Examining the role of nickel and NiTi nanoparticles promoting inflammation and angiogenesis

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\textbf{ABSTRACT}

Nickel titanium (NiTi, or Nitinol) alloy is used in several biomedical applications, including peripheral vascular stents and fallopian tube stents. There are significant biocompatibility issues of metallic implants to nickel ions and nano-/micro-sized alloy particles. Our laboratories have recently shown that microscale CoCr wear particles from metal-on-metal hips crosslink with the innate immune signaling Toll-like receptor 4 (TLR4), prompting downstream signaling that results in interleukin (IL)-1\textbeta and IL-8 gene expression. \textit{In vivo}, NiTi alloy can also generate wear particles on the nanoscale (NP) that have thus far not been studied for their potential to induce inflammation and angiogenesis that can, in turn, contribute to implant (e.g. stent) failure. Earlier studies by others demonstrated that nickel could induce contact hypersensitivity by crosslinking the human, but not the mouse, TLR4. In the present work, it is demonstrated that NiCl\textsubscript{2} ions and NiTi nanoparticles induce pro-inflammatory and pro-angiogenic cytokine/chemokine expression in human endothelial and monocyte cell lines \textit{in vitro}. These observations prompt concerns about potential mechanisms for stent failure. The data here showed a direct correlation between intracellular uptake of Ni\textsuperscript{2+} and generation of reactive oxygen species. To determine a role for nickel and NiTi nanoparticles in inducing angiogenesis \textit{in vivo}, 1-cm silicone angioreactors were implanted subcutaneously into athymic (T-cell-deficient) nude mice. The angioreactors contained Matrigel (a gelatinous protein mixture that resembles extracellular matrix) in addition to one of the following: PBS (negative control), VEGF/FGF-2 (positive control), NiCl\textsubscript{2} or NiTi NP. The implantation of angioreactors represents a potential tool for quantification of angiogenic potentials of medical device-derived particles and ions \textit{in vivo}. By this approach, NiTi NP were found to be markedly angiogenic, while Ni\textsuperscript{2+} was less-so. The angioreactors may provide a powerful tool to examine if debris shed from medical devices may promote untoward biological effects.

\textbf{Introduction}

Nickel titanium (NiTi) alloy is used extensively in a number of biomedical applications, including peripheral vascular stents (Nathan et al. 2017), septal occluders (Lertsapcharoen et al. 2009), abdominal aortic aneurysm stent grafts (Mensel et al. 2014), mitral heart valves (Berreklouw et al. 2011; Saia et al. 2012), and aortic heart valves (Saia et al. 2012). NiTi alloy has very useful shape-memory super-elastic properties that can be controlled by temperature. This permits the fabrication of devices that can be crimped down onto catheters and deployed via vascular access sites. Surface treatment and passivation to produce a stable oxide layer are critical to ensuring NiTi alloy corrosion resistance; however, nickel oxide is more likely to dissolve than the titanium dioxide layer, releasing Ni ions (Shabalovskaya 2002). Corrosion is undesirable because it leads not only to release of Ni ions, but also to fractures and release of nano- and microscale particles. Stent fracture is an issue particularly in femoral popliteal arterial applications. Advanced designs have resulted in better radial strength and conformance to arterial structures (Aghel and Armstrong 2014) in peripheral and coronary stents (Chininak and Sadeghipour 2014). Notably, coating the surface of NiTi alloy with polystyrene mitigates corrosion and acts as an insulator (Quinones and Gawalt 2008).

A critical biocompatibility concern is to determine the local nickel concentrations around NiTi implants, which are known to vary with time and distance on the order of mm, as shown in studies lasting over seven days (Wataha et al. 2013). By following ion release under both static and dynamic conditions into cell culture media, an initial bolus of nickel ion release from NiTi alloy was observed on Day 1. It dropped by 50\% on Day 3 and again on Day 7 and was unchanged from Day 7 to Day 42 (Haider et al. 2011). From the above and other studies, it is known that nickel release from NiTi results in localized tissue concentrations in a fairly rapid fashion and is related to the thickness of the oxide layer. Depending on deployment site,
nickel could be released into surrounding tissue and/or into circulating blood (Haider et al. 2009).

Although many biocompatibility studies use rodents, this is a poor model for nickel and NiTi alloy implants. Toll-like receptors (TLR) are pattern recognition molecules on immune cells that sense danger signals from pathogens, resulting in activation of the immune system. The human (hu) TLR-4 can directly be activated by nickel, in contrast to the rodent counterpart (Schmidt, Raghavan, et al. 2010). Importantly, our research has shown that huTLR-4 is activated by CoCr metal-on-metal hip wear particles from orthopedic devices (Potnis et al. 2013; Dutta et al. 2015). Special (transgenic) mice expressing the huTLR-4 gene have been generated which provide significant insight into NiTi alloy toxicity (Schmidt, Raghavan, et al. 2010).

Critically, monocytes express TLR-4 receptors, which activate nickel-specific signaling with the generation of nuclear factor kappa-light-chain-enhancers of activated B-cells (NF-κB). Nickel ions and CoCr wear particles enhance monocyte differentiation. Importantly, hypoxia-inducible factor (HIF)-1α can be cross-linked by nickel, mimicking low oxygen concentrations, resulting in stabilization of HIF-1α, dimerization, and nuclear entry. This leads to expression of target genes with hypoxia responsive elements (HRE) including matrix metalloproteases (MMP) and vascular endothelial growth factor (VEGF) (Salnikow et al. 2002; Maxwell and Salnikow 2004). Transcriptional analysis of human monocytes following nickel exposure identified a dose-dependent up-regulation of 1385 genes (Golz et al. 2016). In another study, nickel induced NF-κB in human monocyte cell lines, leading to release of interleukin (IL)-8 (Freitas and Fernandes 2011).

There are significant biocompatibility issues in metallic implants that may be missed because humans respond to nickel ions and nano-/micro-sized NiTi particles much differently than rodents in the context of immune-signaling TLR-4. NiTi alloy can generate nanoparticles (NP) in vivo (i.e. “wear” particles) that have not previously been studied for their potential to induce inflammation and angiogenesis, which can lead, for example, to stent failure. Previous studies demonstrated that nickel could induce contact hypersensitivity by crosslinking the human (but not the mouse) TLR4 and activating downstream inflammasome pathways. The EA.hy926 cell line retains characteristics of human umbilical vein endothelial cells (HUVEC) and can respond to metal-on-metal hip implant wear particles (Potnis et al. 2013; Dutta et al. 2015).

Medical devices often fail due to inappropriate inflammation and angiogenesis. The implanted device or wear/breakdown materials may be highly inflammatory and thus potent initiators of blood vessel formation. The current study focused on NiTi nanoparticles that may be generated by the corrosion and wear of NiTi devices. The research strategy used encompassed an in vitro approach to catalog the endothelial and macrophage responses to NiTi NP on a cellular and molecular level and to extend these observations to an in vivo implantation model.

### Materials and methods

#### Cell culture

Immortalized EA.hy926 human vascular endothelium cells (ATCC, Manassas, VA) were cultured in DMEM with high glucose (ATCC) supplemented with 10% heat inactivated-fetal bovine serum (HI-FBS), penicillin-streptomycin, and 2 mM L-glutamine (all Gibco, Life Technologies, Grand Island, NY). THP-1 cells (human monocytes, ATCC) were cultured in RPMI-1640 (ATCC) containing HEPES, high glucose, and L-glutamine. The THP-1 cells were differentiated with 100 nM phorbol myristate-13 acetate (PMA; Sigma, St Louis, MO, USA). The stimulated THP-1 cells were plated into 24-well flat-bottom plates (500,000 cells/well) for 24 hr to completely differentiate into macrophages. The media was replaced after 24 hr and the cells then incubated with new media for a further 1 hr.

#### Chemicals

**Nickel chloride**

Nickel (II) chloride anhydrous powder (Sigma) was reconstituted as a 1 M stock in low-endotoxin Limulus amebocyte lysate (LAL)-tested water (Associates of Cape Cod, Falmouth, MA, USA).

**NiTi alloy nanoparticles (NiTi NP)**

NiTi Alloy Nanopowder (Ni:Ti = 50:50, 99%, average size 60 nm), Stock #US1369, US Research Nanomaterials Inc., Houston, TX). The NP were prepared by laser evaporation; the certificate of analysis is shown in Table 1. The NP were heat-treated in a 250°C oven for 2 hr, cooled, and re-suspended at 1.0 mg/ml in LAL reagent water. A soluble Limulus kit (Associates of Cape Cod) was used to assess endotoxin levels in all NiTi alloy particle suspensions.

**NiTi NP analysis**

**Transmission electron microscopy (TEM) analysis**

An aliquot of the NiTi NP (10 µl) was dispersed in distilled water (1 mg/ml), deposited onto a 400-mesh Formvar/carbon-coated TEM Copper grid (Electron Microscopy Sciences, Hatfield, PA), then blotted off with a piece of filter paper after 5 min. The grid was air-dried for 1 hr prior to TEM imaging in a JEM-1400 TEM (JEOL, Tokyo, Japan) with an acceleration voltage of 80 kV. The NP size was then analyzed using Image J software (NIH, Bethesda, MD, USA).

**Scanning electron microscopy (SEM) energy dispersive X-Ray spectroscopy (EDS) analysis**

A Mira 3 field emission SEM (Tescan, Warrendale, PA, USA) equipped with an X-Max 80 EDS spectrometer (Oxford, Concord, MA, USA) was used to image the NiTi particles and determine their elemental profile.

**Particle tracking analysis**

NiTi NP (0.1 ng/ml) were suspended in either complete RPMI or DMEM media for 0.24, and 48 hr. A NanoSight NS500 particle tracking analyzer (Malvern Pan-Analytical Products, Westborough, MA, USA) was used to determine the NiTi NP hydrodynamic size.

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*Table 1. NiTi certificate of analysis.*

| Ni-Ti alloy nanoparticles certificate of analysis (%) | Ni | Ti | Co | Fe | Cu | Cr | Residue |
|---------------------------------------------------|----|----|----|----|----|----|---------|
| >54.18                                            | ≥45.56 | ≤0.018 | ≤0.023 | ≤0.020 | ≤0.02 | ≤0.0002 |
Release of nickel from NiTi NP

NiTi NP (0.1 mg/ml) were sonicated for 15 min and then added to 2 ml of 0.2 μM-pore-filtered 10 mM MOPS (3-(N-morpholino) propane sulfonic acid) buffer (pH 8.0) and incubated for 0, 24, and 48 hr at 37°C in a humidified incubator with 5% CO2 gas. At the end of each incubation period, the NiTi particles were dispersed with vigorous pipetting, transferred to 1.8 ml polypropylene centrifuge tubes, and then centrifuged (8000 × g) for 1 hr at room temperature. The resulting supernatant was carefully removed for analysis. Note that the collected supernatants were negative for NiTi particles using a Zetasizer (Malvern, UK) (data not shown). Supernatants were diluted in 10 mM MOPS and assayed (in triplicate) in 96-well flat bottom plates. Samples received 2 μM Newport Green (Molecular Probes) and were then incubated 60 min at room temperature prior to analysis (Hahn et al. 2012). Fluorescence was recorded using a microplate reader ( Molecular Devices, San Jose, CA) set at an excitation of 485 nM and an emission of 538 nM.

Flow cytometry

Flow cytometric analysis was performed on an LSR Fortessa system (BD Biosciences, San Jose, CA). A minimum of 10,000 events were acquired for each sample. Cells were first gated based on their forward and side-scatter profiles. Untreated and unstained cells were used as references for gating positive fluorescence signals. Each experiment was repeated three times. Data was further analyzed using FloJo software (FloJo LLC, Ashland, OR, USA).

Live/dead cell assay

Viability of the THP-1 and EA.hy926 cells were evaluated by flow cytometry using Live/Dead Fixable Violet Dead Cell Stain (Molecular Probes, Life Technologies, Eugene, OR, USA). PMA-differentiated THP-1 and EA.hy926 cells were first exposed for 24 hr to increasing concentrations of NiCl2 or lipopolysaccharide (LPS) from Escherichia coli (20 ng/ml, Type 0111:B4; Sigma). The cells were then harvested and stained according to manufacturer directions. The stained cells were then washed twice and analyzed by flow cytometric analysis.

Intracellular nickel ion detection

Measures of Ni uptake by the THP-1 and EA.hy926 cells (via measures of intracellular Ni2+) were performed using Newport Green DCF diacetate (Molecular Probes). The PMA-differentiated THP-1 and EA.hy926 cells were first exposed for 24 hr to increasing concentrations of NiCl2. The cells were then harvested and fixed with Cytofix/Cytoperm Fixation and Permeabilization solution (BD Biosciences) for 15 min. After two washes with HBSS, the fixed cells were stained following manufacturer protocols. The stained cells were then washed twice with HBSS and subjected to flow cytometric analysis using a 488 nm excitation with a 530/30 band pass filter.

Reactive oxygen species (ROS) production

Production of ROS by THP-1 and EA.Hy926 cells was evaluated using CellROX Deep Red Reagent (Molecular Probes). The PMA-differentiated THP-1 and EA.hy926 cells were first exposed for 24 hr to increasing concentrations of NiCl2 or LPS (20 ng/ml). The cells were then harvested and stained according to manufacturer directions. The stained cells were then washed twice and analyzed by flow cytometric analysis.

Real-time PCR

The EA.hy926 and THP-1 cells were exposed to NiCl2 (0.2–1.5 mM), LPS (20 ng/ml; positive control), or medium alone (negative control; NC), and then washed/processed for qRT-PCR. Total RNA from cultures was isolated and purified using a RNeasy Plus Mini Kit (Qiagen Technologies, Gaithersburg, MD, USA). Total RNA concentration and purity was determined using a Qubit 2.0 Fluorometer (Life Technologies). Total RNA was collected from two independent experiments (n = 3) and then RNA (0.5–1 μg) treatment was reverse transcribed into cDNA using a QuantiFast SYBR Green PCR Kit (Qiagen) and gene-specific primers (Invitrogen) (Table 2). Results were obtained using a LightCycler 480 System (Roche Diagnostics Corporation, Indianapolis, IN). All mRNA levels for the analyzed genes were normalized to the housekeeping gene GAPDH and relative expression was then calculated.

Mice

Nude mice (female, 6–8 wk-of-age) were purchased from The Jackson Labs (Bar Harbor, ME, USA) for use in Directed In vivo Angiogenesis Assays (DIVAA). To determine a role for NiTi NP in stent re-vascularization in vivo, 1-cm silicone angioreactors (Trevenig Gaithersburg, MD) were subcutaneously implanted into athymic (T-cell-deficient) nude mice. The angioreactors were initially filled with Matrigel (Trevenig) along with either PBS (negative control) or VEGF/FGF-2 (Trevenig) (positive control). The VEGF/FGF-2 was prepared by adding 60 μl of rFGF-2 (1.8 μg)/VEGF (600 ng) and 12 μg heparin (2 mg/ml) to 200 μl of PathClear® basement membrane extract (BME; Trevenig). For the evaluation of the NP, an aliquot of the 1 mg/ml NiTi NP stock was diluted in Matrigel by a factor of 10, and then 20 μl of this solution was loaded into each reactor (Guedez et al. 2003). After 15 days, each angioreactor was removed and the contents were extracted and digested with Cellisperse (Trevenig) for cell isolation. Recovered cells were stained using fluorochrome-labeled anti-MAdCAM-1 (Lifespan BioSciences, Seattle, WA, USA) and fluorescein isothiocyanate (FITC)-labeled anti-CD31 (BD Biosciences). After washing, the labeled cells were then subjected to flow cytometric analysis.

Table 2. Characterization of NiTi NP.

| Characteristic               | Value          |
|-----------------------------|----------------|
| Average particle size       | 64.60 nm       |
| Range                       | 25.73 – 272.90 nm |
| Average aspect ratio        | 1.056          |
| Average roundness           | 0.950          |
| Average circularity         | 0.990          |
| Average perimeter           | 193.60 nm      |
| Shape                       | Spherical      |

Statistical analysis

All data are expressed as means ± SD. Individual experiments were performed at least in triplicate (n ≥ 3). Instat 2 software (Prism v.5; Graph Pad Software, San Diego, CA) was used for all
statistical analyses. Significant differences between groups were determined using a Student’s t-test or a one-way analysis of variance (ANOVA) followed by a Dunnett’s or Bonferroni’s post-hoc test to compare the significance of any differences between means. A p-value ≤ 0.05 was considered significant.

Results

Initial experiments focused on characterizing the 60 nM NiTi NP. The certificate of analysis (Table 1) shows the alloy was 54% Ni and 45% Ti. As shown in Figure 1(A,B), both the SEM and TEM images reveal the generally spherical form of the NiTi NP. The size determined by TEM was around 64 nm and the average hydrodynamic diameter measured by PTA was 166.4 nm. Further analysis of the NP using SEM-EDS revealed an even distribution of Ni and Ti atoms on the surface of the particles and prominent Ni and Ti signals. Importantly, in the elemental analysis, no other metals were detected in the NP samples (Figure 1(C,D)). Physical geometry of the particles was also estimated. The average aspect ratio was 1.056, the average roundness 0.9, the average circularity 0.99, and the average perimeter 193.6 nm. The NP were spherical in shape (Table 3).

Endotoxin is a serious concern in biological assessments of NP. To address this issue, NiTi NP were heat-treated and re-suspended in endotoxin-free water. The NiTi particles were tested using a commercial endotoxin kit and were negative for LPS. Results were obtained using a lysate sensitivity of 0.03 EU/ml (k = 0.03 EU/ml). Clots for PPC indicated there was no significant interference. Therefore, all three concentrations of Ni/Ti NP used here contained less than 0.03 EU/ml (the labeled sensitivity of the lysate reagent) endotoxin (Table 4).

The amount of nickel ions released from NiTi NP in vitro was assayed using Newport Green florescence. As shown in Table 5, when NiTi NP were incubated in MOPS buffer (pH 8.0), the nickel concentration rose quickly to 0.5 mM at 24 hr and plateaued at 0.61 mM at 48 hr. Attempts to perform this assay in compete culture media were unsuccessful. Clearly,
treatment of cells with the NiTi NP is a combination of nickel ions and the NP.

The hydrodynamic size of the NiTi NP was assessed using dynamic light scattering. As shown in Table 6, suspending the NiTi in RPMI complete media resulted in immediate agglomeration and so the NiTi NP size remained unchanged over time. In contrast, the hydrodynamic size of NiTi NP increased 8-fold when incubated in DMEM for 24 hr. At 48 hr, no further increases in size were seen, however, the mean hydrodynamic size was significantly different from the “0” time control.

Because nickel is much more toxic than titanium (Ortiz et al. 2011), this study focused on assessing the effect of nickel on viability of both the THP-1 and EA.hy926 cells. In these experiments, PMA-differentiated THP-1 monocytes, and EA.hy926 cells were exposed to increasing concentrations of NiCl2 or LPS (20 ng/ml) for 24 hr. Histograms showed separation of live (left) and dead (right) cells treated with increasing concentrations of NiCl2 or the single dose of LPS (Figures 2 and 3). In each figure, the X-axis represents dye fluorescence, and the Y-axis represents cell counts. For the THP-1 cells, 10, 0.25, 0.5-, and 0.75-mM nickel had no effect on viability (values ranged from 10 to 15%). However, the measured cytotoxicity increased to 21% with 1 mM nickel. Importantly, the LPS positive control worked as expected, inducing 22% cell death. In contrast, in EA.hy296 cells, there was a concentration-dependent decrease in viability of 6–13% at 1.0 mM nickel, a level that reached the LPS control value.

With these noted changes in cell viability induced by nickel, the amount of nickel taken up by the THP-1 and EA.hy926 cells over the 24-hr period was next evaluated. In this experiment, cells were exposed to increasing concentrations of nickel for

| Table 5. Nickel release from NiTi particles in vitro. |
| --- |
| Time (Hr) | Ni Concentration (mM) |
| 48 | 0.61 ± 0.07 |
| 24 | 0.50 ± 0.09 |
| 0 | 0.00 |

Nickel release was assessed fluorescently using Newport Green. Assay was performed in triplicate. Data shown are mean ± SE of triplicate measurements.

| Table 6. NiTi NP agglomeration in RPMI and DMEM complete media at selected timepoints. |
| --- |
| Time (Hr) | RPMI complete media | DMEM complete media |
| 0 | 194 ± 50 | 80 ± 36 |
| 24 | 235 ± 50 | 450 ± 113 |
| 48 | 261 ± 53 | *450 ± 113 |

*Value significantly different from RPMI system.

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Figure 2. Analysis of viability of PMA-differentiated THP-1 monocytes exposed 24 hr to increasing concentrations of NiCl2 or 20 ng LPS/ml. Treated and untreated cells were labeled with live/dead fixable dead cell stain and analyzed by flow cytometry using 405 nm excitation with a 450/50 nm emission band-pass filter. Histograms show separation of live (left) and dead (right) cells treated with increasing concentrations of NiCl2 or the LPS. X-Axis represents dye fluorescence and Y-Axis represents cell counts. Each histogram presents the mean ± SE of three experimental samples from three independent experiments, n = 3.
24 hr and then labeled with Newport Green dye to assess intracellular nickel concentrations by flow cytometry. The data show that THP-1 cells internalized roughly twice the amount of the dye label than the EA.hy926 cells. In both cell types, there was a linear increase in dye signal (MFI; mean fluorescence intensity) with time as the nickel concentration increased from 0.25 to 0.75 mM. Between 0.75- and 1-mM nickel, there was an enhanced uptake of nickel by the THP-1 and EA.hy926 cells (Figure 4(A,B), respectively). With the highest concentration of NiCl₂, there was a 3.3- and 7.1-fold increase in Ni²⁺ uptake by the EA.hy926 and THP-1 cells, respectively.

The entry of nickel into cells may induce oxidative stress; this was examined here via a flow cytometric assay to detect reactive oxygen species (ROS) formation in the cells. Generation of ROS induced by nickel was seen to mirror closely with the nickel uptake data for the cells (Figure 5(A,B)). Specifically, there were 3.8- and 7.4-fold increases in ROS generation in the THP-1 and EA.hy926 cells, respectively. Interestingly, at the highest nickel concentration, both the Newport Green- and Cell Rox-based assays seemed to reflect a nonlinear response in the THP-1 cells. In contrast to the THP-1 data, the generation of ROS in the EA.hy926 cells was more dose-related.

The effect of nickel on gene expression was examined by monitoring the expression of more than 40 genes for responsiveness to nickel (Table 2). Several genes responded in a dose-related fashion in the initial screening. In THP-1 cells exposed to nickel, the mRNA for the pleotropic transcription factor HIF-1α increased in a linear fashion with 0.25 to 0.75 mM nickel but decreased at 1.0 mM nickel. Interestingly, there was no LPS induction of HIF-1α. Levels of BCL-2 (regulator of apoptosis) mRNA accrued in a dose-related manner over the range of 0.25 to 1.0 mM nickel. There was no induction of BCL-2 by LPS in the THP-1 cells. Levels of intracellular cell adhesion molecule-1
(ICAM-1) and cellular inhibitor of apoptosis protein (c-IAP) mRNA increased linearly from 0.25 to 1.0 mM nickel. In this case, mRNA levels for ICAM-1 and c-IAP were also induced by LPS (Figure 6(A)).

Nickel-induced gene expression profiles in the EA.hy296 cells were very different than in the THP-1 cells. There was little mRNA induction for HIF-1α and ICAM-1 after nickel treatment. At only one dose, i.e. 1.0 mM, was there a significant induction of gene expression in the EA.hy296 cells. With regard to BCL-2 and c-IAP, mRNA levels did increase with rising levels of nickel treatment; however, the changes were not statistically significant.

A characterization of the NiTi particles was also undertaken. Initially, how the particles may alter the generation of reactive oxygen species was examined. EA.hy926 cells were exposed to Ni/Ti-0.1 mg/ml nanoparticles or medium control (negative control-NC) for 24, 48, and 72 hr. At the indicated times, cells were harvested and incubated with Cell Rox and analyzed by Flow Cytometry. As shown in Figure 7, The maximal effect was seen at 24 hr, with 32% of the cells exhibiting a positive signal. By 48 hr, this number of cells generating a fluorescent signal dropped by one-third and remained low at 72 hr. Clearly, the majority of oxidative generation occurred early in the first 24 hr.

The oxidative burst may be due to the internalization of NiTi particles in the EA.hy926 cells. Again, Newport Green was employed to examine this in more detail. A time-course experiment revealed that at 24 hr, NiTi treatment of EA.hy926 cells resulted in almost uniform uptake of the cells as measured by MFI as shown in Figure 8. This was sustained at both the 48 and 72 hr timepoints as well. This suggested that the subsequent cellular events were due to uptake of NiTi into the endosomal compartment of the EA.hy926 cells.

The critical issue following NiTi exposure was to identify which genes were amplified and if they were pro-angiogenic and/or pro-inflammatory. EA.hy926 cells were exposed to Ni/Ti-0.1 mg/ml nanoparticles or medium control (negative control-NC) for 24, 48, and 72 hr and then processed for qRT-PCR. mRNA levels of respective genes were normalized to the GAPDH housekeeping gene and relative expression calculated. Importantly, a number of pro-angiogenic factors were up-regulated (Figure 9(A–K)). Angiopoetin-4 like 4 (Ang-like-4), a positive driver of endothelial cell migration and a negative regulator apoptosis, peaked at 48 hr; in parallel, both aspartate synthetase and hypoxia inducible factor-1 (HIF-1) exhibited the same induction kinetics and were clearly pro-angiogenic. In addition, two inhibitors of apoptosis, i.e. C-IAP-2 in conjunction with BCL-2 were induced as well and peaked at 48 hr. The appearance of the monocyte chemotactic factor-2 (CCL-2, a positive regulator of angiogenesis) spanned the 48–72 hr timepoints. At 72 hr, toll like receptor -4 (TLR-4), critical for conveying nickel, cobalt, and metal particle signals into cells, was maximally induced.

![Figure 5](image1.png)  
**Figure 5.** Flow cytometric analysis of reactive oxygen species production in PMA-differentiated THP-1 monocytes and EA.hy296 cells exposed to increasing levels of NiCl₂ or 20 ng LPS/ml for 24 hr. (A) THP-1 cells. (B) EA.hy296 cells. Treated and untreated cells were labeled with CellRox Deep. Each bar represents the mean ± SE (n = 3). *Value significantly different vs. NC (*p < 0.05; **p < 0.01; ***p < 0.001).

![Figure 6](image2.png)  
**Figure 6.** Quantification of mRNA expression using qRT-PCR with cells exposed to NiCl₂ for 24 hr. (A) Differentiated THP-1 cells. (B) EA.hy296 cells. Quantification of mRNA expression was performed using qRT-PCR. mRNA levels of respective genes were normalized to GAPDH housekeeping gene and relative expression was calculated. Each bar shows the mean ± SE. *Value significantly different vs. NC (*p < 0.05; **p < 0.01; ***p < 0.001).
Most importantly, IL-8, the critical driver of angiogenesis, was maximally induced at 72 hr. The negative regulator of angiogenesis, TNFα-related apoptosis-inducing ligand (TRAIL), peaked at 72 hr. Surprisingly, there were no changes in expression of intracellular adhesion molecule-1 (ICAM-1) at any timepoint.

Several other notable genes were not changed (Figure 9(L–S)). This included CCL20 which regulates monocyte chemotaxis; Cyclin G2, the controller of cell cycle progression; CXCL3, a pro-angiogenic cytokine; the pro-angiogenesis enzyme hemeoxygenase (HO)-1; and, the growth factor m-CSF. The chemokine CXCL2 was induced in a dose-related manner; however, this induction was not significant. In parallel, IL-1β (which can alter vascular permeability) was also dose-relatedly induced, but again this effect was not significant. Lastly, no change was seen in Cox-2 expression. Taken together, the NiTi particles induced several key up-regulators of angiogenesis and inhibitors of apoptosis within a 72 hr window and these results suggest their potential for potentiating angiogenesis in vivo.

It should be emphasized that the in vivo concentration of nickel following intramuscular implantation of nickel wire was around 1 mM. The Newport Green and ROS experiments were performed on all cells, both live and dead. The large increase in the MFI following 1 mM nickel exposure may be due to the cells undergoing apoptosis (as the Live-Dead flow cytometry is decreased and there is prominent upregulation in the BCL2 and c-IAP genes). In sum, the flow cytometric analysis of nickel uptake, as well as ROS generation and expression, all point to significant apoptosis that could result in inflammatory and angiogenic reactions in vivo.
To assess the angiogenic potential of nickel and NiTi NP, angioreactors containing Matrigel, nickel ions, or NiTi NP were implanted for 15 days. As shown in Figure 10(A,D), the number of MADCAM$^+$CD31$^+$ double-positive cells found in the negative control angioreactor was 2.3%, while the number of MADCAM$^+$CD31$^+$ double-positives found in the positive control angioreactor was 42%. With the nickel or NiTi NP angioreactors, 11 and 15% of the cells were MADCAM$^+$CD31$^+$ double positives (Figure 10(B,C), respectively).

In situ photographs of the angioreactor prior to excision (Figure 10(E)), the excised negative control (Figure 10(F)), and the excised positive control (Figure 10(G)) are provided for context. In sum, this in vivo experiment showed that both nickel and NiTi NP were pro-angiogenic and could alter endothelial function in vivo.

Discussion

The present study demonstrated that NiTi NP and nickel ions enter cells, trigger ROS formation, and also induce expression of cytokines/chemokines that are both pro-inflammatory and pro-angiogenic. Nickel also activates HIF-1α that, in turn, induces expression of numerous genes and signaling pathways that are pro-angiogenic in both endothelial cells and macrophages (Maxwell and Salnikow 2004). Importantly, human TLR-4 plays a significant role in activating cells and inducing inflammatory signaling, which is absent in the murine TLR-4 (Schmidt and Goebeler 2011, 2015).

The interaction of stent surfaces with endothelial cells is mediated through pattern recognition receptors and toll-like receptors (TLR), which are an element of innate immunity. TLR bind protein and lipid ligands of bacteria, fungi, and helminths (Dowling and Mansell 2016). Endothelial cells express TLR4 receptors that bind to bacterial LPS (lipopolysaccharide, a cell wall component of Gram-negative bacteria). LPS signaling requires that a complex of MD2 and TLR4 be present on the membrane for receptor dimerization. Similar to bacterial activation, exposure to 1.5 mM nickel for 8 hr crosslinks the receptor in endothelial cells. Past studies showed that the nickel/TLR4 dimerization acted independent of MD2 (Raghavan et al. 2012).

One important issue in metal toxicology pertains to localized in vivo concentrations of nickel. This was investigated by Wataha et al. (2013) in a rat model following intramuscular implantation of nickel wire. Using a combination of laser capture microdissection and quantitative mass spectroscopy, the tissue concentration of nickel adjacent to the implant was found to be 827 μM. This value is critical in that the highest concentration of nickel utilized in the present study was 1 mM. In primary human umbilical vein endothelial cells (HUVEC), TNFα-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis was enhanced by pre-incubation of the cells with nickel. This effect was dose-dependent when the concentration tested ranged from 0.25 to 1.5 mM (Schmidt, Hupe, et al. 2010). In an earlier study, treatment with nickel at 85 μg/ml (658 μM) for 24 hr resulted in a 75% reduction in viability in THP-1 cells (Miyazawa et al. 2007). Similarly, 25-400 μM nickel treatment for 48 hr resulted in a dose-dependent lowering of THP-1 cell viability (Zhang et al. 2015). Thus, the doses used in the current study are well in line with those of many others that showed the impact from nickel on cell viability/function.
The wire implantation studies revealed that \( \approx 1 \text{ mM} \) nickel was found to be adjacent to the implant. This value is approximately where in the present study (and others) the majority of the biological effects were seen. Another critical issue addressed in the present study was an evaluation of the level of uptake of nickel into the treated cells. Others have shown an earlier study, oxidative stress (assessed using Dichlorofluorescein dye) was enhanced in HUVEC that were cultured on mechanically polished (3–400 °C) surfaces that contained metallic nickel or NiO (Plant et al. 2005). In epithelial cells, Ni ions were also detected using Newport Green dye 24–48 hr after treatment with metallic Ni and NiO (Pietruska et al. 2011). Significantly,
micron-sized particles did not generate a presence of intracellular Ni ions. The finding in the current study of induced formation of reactive oxygen species following uptake of nickel and NiTi NP (mirrored via Newport Green fluorescence) reiterates findings shown in previous work (Duan et al. 2015).

The present study also examined effects of nickel on gene expression in both the THP-1 and EA.hy926 cells. Importantly, this experiment screened more than 25 genes that were found impacted on by nickel treatment (Viemann et al. 2007), as well as those that were expressed in a dose-dependent manner. HIF-1α is a pleotropic transcription factor with multiple gene targets that plays a critical role in wound healing, inflammation, and angiogenesis (Krock et al. 2011). In both the EA.hy926 and THP-1 cells, HIF-1α expression was induced by nickel. This was consistent with previous reports with HUVEC and monocytes wherein HIF-1α was induced by nickel (Viemann et al. 2007). B-Cell lymphoma (BCL)-2 which regulates cell death as an anti-apoptotic mitochondrial protein was also induced by nickel in both the EA.hy926 and THP-1 cells. This was not surprising given that BCL-2, a significant driver in cellular proliferation, is under the transcriptional control of HIF-1α (Sendoel and Hengartner 2014).

It was also seen here that cellular inhibitor of apoptosis protein (c-IAP) was induced significantly in the THP-1 cells by nickel. cIAP plays a central role in gene expression and cell survival (Corliss et al. 2016) and is also critical to endothelial function and vascular patency during angiogenesis. Among macrophages, cIAP is found to be elevated in the M2a subtype (Morón-Calvente et al. 2018). This would be in keeping that among macrophages broadly separated into M1 and M2 subtypes, the M1 are primarily inflammatory while the M2 promote angiogenesis (Wang et al. 2014). ICAM-1 was also upregulated significantly in the THP-1 cells by nickel. ICAM-1 is expressed on both endothelial cells and immune cells and is responsible for transmigration of cells into inflamed tissues. The nickel-induced gene expression profiles in the EA.hy926 cells were very different than in the THP-1 cells. Specifically, there was little HIF-1α or ICAM-1 mRNA induction due to nickel treatment. With regard to BCL-2 and c-IAP, mRNA levels for each did increase with increasing concentrations of nickel used for the exposures, but the changes overall were not statistically significant. Taken together, this data suggests that nickel is capable of driving both the endothelial cells and monocyte cells toward anti-apoptotic and pro-angiogenic states, but that the effect on the inflammatory cells seems to be greater.

In the EA.hy926 cells, it was also seen that the NiTi NP were potent activators of several genes that are associated with angiogenesis, including Ang-like-4, HIF-1α and IL-8. Critically, TLR-
was induced as well. TLR-4 is critical in conveying nickel metal ion signals to the inflammasome. In parallel, two anti-apoptotic genes were induced in the cells, i.e. C-IAP-2 and BCL-2, both of which enhance endothelial cell survival during angiogenesis. As shown in Table 5, NiTi NP release nickel ions in vitro, and as a result, the in vitro events are most likely a combination of NiTi NP and ions.

Critically, the NiTi NP are agglomerated in vitro, about 2-fold more so in the complete DMEM media than the RPMI. This may also account for the more robust oxidative response endothelial than the THP-1 cells.

To build on these findings and to better characterize the angiogenic potential of NiTi NP, the current study employed the DIVVA system. Here, this system concentrated angiogenesis within silicone tubes filled with a mixture of NiTi NP and Matrigel. The DIVVA system has been critical in demonstrating that the CD97 protein is required for adhesion and inflammatory cell migration as well as tumor progression and neoangiogenesis (Guedez et al. 2003; Wang et al. 2005). The system has also been used to assess effects of anti-angiogenic agents (reviewed in Napoli et al. 2011). What is novel in the present work is use of the DIVVA system in the context of metallic wear particles rather than its historic use to evaluate effects of drugs or biologics.

The present study focused on determining the presence in angioreactors of cells bearing CD31, a platelet endothelial cell adhesion molecule that is a well-characterized mecano-sensor of shear stress. In addition, cells were counterstained for MADCAM (mucosal vascular addressin cell adhesion molecule-1) protein that is also expressed on high endothelial venules and binds to counter receptors and VLA-4 (α4β1), L-selectin, and leukocyte β7 integrin LPAM-1 (α4β7) (Rivera-Nieves et al. 2008). The data here showed NiTi NP induced angiogenesis to a level 4–5-fold above background and that this was most likely associated with inflammatory processes triggered by cytokine release. Neovessels development by endothelial cells is driven in great part by VEGF and FGF cytokines (Cao et al. 2004). Of note, each of these cytokines are induced to be released from M2 macrophages by hypoxia (and thus, HIF-1α). Both are also released from activated macrophages and endothelial cells. Based on the findings here, it is clear that biomaterials/particulates have a potential to induce profound effects on neo-angiogenesis and re-vascularization processes. The results here are the first to show that nickel (in the context of an NP form) can impact on both processes, and actually act to stimulate angiogenesis in situ.

To date, there is limited information on biological responses to NiTi NP and their overall impact upon vascular tissues (Luo et al. 2015). There is a critical need to address this gap more fully, since NiTi alloys are widely used in numerous cardiovascular applications, including septal occluders, heart valves, and peripheral stents. Fractures of implants containing the alloy do occur (Aghel and Armstrong 2014; Chinikar and Sadeghipour 2014) and these give rise to a variety of immune-related problems. For example, fracture of a NiTi stent used in peripheral vascular deployment in the leg was seen to give rise to cases of allergy that manifest as severe pruritus and eczematous dermatitis (D’Arrigo et al. 2014). In an affected patient, patch testing revealed nickel ion sensitization; following stent explantation, the symptoms resolved. Histologic examination of the explanted device revealed significant infiltration of macrophages, plasma cells and lymphocytes. In a popliteal artery deployment, a fractured NiTi stent induced severe thrombosis, necessitating its removal and replacement with a by-pass graft (Guerra and Kirkwood 2017). Nickel allergic reactions have also been seen with the Amplatz septal Occluder device (St Jude Medical) (Jain et al. 2013) and with NiTi vena cava filters (Jia et al. 2015). Most importantly, sent fractures may be the nidus of hypersensitivity reactions as well (Mori, Kutys, et al 2017).

Conclusions

The present study with THP-1 monocytes and EA.hy926 human endothelial cells showed how nickel and NiTi NP could alter viability, increase oxidation, and promote changes in gene expression associated with pro-inflammatory and pro-angiogenesis outcomes. Taken together, the results here demonstrated that nickel and NiTi NP can enter both types of cells, with deleterious consequences. With the widespread use of NiTi devices, more attention to localized tissue reactions is warranted to address concerns inspired by nickel ion release due to surface finish and/or NiTi NP that may be generated from corrosion or wear. The use of angioreactors here provided a unique means of evaluating in vivo potential untoward events of such medical devices wear particles.

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Ethical approval

All animal studies were approved by the Animal Care and Use Committee. The information in these materials is not a formal dissemination of information by the FDA and does not represent agency position or policy.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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