signal of the implant region. Amounts of bioluminescent signal were shown maximum value after SUS316L implanted site for 3 weeks. In contrast, the emission intensity of the HNS was nearly constant, even after implantation, which is consistent with the fact that the HNS does not elute Ni ions. After 3 weeks, the bioluminescent signal of the HNS ($0.28 \times 10^8 \pm 0.18 \times 10^8$ p/s) was significantly lower ($p < 0.05$) than that of the SUS316L ($1.37 \times 10^8 \pm 0.76 \times 10^8$ p/s), suggesting that inflammation may be induced by Ni ions derived from SUS316L. After 8 weeks, the bioluminescent signal of SUS316L markedly decreased, and no significant differences were observed between HNS and SUS316L. This finding may reflect encapsulation of SUS316L within the subcutaneous tissue at this time point. The toxic and allergic effect of some metal ions released from used these metals has been previously described.[35] Indeed, Ni ion triggers expression of the surface adhesion molecules, such as vascular cell adhesion molecule-1 and intercellular adhesion molecule 1 on endothelial cells[36] and induces an increase in NF-κB DNA binding in endothelial cells. These data show that HNS had excellent biocompatibility when compared with SUS316L when used in a coronary artery stent.

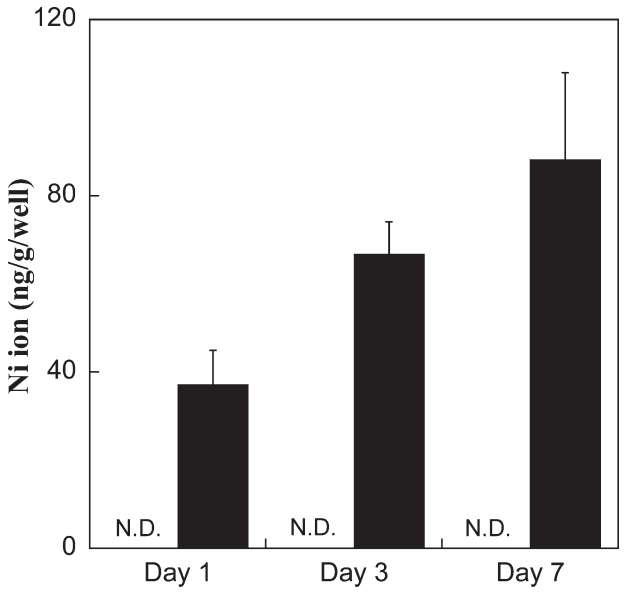

FIGURE 3. Concentration of Ni ions eluted from HNS (gray bar) and SUS316L (black bar) after immersion in PBS at 37°C for 1, 3, and 7 days. Data are the average ± SD of five samples. N.D. means not detected.

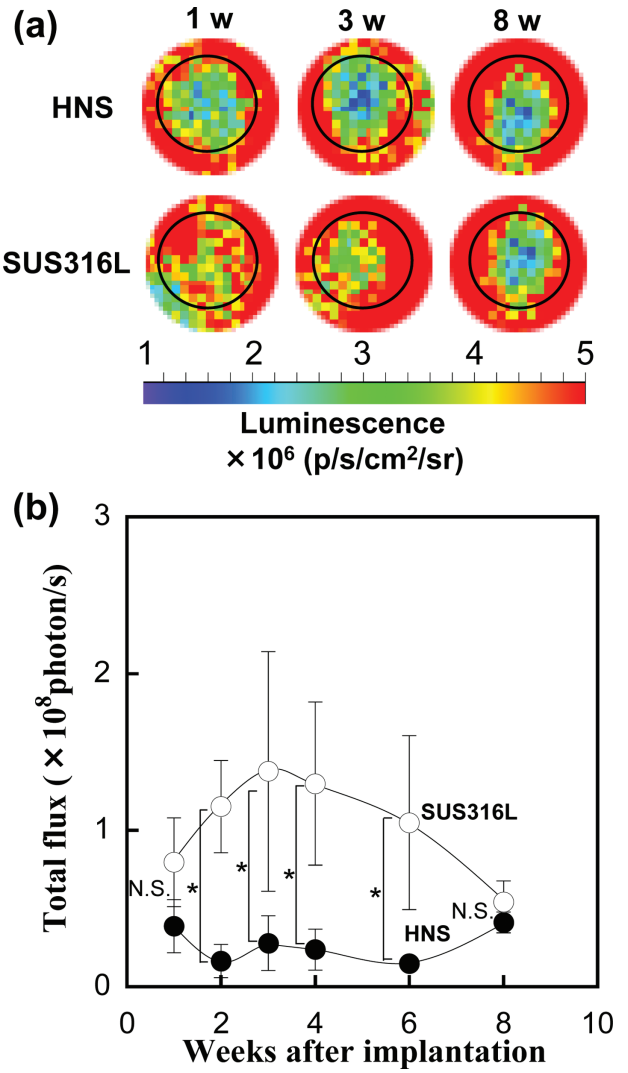

FIGURE 4. NF-κB-dependent bioluminescence in living mice implanted with HNS and SUS316L. (a) Diagrams show the bioluminescent signal within 10 mm of the implanted region. The color overlay on the image represents the photons/s emitted from the animal, as indicated by the color scales. The metals embedding part are surrounded by a dotted line. (b) Quantitative analysis of inflammation of HNS (•) and SUS316L (○) after implantation for various days. Data are the average ± SD of five samples, with (*) indicating $p < 0.05$. N.S. means no significant difference.

CONCLUSIONS

Because NHS does not contain Ni element, endothelialization property of HNS is higher than that of SUS316L. The inflammatory response of HNS was 4.89 times lower than that of SUS316L using bioimaging and NF-κB responsive transgenic mice. These data concluded that the HNS have excellent endothelialization property and biocompatibility compared with SUS316L as used stent materials. HNS therefore promises to use for coronary artery stents.

ACKNOWLEDGMENTS

We thank Ms. M. Ueno and Ms. T. Ishizuka, Biomaterials Unit, National Institute for Materials Science, Japan for their technical support.

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