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Analysis of the effects of surface treatments on nickel release from nitinol wires and their impact on candidate gene expression in endothelial cells

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Abstract Nitinol has many applications in the medical device industry, however the large amount of nickel, a known allergen and carcinogen remains a serious concern. Studies have already shown that nickel ions induce the differential expression of a range of genes, including cell adhesion molecules. This study sought to determine the level of nickel ions released from nitinol wires that had been surface treated by etching and mechanically polishing or etching and pickling compared to untreated wires and determine the biological impact of the wires on human umbilical vein endothelial cells (HUVECs) at the transcriptional level by real-time PCR. The four different wire types were incubated in media and the amount of nickel eluted after 24, 48 and 72 h was determined. HUVECs were then cultured and incubated with the four different wire types for 24 h. Cells were harvested, RNA isolated and real-time PCR was carried out to measure the expression levels of ICAM-1, VCAM-1 and E-selectin, three known inflammatory mediators, compared to control cells. E-selectin, a marker of endothelial cell injury and activation was found to be significantly up-regulated in cells incubated with wires that released the highest amount of nickel ions. Nickel ions are released from nitinol wires with certain surface characteristics and these ions have a biological effect on HUVECs in vitro.

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Introduction

Over 1 million percutaneous coronary revascularisation procedures are carried out worldwide annually and approximately 50% of such patients undergo stent implantation [1]. To a large extent the development of intracoronary stents has tackled a number of problems associated with percutaneous transluminal coronary angioplasty (PTCA), with stent implantation resulting in a decrease in mortality and in the necessity for emergency bypass surgery [2]. Despite their benefits, however, vascular stents have limitations, with stent implantation resulting in vessel damage and associated inflammatory and repair reactions [3]. In addition, damage to the endothelial cell (EC) layer caused by the stent can lead to intimal hyperplasia, thus leading to in-stent restenosis, defined as 'loss of greater than or equal to 50% of the gain produced at angioplasty or a >50% stenosis at follow-up angiography' [4]. Restenosis remains a significant problem, with 15–20% of patients being affected after primary stenting. Stent associated problems result from the mechanical, biological and physical properties of the stent.

After over 15 years of clinical practice in the area of biomaterials research, however, no single commercially available stent fits all the requirements of the 'ideal' stent, thus extensive multi-disciplinary research is required, to improve the physical and mechanical properties of stents and to improve the biocompatibility of the implant [5]. Research in this area has endeavoured to improve stent design through the development of novel materials,

surface treatments and coatings for use in stent manufacturing [3].

Currently, the majority of vascular stents are made from 316L stainless steel [6]. 316L consists of 0.03% Carbon, 60–65% Iron mixed with 17–18% Chromium and Nickel 12–14%. The properties of 316L offer excellent mechanical properties and the chromium content confers resistance to corrosion and provides strength and hardness [3]. However the effects of blood cells and proteins of the circulatory environment on the stents are associated with ion release from the implant material, which can lead to allergic reactions, resulting in fibroproliferative disorders around the stent, a feature common in restenosis [6]. Surface oxides produced during manufacturing also provide an ideal substratum for thrombus formation in some cases [7].

Nitinol is an alloy consisting of almost equal parts of nickel and titanium. It has recently emerged as an alternative to stainless steel in stent manufacturing, due to increased biocompatibility. Nitinol belongs to a group of metals known as shape-memory alloys (SMA), with metals in this group having the unique properties of shape-memory and superelasticity [8]. Nitinol stents can therefore be heat-set in a specific shape during manufacturing and as result, if they are deformed as a result of any stress they can revert to their original shape once that stress is removed [8] In addition, the surface naturally forms a coating consisting primarily of titanium oxide, although impurities may be present [9]. In a previous study carried out to investigate the biocompatibility of nitinol, in which the nitinol samples used were mechanically polished prior to analysis, no cytotoxic, allergic or genotoxic effects were demonstrated in vitro [10]. However this does not accurately reflect the state of nitinol wires used in stenting procedures that are often not polished prior to implantation. Thus there is still a requirement for appropriate studies on this alloy to be carried out.

Despite the demonstrated biocompatibility of nitinol, the large concentration of nickel in the alloy remains a serious concern [8]. It has already been identified that exposure to nickel compounds can induce allergic reactions and approximately 16% of the population are allergic to nickel [11]. In addition, long-term exposure to nickel compounds has been associated with carcinogenicity and nickel has been found to cause altered gene expression profiles for a range of genes, including activating transcription factor 1, cap 43, a hypoxia regulated gene and thrombospondin [12]. Nickel (II) was found to upregulate ICAM1, VCAM1 and E-selectin in cultured HUVECs and these adhesion molecules play a key role in leukocyte recruitment in post-implantation pathways [13]. These findings have serious implications for the use of nitinol in the medical device industry and as a result it is vital to fully characterise the alloy and also to determine if nickel is being released from the nitinol and if it is, investigate the effect it has at the site of implantation.

This study sought to investigate nickel release from four different nitinol wire types and the consequence of this release at the biological level using quantitative real time PCR. In this study the wires were analysed with surface conditions identical to those used for stent implantation. In addition, the effect of different surface treatments on the wires was examined.

Materials and methods

Materials used

Nitinol wires with a diameter of 0.762 mm were manufactured and supplied by Fort Wayne Metals, Indiana, USA for this study. The wires were produced from binary nickel-titanium alloy with a nominal composition of 50.8 atomic percent nickel. Samples were cold drawn and then heat-treated at 500°C under various argon/oxygen conditions, resulting in a light oxide layer or a heavy oxide layer. Wire samples with a heavy oxide and a light oxide then underwent subsequent treatments according to the manufacturers protocols, resulting in four different wire types to analyse (see Table 1). Wires that were treated by etching were etched using a solution proprietary to Fort Wayne Metals Research Products Corporation (FWMRPC) with the aim of removing the oxide layer. Pickling was carried using a solution proprietary to FWMRPC, with the end goal of attacking the base metal. Mechanical polishing involved treatment with a mechanical wire-polishing machine fixed with abrasive pads. The atomic percentage of nickel (Ni at %) in the oxide layer is also outlined in Table 1 as determined in a previous study by X-ray photoelectron spectroscopy (XPS). Surface characterisation of the wires under investigation was performed in a previous study [14].

Table 1 Summary of surface treatments and nickel measurements in oxide layer and eluted into media

Wire	Surface treatment	Ni at %	24 h mg/l	48 h mg/l	72 h mg/l
LO	Compax/light oxide	15	0.65 ± 0.015	0.74 ± 0.01	0.97 ± 0.12
HO	Compax/heavy oxide	3.6	0.01 ± 0.004	0.01 ± 0.007	0.03 ± 0.004
HO-T	Diamond/heavy oxide/etch/ mechanical polish	1.2	<0.005	<0.005	0.009 ± 0.003
LO-T	Diamond/light oxide/etch/pickle	ND	0.007	<0.005	<0.005

Wires were cut to the appropriate length and washed thoroughly by sonication. Wires were then sterilised by autoclaving in preparation for cell studies.

Nickel release study

Eight wires approximately 2 cm in length of each wire type were placed in 6 well plates in duplicate. The resulting surface area of wire in each well is thereby approximately 3.84 cm^2. Three ml of media was placed in each well and wires were incubated for three different time points- 24, 48 and 72 h and wires were incubated at 37°C and in a 5% CO_2 atmosphere. After each time point the wires were removed. The media was then analysed for Ni^{2+} concentration by inductively coupled plasma-optical emission spectroscopy (ICP-OES) (OPTIMA 5300 DV, Perkin Elmer, Massachusetts, USA). The nickel concentration of media alone was also measured.

Cell culture

Human umbilical vein endothelial cells (HUVECs) supplied by Cambrex, UK were cultured in Endothelial Growth Media-2 (EGM™-2) (Cambrex, UK), supplemented with SingleQuots® (Cambrex, UK. All cells used for experiments were between passage 6 and 9.

HUVECs were seeded at a concentration of 40,000 cells/cm^2 in 6-well plates (Sarstedt, Germany) and were left for 24 h to attach. Wires were then washed in Hanks Balanced Salt Solution and were placed in direct contact with the cells. Six wells were assigned for each wire type and 8 wire pieces were placed in each well and 6 wells were left as a control with no wires. After 24 h of incubation the wires were removed and cells were harvested using trypsin for further analysis. Three independent cell culture experiments were carried.

RNA isolation and quantitative real-time PCR

RNA extractions incorporating an on-column DNase step were carried out using the QIAGEN RNeasy mini kit. The integrity of the RNA was checked by running 2 μl of each sample on a formaldehyde gel. RNA was quantified using an ND-1000 Spectrophotometer (Nanodrop Technologies, USA) and 1 μg of each RNA sample was used to make cDNA. The RNA samples were denatured at 70°C for 5 min. Reverse transcription was performed at 42°C for 60 min in a reaction volume of 20 μl containing the following: oligo dT primer, Moloney murine leukaemia virus (M-MLV) reverse transcription buffer [50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT)] (Promega, UK), PCR grade water (Sigma Aldrich) deoxyribonucleotide triphosphates (dNTPs) (Promega, UK) and M-MLV reverse transcriptase (Promega, UK). Reverse transcriptase was inactivated by heating samples at 65°C for 10 min. Control RNA samples, in which no reverse transcriptase was added, were included to confirm that no genomic DNA contamination was present and cDNA was also checked by standard RT-PCR. Primers to β-Actin, E-selectin, Intracellular Adhesion Molecule 1 (ICAM-1) and Vascular Cell Adhesion Molecule 1 (VCAM1) were designed to published mRNA sequences from GenBank using Primer Express software (Applied Biosystems, Foster City, CA) and sequence specificity was confirmed by performing a BLAST (NCBI) search. Primer sets were synthesized by MWG Biotech (Ebersberg, Germany) (Table 2).

cDNA quantification standards, containing a known number of cDNA copies of each gene, were prepared by purifying PCR products for each gene using the QIAGEN gel purification kit. These purified products were then quantified by densitometry and appropriate dilutions were carried out. Standard curves containing a certain number of cDNA copies were generated for each of β-actin (3.75×10^7 cDNA copies, 3.75×10^5 cDNA copies, 3.75×10^4 cDNA copies and 3.75×10^3 cDNA copies), E-selectin (2×10^5 cDNA copies, 2×10^4 cDNA copies, 2×10^2 cDNA copies and 2×10^1 cDNA copies), ICAM-1 (1×10^5 cDNA copies, 1×10^4 cDNA copies, 1×10^2 cDNA copies, and 1×10^1 cDNA copies), and VCAM-1 (3×10^5 cDNA copies, 3×10^4 cDNA copies, 3×10^2 cDNA copies and 3×10^1) gene.

Amplification reactions were carried out in real-time, with separate reactions being set up for each primer set, each containing 12.5 μl of 1 × SYBR Green I PCR Master Mix (Applied Biosystems), 12.5 nanomoles of each primer and 2.5 μl template (1 in 50 dilution of cDNA) in a final volume of 25 μl. Amplification reactions were performed in 96-well optical reaction plates on the ABI 7000. A dissociation curve was generated for each primer set at the

Table 2 Oligonucleotide primers

Gene	Primer	Product size
β-Actin	For: GGGCATGGGTCAGAAGGATT	101
	Rev: AGTTGGTGACGATGCCGTG	
E-selectin	For: GAGTCCAACATTCCCTTGGTAGC	101
	Rev: GCTTTCCGTAAGCATTTCCG	
ICAM-1	For: CCGACTGGACGAGAGGGAT	108
	Rev: AGACACTTGAGCTCGGGCA	
VCAM-1	For: ATCCTGAAGAAAAAAGCGGAGA	101
	Rev: TCATATACTCCCGCATCCTTCAA	

end of each run and PCR products were run on 2% agarose gels (Sigma Aldrich) to confirm the size of the product and the specificity of the primers. cDNA copy numbers for E-selectin, ICAM-1and VCAM-1 were generated from their respective standard curves and normalised to the house-keeping gene β-actin. A fold increase was calculated relative to the control cell's expression levels. Real-time RT-PCR was carried out for each of the three independent cell culture experiments in triplicate and results were then analysed using a one-way ANOVA followed by Scheffe's test using the statistical package SPSS for Windows version 12.0.1 (SPSS Inc., Chicago, Ill, USA). A P value of <0.05 for the ANOVA was considered to be statistically significant.

Results

Nickel release study

The amount of nickel released from the wires is illustrated in Table 1 and results are presented as mean values ± standard deviations. The nickel content of the media alone was below the limit of detection of the machine. The amount of nickel released into the media appears to be related to the amount of nickel in the oxide layer.

Quantitative real-time PCR

Results are presented as the mean + standard deviations.

E-selectin was found to be 1.9 fold up-regulated in cells incubated with LO wires compared to control cells ($P = 0.033$) as illustrated in Fig. 1. E-selectin was also found to be 2.1 times up-regulated in cells incubated with HO wires compared to control cells ($P = 0.01$).

ICAM-1 was found to be statistically upregulated in cells incubated with wire HO-T compared to control cells ($P = 0.031$). There was a 2.4 fold increase in expression of ICAM-1 in cells incubated with HO-T wires compared to control cells as illustrated in Fig. 2.

VCAM-1 was found to be significantly up-regulated in cells incubated with HO wires and in cells incubated with HO-T wires compared to control cells ($P = 0.004$ and $P = 0.031$ respectively). VCAM-1 was upregulated 2.7 fold in cells incubated with HO wires and 2.1 fold in cells incubated with HO-T wires compared to control cells, as illustrated in Fig. 3.

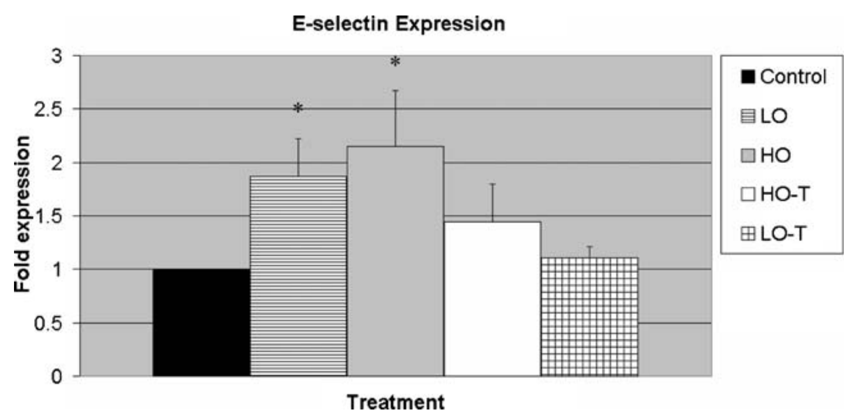

Fig. 1 E-selectin Expression profile (* indicates significance compared to control samples)

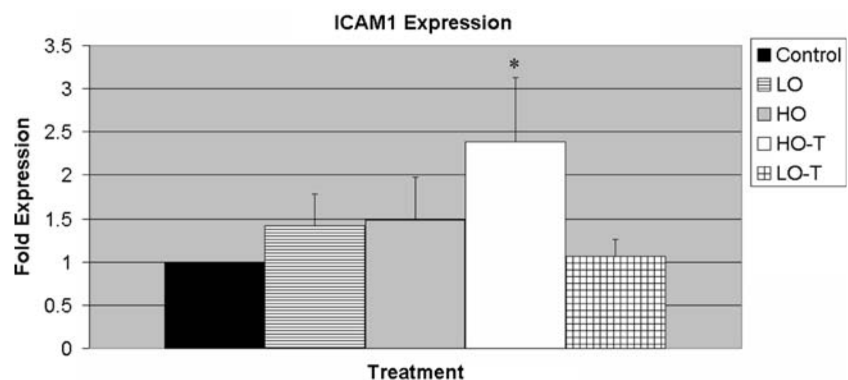

Fig. 2 ICAM-1 Expression profile (* indicates significance compared to control samples)

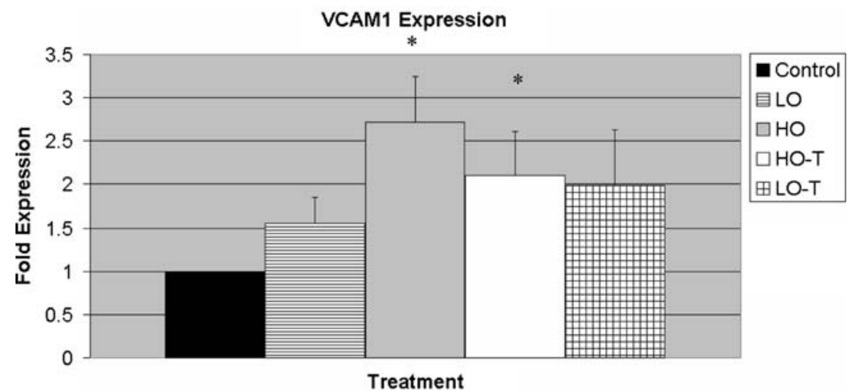

Fig. 3 VCAM-1 Expression profile (* indicates significance compared to control samples)

The error between technical replicates in this study is quite low and the main error occurred as a result of differences between biological replicates.

Discussion

In this study it was demonstrated that nickel is released from nitinol wires and that the amount of nickel released appears to be related in some way to the nickel content of the oxide layer. The atomic percentage of nickel in the oxide layer is higher in the wire samples that did not undergo any additional surface treatments following heat treatment, thus treatments such as etching and pickling may have desirable effects on nickel release from nitinol.

The amount of nickel released from LO and HO wires, although low, had a significant effect on E-selectin gene expression in HUVECs after 24 h incubation with the wires. E-selectin was found to be significantly up-regulated in cells incubated with LO and HO wires only. A detectable amount of nickel was released from only these wire types after 24 h. The surface roughness of the wires under investigation was determined by atomic force microscopy (AFM) in a previous study [14] and LO has the highest Rz value (450 nm) and HO has the lowest Rz value (130 nm) of all of the wires under investigation in this study, thus indicating that the surface roughness does not cause the observed up-regulation of E-selectin. Taken together this data indicates that the nickel released from the nitinol wires after 24 h resulted in an increase in E-selectin gene expression.

E-selectin is an adhesion molecule that plays a vital role in post-implantation reactions. It is involved in the initial tethering of leukocytes during inflammation and instigates slow rolling of leukocytes along the endothelium which is required for transendothelial migration [15]. A number of studies carried out have investigated the significance of soluble E-selectin (sE-selectin) levels in the blood during various disease states and following a number of surgical procedures [16–19]. One study carried out found elevated levels of E-selectin in patients with unstable angina compared to healthy controls and another study found elevated levels in patients with unstable angina compared to those with stable angina [16, 18]. Thus these studies deemed elevated sEselectin levels in circulating blood a suitable marker of atherosclerotic plaque inflammation. Other studies have explored the use of sE-selectin levels as a predictor of restenosis and endothelial damage following balloon angioplasty and stenting. Another study investigated this correlation in patients that had undergone PTCA and stent implantation [19]. In this study a relationship was found between patients with elevated sE-selectin and those that developed restenosis. These studies identify soluble E-selectin as a key player in restenotic pathways and as a potential marker of restenosis. Unlike other markers, E-selectin is expressed by endothelial cells only, thus increased levels of sE-selectin is a direct indicator of endothelial injury.

Earlier studies have investigated the biological effects of nitinol in vitro. One previous study utilising ELISA techniques to investigate the effect of nickel-eluting biomaterials, including nitinol, did not find a significant up-regulation of cellular adhesion molecules (CAMs) when cells were incubated with these materials [20, 21]. This suggests that analysis of transcriptional activity may be a more sensitive approach when examining the effects of nickel in vitro. In one previous study, it was found that the alloy induced monocytes to produce IL-1β at sufficient concentrations to indirectly stimulate endothelial cells to produce ICAM-1 [21]. This may imply that interactions with other cell types are required for nickel ions to induce the transcriptional activation of other CAMs. Thus, co-culture techniques assessing both gene and protein expression levels of CAMs may offer the ideal in vitro assay. In another study investigating nitinol wires at the biological level no cytotoxic, genotoxic or allergic effects were observed [10]. However, this study investigated more long-term effects such as mutagenesis over a short period of time. These techniques may not be sensitive enough to detect cellular responses to small amounts of nickel eluted from a nitinol alloy and therefore this study does not determine that the alloy induced no effect.

Interestingly in this current study, the other adhesion molecules investigated, ICAM-1 and VCAM-1, showed a different pattern of expression after 24-h incubations with the 4 wire types. ICAM-1 was found to be significantly up-regulated in cells incubated with wire HO-T only, while VCAM-1 was up-regulated in cells incubated with HO and HO-T wires only. This may imply that alteration of gene expression occurred as a result of the impact of the heavy oxide layer on the cells in culture. In addition, the treated light oxide wires (LO-T), which have the lowest amount of nickel in the oxide layer, did not cause significant up-regulation of any of the candidate genes under investigation in this study, compared to control cells. These wires underwent etching and pickling surface treatments, leading to the most biologically inert material within the range of this study, which may have implications for the use of nitinol as an implantable-material. It should be noted that all the wires studied caused an up-regulation of the CAMs investigated. Although this differential gene expression was not at a significant level in all cases, the wires must have a contact effect on the cells.

In conclusion, we have demonstrated that nickel is released from nitinol wires in vitro, while nickel release is somewhat related to the various surface treatments the material undergoes. These results indicate that untreated nitinol may have an adverse effect in vivo if the amount of nickel being released, which is having a biological effect in vitro, has an equivalent effect in vivo. In addition, nickel release is not the only important parameter when characterising nitinol as an implant material and the surface characteristics of the material has consequences at the gene expression level. Quantitative real-time PCR has also been identified as a reliable method to characterise the biological stability of a material due to its sensitivity compared to protein expression techniques such as ELISA.

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