Effects of Nitinol Surface Treatments and Ethylene Oxide Sterilization on Human Lymphocyte Proliferation.

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Abstract. Surface treatment and sterilization are those critical protocols in the manufacturing of implant devices that can eventually predetermine the outcome of implantation. Biological performance of well characterized Nitinol surfaces (mechanically polished, chemically etched, aged in boiling water, heat treated, and electropolished) was evaluated using human peripheral blood lymphocyte proliferation test. To assess the effect of sterilization, two types of sterilization were used, UV and ethylene oxide. Incubation of PBL with Nitinol samples demonstrated 15-32% higher proliferation compared to controls (cells + Con A, no metals). In the case of UV sterilized samples, higher Ni surface concentrations resulting in higher Ni release caused statistically more significant stimulation. This stimulation was similar to the observed in the case of ethylene oxide sterilized samples, which, however, induced zero Ni concentration in medium. The control Ti samples also caused a 15% higher proliferation in all individuals compared to stimulation with ConA alone. Pure nickel induced significant suppression that varied from individual to individual in the range 40 to ~100 %**.** Distinct stimulatory and suppressive effects exerted by Ni-containing samples on lymphocytes, cells of immune system fall in the category of the U-shaped dose response to toxic substances. A 15-32% increase in proliferation of ConA stimulated PBL by very low metal ion concentrations induced in biological solutions as well as by EtO sterilized samples is interpreted as an enhanced immune response. The results obtained using lymphocyte stimulation are discussed together with the results of an earlier study on carcinogenic potential of Nitinol alloys in rats (A. Starokha. New technologies in the treatment of rhinosinusitis utilizing superelastic shape memory implants, D.Sc. Thesis, Sankt-Petersburg, 1998).

Key words: Nitinol, nickel, titanium, surface treatment, biocompatibility, toxicity, carcinogenicity, lymphocyte stimulatory effects, UV sterilization, ethylene oxide sterilization, U-shaped dose response.

INTRODUCTION

Due to unique shape memory and superelastic properties associated with thermoelastic martensitic transformations taking place around room temperature, the nearly equiatomic alloys of Ni and Ti or Nitinol are being intensively explored for the design of permanent implants. Because of high Ni content of Nitinol medical alloys (~ 56 % Ni by weight), Nitinol biocompatability was challenged both in *in vivo* and *in vitro* studies most of which were reviewed recently.[1-3] In contrast to pure Ni, a cause of severe inflammation, necrosis of tissues surrounding implants, and tumor growth at implant sites,[4-7] Nitinol demonstrated rather remarkable biocompatibility *in vivo.*[7-10] However, a few *in vitro* studies yet indicates the

possibility of Nitinol toxicity even during short term exposures.[11-15] Ni release from Nitinol, ten-sixteen time higher than from stainless steel during the first day of immersion into cell cultures or corrosive solutions, decreases to the level of stainless steel by the third day of immersion.[16,17] Although it is not clear at present whether this drop is associated with the growth of a thicker passive oxide in the biological environment or with the formation of surface complexes inhibiting corrosion, it is obvious that the first two-three days of exposure to Nitinol may be critical for the outcome of implantation or *in vitro* experiments. In our earlier study, the effect of Nitinol on Con A stimulation of Lewis rat lymphocyte was evaluated to assess its toxic potential.[11-12] It was shown that Ni rich surfaces (~28 at. % Ni) obtained due to oxidation in hydrogen peroxide exerted severe toxic effect (90-95% suppression) similar to that of pure Ni on cell proliferation, while Nitinol samples with autoclaved surfaces (2-4 at. % Ni surface concentration) induced up to 25% stimulation. The nature of observed stimulation was not explored, and it was not clear whether it was a specific of the Lewis rat lymphocytes.

The purpose of this study was to comparatively evaluate responses of human peripheral blood lymphocytes to various Nitinol surfaces, and, thereby, begin assessing immune responses. The surface finishes of Nitinol samples in this study were selected based on the following rationale. A mechanically polished (Mp) surface is used routinely in both *in vitro* and *in vivo* studies in the absence of a standard procedure for Nitinol surface treatment. This surface, however, demonstrates inconsistent corrosion behavior[18] and a propensity for chemical heterogeneity upon heating.[19] In contrast, Nitinol which is chemically etched and aged in boiling water demonstrates excellent resistance to general and localized corrosion.[20] Smooth surfaces resulting from electropolishing (Ep) are under development for medical devices. Due to the chemical heterogeneity associated with the inclusions inherited from the bulk of Nitinol, Ep surfaces have corrosion resistance inferior to chemically etched surfaces.[21] Nitinol surfaces heat treated acquire a spectrum of colors that makes them very attractive for the design of jewelry. The latter, in turn, raises the question of Ni release and is of concern for people with Ni sensitivities. A brief ~ 500^{o}C heat treatment is also a step necessary to set the shape of a Nitinol implant device. Although it has been shown that this type of heat treatment causes a dramatic drop in the corrosion resistance of Nitinol,[22-23] the resulting blue oxide is occasionally used as a final surface finish for Nitinol implants.

Sterilization protocols may also negatively affect the implant surfaces. Thus, steam sterilization in an autoclave alters surface Ni content, surface energy, oxide thickness and causes occasional pitting of Nitinol surfaces.[24-25] Ethylene oxide sterilization (EtO) was introduced as an alternative sterilization method for gamma ray sterilization that causes degradation of polyethylenele, an orthopedic bearing material. EtO, however, is a toxic gas which leaves residues on the implant surfaces.[26] During this procedure metal is exposed to the temperatures ~ 30-60^{o}C and 40-90% humidity.[27] Also, it has been shown that EtO sterilization promotes the growth of oxide film[25] which makes Nitinol surface more hydrophobic and less attractive for cell adhesion. EtO sterilization is also known to impair the biological performance of bone inductive implants[28] and cause strong inflammatory reactions around grafts.[29] We hypothesized that EtO can react with Nitinol surface elements and, thereby, alter the surface. Ultraviolet (UV) light sterilization that does not employ heat, humidity or toxicity may be more appropriate for Nitinol implants whose surface chemistry can be easily modified. It has been shown that UV sterilization results in a high energy surface that promotes cell adhesion and implant fixation.[30] UV sterilized surfaces have been reported to result in 20-40% increase in the temporal accumulation of calcium in primary osteoblast - like cells compared to argon plasma-cleaned Ti

surfaces.[31] In order to minimize negative effects associated with sterilization, most of the samples for this study were sterilized with ultraviolet (UV) light. One group of samples, however, was sterilized with ethylene oxide to evaluate its impact on Nitinol surface chemistry and on biological performance.

MATERIALS AND METHODS

Material and sample preparation

Nitinol alloy (55.91 Ni by weight, with shape recovery temperatures $A_s \sim -11^oC$ (start) and $A_f \sim 0^oC$ (finish)) in the form of rods, obtained from Memry Corporation was spark cut in disks of 5.5 mm diameter and 1.8 mm thickness. Surface of the discs were wet polished using SiC paper of 320, 400, and 600 grit (Mp). One group of discs was chemically etched in the 1HF + 4 HNO_3 acid solutions deluted by 5-10 parts of water (Ce) and aged in boiling endotoxin free Nanopure water for 30 min (CeWb). Heat treatment, mimicking a shape setting procedure, was performed at 500^oC for 15 min in air in a muffle furnace (CeWbHt). Two other groups of samples were electropolished using different electrolytes. The first electrolyte (Ep1) performed better at room temperature. The second electrolyte (Ep2) allowed Nitinol electropolishing in the martensitic phase at the temperatures below -45^oC. Both electrolytes provided shiny surfaces with inclusions retained in the surface.[21] Occasional pits of ~ 0.1- 0.2 μm size were revealed after electropolishing in the room temperature electrolyte. As far as the smoothness of the surface is concerned, samples electropolished at low temperatures in martensitic phase revealed surface relief associated with the recovery of austenite phase at room temperatures. Pure Ni and Ti were selected as the negative and positive controls. The surface of Ni samples was prepared by electropolishing in the room temperature electrolyte (Ep1). Ti samples were immersed for 10 min in a 30% HNO_3 solution according to a standard surface passivation protocol,[32] and aged in endotoxin free Nanopure water for one hour to form a more compact surface oxide providing better corrosion resistance and lower metal release.[33,34] After surface treatment, UV sterilization was performed on all samples (except EtO) in a laminar flow hood for 30 min each side. The samples were 20-30 cm from a UV bulb with the intensity of 150 $\mu W/cm.^2$

Ethylene oxide sterilization

In total four trials were performed to investigate the effect of ethylene oxide sterilization. Two sterilizations were performed in a sterilizer rebuilt from a steam autoclave. Another two were performed in a commercial sterilizer in a local hospital under standard conditions of warm cycle (55^oC) as indicated by the manufacturer. For ethylene oxide sterilization chemically etched and aged in boiling water Nitinol samples were placed in glass petri dishes that were sealed in the plastic pouches. Color controls were inserted in the pouches to ensure sterilization. Surface analysis, as described below, was conducted on three samples from each sterilization.

Surface analysis

X-ray photoelectron (XPS) and Auger spectroscopy in combination with scanning electron microscopy (SEM) were used for surface analysis of Nitinol samples. Thickness of

surface oxide was defined based on the Ar^+ sputtering rate for SiO_2 (11nm/min) at the point where the intensity of the 1s peak of oxygen dropped twice compared to an initial. Detailed description of the surface analysis techniques can be found in our recent publication.[19]

Atomic absorption spectroscopy.

Ni concentrations in the media after exposure to metal samples were determined using flame atomic absorption spectroscopy on two parallel samples that were prepared for the study of human microvascular endothelial cells (HMVEC). Samples were submerged in the 1.5 ml polypropylene tubes containing the HMVEC commercial medium (Cell Systems Corp., Kirkland, WA) with 10% of fetal bovine serum. Medium (0.75 ml per sample of a ~ 2 cm^2 surface area) and samples were incubated at 37°C in a 5% CO_2 environment for 72 hrs, parallel to a HMVEC cell culture assay. After samples were removed, media was analyzed for Ni concentration. The HMVEC medium with serum had a background Ni concentration of 6.1 ng/ml which was subtracted from the total Ni concentrations measured after sample exposure. Mean Ni concentrations and standard deviations were determined.

Lymphocyte proliferation test

Peripheral blood mononuclear (PBL) cells were collected from freshly drawn human blood (four individuals of close age) using Ficoll-paque density gradient medium, washed three times, and enumerated using a Hemavet® 850 (CDC Technologies, Inc; CN). Based upon the white blood cell count, the cells were diluted to 5 x10^6/ml in RPMI-1640 medium supplemented with 10 mM HEPES, 2 mM L-glutamine, 50 μg/ml gentamicin, (all from Life Technologies, Gathersburg, MD) and 10% heat inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS). Diluted cells (100 μl) were plated with an equal volume of Concanvalin A (ConA), a T-cell mitogen, or with supplemented media in 96 well flat bottom plates (Costar, Fisher). The final concentration of ConA was 2.5 μg/ml, and the total content of one well was 0.2 ml. Metal samples prepared as described above were loaded into 5 parallel wells containing ConA (one tissue culture plate for each individual). Plates were incubated at 37°C in 7% CO2 for 72 hr. During the last 6 hours each well was pulsed with 1 μCi 3H-thymidine. Cells from the individual cultures were harvested onto glass fiber filters using a Skatron Combi Cell Harvester. Radioactivity was enumerated on dried filters using CytoScint scintillation cocktail and a Packard liquid scintillation counter.

Endotoxin test

Parallel samples of NiTi prepared as described above were tested for endotoxin using a Limulus amebocyte lysate ELISA kit (QCL-1000, BioWittaker, Walkersville, MD) with a sensitivity to 0.1 EU/ml. Samples were immersed in 0.2 ml of endotoxin free water supplied with the kit in a 96 well tissue culture plate similar to those used for the lymphocyte proliferation assay. The samples were incubated for the same time as the proliferation assay (72 hr.) Each sample was placed in an individual well and after 72 hr the water was assayed for endotoxin. Absorbance measurements were compared with a standard curve provided with the assay. All samples measured gave absorbance readings similar to endotoxin free water < 0.1 EU.

Statistical Analysis

In order to determine whether the differences in proliferation among samples were statistically significant, the data were analyzed using a general ANOVA and a least significant differences (LSD) test.

RESULTS

NiTi surface chemistry

Nickel surface concentrations and Ti/Ni ratios obtained using XPS analysis together with the Ni concentrations found in media after 72 hrs of exposure to Nitinol samples are presented in Table 1. One can see that the maximal Ni concentration of ~7 at. % was observed on the surface of chemically etched samples. For chemically etched samples which were also aged in boiling water for 30 min the Ni surface concentration dropped to 2.2%, and was only 0.9% for those samples that were heat treated after chemical etching and aging in boiling water. Electropolishing in the room temperature electrolyte resulted in a slightly higher Ni surface concentration than in the low temperature Ep2 electrolyte (3.3 and 1.9 at %, respectively). Ni on the surfaces of Mp and chemically / electrochemically treated Nitinol samples was only partially oxidized, in contrast to the heat treated surface that manifested Ni only in an oxidized state.[19]

The major Ni $2p_{3/2}$ peak was located at ~ 856.4 eV. The latter peak could be equally assigned to Ni_2O_3 oxide or Ni hydroxide.[35] Titanium dominance on the surface was obvious. The minimal Ti/Ni ratio observed on various Nitinol surfaces was 2 for freshly chemically etched samples, and the maximal was 23-24 for the CeWbHt and Ep1Wb samples. Ti on the studied surfaces was observed only in the +4 oxidized state with the $2p_{3/2}$ peak located at 458.9 eV (TiO_2).[35] Thickness of the oxide film obtained from Auger depth profiles ranged from 1.5 to 1.8 nm for chemically etched and EP samples. It increased to 2.1 nm after EtO sterilization and to 62 nm after heat treatment in air. Thickness of surface oxide film formed during mechanical polishing varied significantly (2.0 - 4.0 nm). Samples of pure Ni revealed ~ 25% Ni on the surface that was mostly in oxidized state typical for Nitinol surface.[24]

Although UV sterilization did not affect Nitinol surface chemistry upon a short exposure used for sterilization of samples for PBL study, it caused a slight change in color of the surface towards blue upon 12 hrs exposure to light in a laminar culture hood. A very light change in color corresponded to the increase in the thickness in surface oxide by ~ 5 Angstrem.

Ethylene oxide sterilization effects on Nitinol surface

Examination of EtO sterilized surfaces with SEM revealed surface discoloration which appeared as black stains of various shapes (Fig. 1a, b, and c) and a uniform coating. While certain stains obviously represented new deposits (Fig.1a,b), the others appeared reminiscent of residues of water stains (Fig. 1c) most probably resulting from condensation of steam during sterilization. These latter stains together with a uniform surface coating acquired during EtO sterilization would fade after exposure to electron beam (Fig. 1d) indicating their organic nature. Black stains like those presented in Fig. 1a, and b, however, did not fade.

Representative Auger spectra taken from Nitinol surfaces after EtO sterilization are shown in Fig 2. One can see that the stained surface (Fig. 1c) that also had a uniform

discoloration revealed at least three types of survey spectra (Fig.2 a,b,c). These spectra imply that there are surface areas that carry deposits of carbon or carbon rich compounds, Fig. 2a,b as well as areas typical of regular NiTi surfaces (Fig.2c). This is an indication of extremely heterogeneous surface conditions resulting from EtO sterilization. After a brief Ar^+ ion etching surface discoloration (stain and uniform coating) faded, and resulting survey spectra (Fig. 2d) acquired a form typical for NiTi that always carries some oxygen.

Auger maps obtained from surface areas identified using SEM as affected by sterilization supported observations described above. Thus, in contrast to a regular fragment of the surface (Fig. 3 a) that exhibits uniform elemental distribution (Fig.3 b-d), stained surface areas (Fig. 3e) revealed strip-like patterns in Auger maps (Fig. 3f-h). Overlapping Ti and oxygen patterns reflect titanium surface oxide. Ni strips complementary to titanium ones indicate Ni segregation on the surface. Auger maps obtained from a surface image presented in Fig. 1 b imply that moss-like black stains observed on the surface after EtO sterilization in a rebuilt autoclave is mostly carbon (Fig.3j).

XPS analysis also pointed at altered surface chemistry. Two observations related to EtO sterilization effects on Nitinol surfaces are worth noting: inconsistency in the location of the 2p peak of Ni and presence of three nitrogen peaks at 407.6, 400 and 396 eV instead of one. A regular nitrogen peak typically observed on Nitinol surfaces at 400 eV is of organic origin. The peaks of nitrogen at 396 eV and 407.6 eV, however, can be assigned to metal nitrides, and to the compounds of nitrogen with nickel: $Ni(NO_3)_2$ and $Ni(NO_3)$ x 6 H_2O.[35] The Ni peaks on the surface could be found occasionally at 853.7 eV instead of 852.8 eV, characteristic of elemental Ni commonly observed on NiTi surfaces.[24] The Ni peak at 853.7 eV could be associated with Ni in the bulk. However, since no Ti was detected from the bulk at any electron escape angles (20-80^o), the Ni peak observed at 853.7 eV also could not be assigned to the bulk. This Ni peak, at 853.7 eV, however, could be assigned to nickel in the compounds like $Ni(C_5H_5)_2$ or those mentioned previously.[35] The presence of extra nitrogen and nickel peaks associated with various chemical compounds is an indication of reactivity of Nitinol surface. A source of nitrogen can be questioned, however, we believe that both EtO and steam could be contaminated by nitrogen.

Ni release

Ni concentrations detected in medium after exposure to Nitinol (0 – 11 ng/ml) and Ni samples were very low, Table 1. We would like to point out that these concentrations were significantly lower than in our earlier study were only two Nitinol surfaces (steam autoclaved and oxidized in hydrogen peroxide were tested.[11] The observed difference in Ni release cannot be assigned to the different techniques used in these two studies for the detection of Ni ions, inductively coupled plasma analysis and flame atomic absorption spectroscopy because sensitivities of the methods were similar, < 5 and < 2 ng/ml, respectively. In contrast to our earlier study,[11] in the present study, the background, Ni occurring naturally in medium (61 ng/ml) was measured and subtracted. This background Ni concentration, which is significantly higher than the Ni concentrations induced by Nitinol samples (Table 1), could contribute to the higher Ni concentrations observed in Ref. 11. And also in the latter study, the valves with the medium and metal samples were periodically shaken though the PBL test was performed under static conditions. It is obvious that shaking of medium with samples inhibited formation of a stable organic layer on the surface that could serve as a barrier for metal ion release, and, thereby, also contributed to the higher numbers observed in that study.[11]

The highest Ni release (1080 ng/ml) was detected from electropolished pure Ni samples that revealed ~ 25% Ni on the surface. It was 100-1000 times higher than from Nitinol samples with Ni surface concentrations in the 2-7 % range. The highest Ni release among Nitinol surfaces was manifested by chemically etched samples (11 ng/ml) that revealed the highest surface Ni content (~ 7%). Surprisingly, Ni release from Nitinol with electrochemically treated smooth surfaces (6-7 ng/ml) was similar to that of Mp (600 grit finish) samples that have a rougher surface with residual deformation induced by grinding. Nickel was not detected in medium exposed to the CeWb, Ep1Wb and EtO sterilized samples. Surfaces of the CeWbHt samples also induced negligible amount of Ni in medium. Zero Ni release in the case of CeWb and Ep1Wb samples can be assigned to a slightly thicker and more compact oxide formed during aging in boiling water.

EtO sterilized samples revealed Ni surface concentration similar to that of Mp samples, but induced no Ni release. This could be explained by the difference in the chemical state of Ni on the surfaces of these samples. While in the case of Mp finish surface, Ni was present in both oxidized and elemental states, in the case of EtO sterilized surfaces, Ni was rather bond in various chemical compounds discussed in the previous sections. Thicker surface oxide resulting from EtO sterilization (2.1 nm vs 1.5-1.8 nm in all other cases) and from heat treatment (62 nm) contributed to the inhibition of Ni release as well. There is another factor that could equally contribute to a significantly higher Ni release induced by Mp surface. Mp ground (600 grit finish) surface is structurally non-uniform and it carries residual plastic deformation.[19] Lattice defects resulting from plastic deformation and the non-uniform character of surface deformation (peak-and-valley topology) make easier metal ion release. This is especially true for Ni that has smaller atomic diameter and low energy of activation of diffusion (Ref), and is present in the surface sublayers of Nitinol in free state in contrast to titanium that is always oxidized. Great variability in the thickness of Mp samples is another indication of their extremely heterogeneous conditions.

We would like to point out that Ti release was not estimated in the present study, however, according to our earlier data it was either at least ten times lower than that of Ni[12] or negligibly small[36] despite Ti dominance on the surface.

Lymphocyte proliferation test

During the incubation of lymphocytes with ConA and metal samples (72 hr) neither discoloration of biological media nor alteration of surfaces of the metal samples was noticed. The average proliferation of cell cultures of peripheral blood lymphocytes (PBL) from four individuals (A, B, C, and D) with various surface finishes of Nitinol as well as positive (Ni) and two negative controls (Ti and cells with no metal) with ConA are presented in Fig.4. Lymphocytes incubated without ConA (media alone) demonstrate the baseline response of non-stimulated cells. There was significant variation in ConA responsiveness between the individuals ($p < 0.0001$), which was expected due to variations in the percentage of lymphocytes found in the isolated PBL.

The averages of lymphocyte stimulation collapsed across all four individuals are shown in Figure 5. As predicted, significant differences in proliferation of lymphocytes in the various incubation conditions (± ConA, ± metal samples) can be seen ($p < 0.0001$). In figure 5, those means with different superscripts are statistically different. Proliferation of lymphocytes incubated with ConA and Ti or NiTi metal samples is significantly greater than proliferation of

lymphocytes incubated with ConA alone. Of particular interest is that lymphocytes incubated with ConA and NiTi samples which were either chemically etched (Ce) or sterilized using ethylene oxide (EtO) had significantly higher proliferation compared to all other lymphocyte cultures. Cells incubated with Ni were significantly suppressed compared to the ConA control ($p < .05$) yet gave an average proliferative response significantly greater than unstimulated cells.

There was a significant interaction of metal samples and individual responses observed in the analysis ($p < 0.0001$), which is due, in part, to the fact that two of the four individuals (A and D) had 87% and ~100% suppression of ConA stimulation in the presence of Ni samples and two other individuals (B and C) demonstrated only partial (40% and 78%) suppression (Fig 4). In addition, the significant interaction of metal samples and individual response is partially due to significant enhancement of proliferation in cultures containing Ti and NiTi (Ep1Wb) for two individuals (C and D), and non-significant enhancement in lymphocyte proliferation with Ti from the others (A and B).

To rule out the possibility of contamination of metal samples with endotoxin that could be a cause of lymphocyte stimulation, possible endotoxin contamination was evaluated on parallel metal samples. This test indicated that endotoxin level in water after exposure to parallel metal samples was below the detectable range (< 0.01 EU/ml). As some metals can inhibit the enzymatic endotoxin test, metal samples were tested also for inhibition of the enzymatic test. No inhibition of the endotoxin test was found. Thus, enhancement of lymphocyte proliferation by Ti and NiTi samples can not be explained by endotoxin contamination.

DISCUSSION

Effect of ethylene oxide on Nitinol surface

Complementary analysis using Auger mapping and SEM were effective in the detection of local surface alterations induced by sterilization. Comparing surfaces resulting from sterilization in rebuilt and commercial EtO sterilizers, it should be pointed out that use of the rebuilt apparatus resulted in increased amounts of black stains and linear discoloration. Samples sterilized in a commercial EtO sterilizer did not reveal a moss-like carbon rich deposits, however, a uniform surface coating as well as occasional stains left by condensed steam were detected. Blue linear discoloration resulting from EtO sterilization was also observed in a study of Ti implants.[37] Presence on the surface of Nitinol of Ni in compounds like $Ni(NO_3)_2$ and $Ni(NO_3)$ x 6 H_2O, Ni $(C_5H_5)_2$ is an indication of alteration of the surface chemistry due to the exposure to EtO (C_2H_4) that also could be contaminated by nitrogen. To pursue the precise chemistry of the surface exposed to EtO, SIMS should be employed to identify the hydrogen status on the surface. However, regardless of the hydrogen status, it is clear that after EtO sterilization the Nitinol surface acquires a uniform surface coating and local discolorations in the form of black stains of various configurations. It was reported[38] that the fatigue life of EtO sterilized Nitinol increased by 20%. This could be due to the deposition of a thin polymer coating similar to a uniform coating we have detected by SEM in the present study that, however, faded after exposure to electron beam.

Elemental segregation, exemplified in Fig. 3 f-h, is reminiscent of the elemental segregation observed on the surfaces of mechanically polished 600 grit finish samples after heat treatment at 500°C.[19] However, in that study the pattern of surface Ni segregation repeated the patterns of residual surface deformation induced by surface grinding, and the Ni segregation

could be explained by preferential diffusion of Ni atoms to the surface through the channels of lattice defects induced by grinding. The patterns of elemental segregation detected in the present study did not follow the patterns of surface deformation induced during sample preparation. These patterns followed the contours that could be formed by evaporation of contaminated water droplets. Water condensed on Nitinol surface provides a solvent for free Ni atoms emerging on the interface and provides the connecting electrolyte needed to activate electrochemical corrosion. For this reason sterilization procedures employing steam and resulting in local water condensation on the surface of Nitinol should be avoided to prevent local enrichment of the surface by Ni and development of heterogeneous surface conditions. It is obvious that surface coating, discoloration and modification of surface chemistry are consequences of EtO sterilization. Mentioned positive effect associated with the prolonged fatigue life[38] that could be in principle explained by the deposition of a uniform polymer coating due to exposure to EtO under pressure during sterilization, need to be verified in special studies.

Lymphocyte proliferation

Ni implants have been associated with an increased incidence of malignant tumor formation in mice.[4,39-40] Tumor development indicates a deficiency of the immune system to detect and respond to transformed cells, which may be due to an inability of the lymphocytes to respond to the tumor. Indeed, previous research has noted that Ni, some forms of Ti, arsenic and cobalt can induce apoptosis in lymphocytes[41-43] which can affect their ability to carry out an immune response. Reduced NK cell activity and depressed *in vitro* T – cell mitogen stimulated responses were reported upon Ni exposure.[44] In the present study we examined the effects of the Nitinol, Ti, and pure nickel samples on peripheral blood lymphocytes stimulated with concanavalin A in order to obtain information on non-specific immune response.

Effect of pure titanium

In contrast to our previous study on rat lymphocytes[11] that revealed an inhibitory effect of pure Ti, in the present study lymphocytes exposed to pure titanium samples proliferated by 10-15% more than the control (cells with ConA). In the previous study[11] the amount of inhibition was dependent on the surface treatment. Thus, a 60% inhibition in lymphocyte proliferation was observed in the case of freshly chemically etched titanium samples. This inhibitory effect was reduced to 30% after chemically etched Ti samples were sterilized in steam for one hour when surface oxidation became more complete. We postulate that the remaining 30% inhibition could be assigned to the rougher surfaces of Ti samples that were used in that study rather than to a specific response of rat lymphocytes to Ti. In the present study, the surface finish after mechanical polishing was similar for all samples studied. Additional passivation in concentrated nitric acid followed by aging in water resulted in Ti surfaces that caused a slight 10-15% stimulation of human PBL. These observations indicate that Ti could also contribute to the observed enhanced proliferation of PBL exposed to Nitinol samples. Obviously, Ti is not a biologically neutral element that is in agreement with earlier studies reviewed in Refs. 3, 45 and surface conditions of titanium implants are vital for the biological performance. Our studies also demonstrate that Ti surface may cause either suppression or a slight stimulation of PBL depending on surface conditions.

Effect of pure nickel and Nitinol

Ni concentrations induced by Nitinol in the present study do not fall in the toxic range. These Ni concentrations are in the range of those defined for human serum (0.1-1.3 ng/ml).[46] Also different cells have different sensitivity, Ni concentrations between 400 and 900 ng/ml did not affect the behavior of bone morrow[47] and smooth muscle cells,[48] respectively. Although Ni concentrations obtained upon exposure to Nitinol samples in the present study did not cause a toxic effect on human PBL, these concentrations were high enough to induce mild to moderate (68% of SDH activity compared to controls) toxicity on HMVEC in our parallel study. Higher Ni concentration, 1080ng/ml (~1 : g/ml) resulting from the exposure to pure Ni samples induced a toxic effect on PBL. A similar Ni concentration obtained in another study[41] induced a ~ 20% cytotoxic inhibitory effect on the proliferation of T-lymphocyte Jurkat cells. A similar response *in vivo* could be a cause of a slow and inefficient immune reaction to infection or tumors. An inefficient immune response to infection in the presence of pure Ni or Ti implants (with non-passive surface[11,12]) could trigger those mechanisms that eventually would result in the failure of implants.

There is one more interesting observation associated with the effect of Ni concentrations induced by pure Ni samples. The lymphocyte proliferation was completely suppressed in the case of the study when four Lewis rats were used. Human lymphocytes, however, exhibited variable responses in the present and an earlier study.[49] Only in one individual, cell proliferation was almost completely suppressed (A). Other individuals, especially B and C responded quite differently. The different lymphocyte responses to Ni concentrations induced by pure Ni may be associated either with different Ni sensitivity of human and rat lymphocytes or with preliminary exposures to Ni. We hypothesize that minimal suppression of PBL proliferation in the B and C individuals observed with pure Ni samples is associated with the preliminary exposure of these individuals to Ni. Individuals B and C each had a history of Ni exposure through leaching from dental and orthodontic devices. In the case of individual C, no complications were observed when stainless steel orthodontic brackets were used in her teen age. In the case of individual B who responded to the pure Ni samples with only 40% PBL suppression, a Rx 96E (62% Ni, 22% Cr, etc) dental bridge was installed in a direct contact with another metal filling. This individual experienced sensations of current flow in the mouth and developed inflammation of frenum tissue behind upper lip next to a bridge, as well as other symptoms in remote organs during exposure to the bridge from the Ni rich alloy. All these symptoms cleared after the removal of the bridge.

Nano- concentrations of Ni detected in medum exposed to Nitinol samples induced significant (15-32%) enhancement in the proliferative response of activated by Con A human lymphocytes. This response was similar to that observed in an earlier study with Lewis rats when up to ~ 25% stimulation was detected.[11,12] According to statistical analysis, most of Nitinol samples induced the same level of enhanced proliferation ("b" group). Only two other subgroups of enhancement ("a" and "d") can be distinguished. The "b" group includes Nitinol surfaces whose Ni concentrations range from 0.9 to 2.2 at. %. These surfaces caused 0 - 7 ng/ml Ni release. It is interesting that the Ep1Wb Nitinol samples performed in the biological study similarly to the pure Ti samples (group "d") than to Nitinol samples. This may be an indication of formation of more passive and Ti-like Nitinol surface upon aging in water of electropolished samples.

The greatest enhancement of PBL proliferation (group "a") was observed with two types of samples, the chemically etched and ethylene oxide sterilized. This enhancement was not linearly related to the Ni surface concentrations as the chemically etched samples had four times higher Ni surface concentration than EtO sterilized. Ni release into medium observed from chemically etched samples was the greatest among the samples studied, while the release from EtO sterilized samples was not detected. These two types of samples had only one common feature, a higher concentration of potentially toxic substances on the surface (Ni atoms and EtO residues) compared to other metal samples. It was demonstrated that residual ethylene oxide measured on sterilized metal surfaces decreased from 23, 15, 12, 9 to 5 ppm after 3, 5, 8, 9, and 26 days, respectively.[26] Metal samples used in our study were exposed to cells 5 -7 days after sterilization. This means that the residual EtO may be in the range 12-15 ppm.

We did not examine the immunological mechanism responsible for this more robust proliferative response of lymphocytes induced by Nitinol. It may be associated either with enhanced production of cytokines by macrophages or by T-cells.[36,50,51] A slight stimulation effect on cell proliferation could be due simply to the physical presence of the surface. However, our earlier study had shown that lymphocyte proliferation rate was statistically similar between cells stimulated with ConA in Nitinol extracts (no surface) and in the physical presence of metal samples.[49] Although a neutral cell response is desirable, lymphocytes are cells of immune system which need to proliferate in order to mount a robust specific response. Stimulation of immune cells by Ni concentrations close to those found in the human blood plasma may be a positive sign reflecting normal activity of immune system. The fact that a similar stimulation effect was observed in the case of rat lymphocytes, excludes the possibility to interpret it as a kind of hypersensitivity or allergic response. In studies of allergic responses, the PBL of Ni sensitive individuals proliferate without Con A present at Ni doses in the range 3-78 : g/ml.[52] We did not examine proliferation induction without Con A. For this reason, an alternative explanation of the observed stimulation of PBL could be an enhanced response of immune cells to low doses of metal ions in biological solutions or to residues resulting from EtO sterilization. Lymphocyte stimulation at low Ni doses (nano-scale) caused by Nitinol and inhibition of lymphocyte proliferation at higher doses (~ 1 : g/ml) caused by pure Ni form a U-shaped dose-response, a relationship known as hormesis.[53]

This new perspective could help us to understand an older study[7,40] aimed at assessing the carcinogenic potential of Nitinol alloys, the only *in vivo* study on this important issue. In that study three groups of rats (~100 each) received NiTi, Ti, and Ni implants, and a fourth group of rats was left unoperated (intact). Survival rates by the end of 6, 12, 18, 23, 27, and 30 months after implantation, mean life expectancy, cancer rates in each group, and the day when the first lump was detected were measured. The NiTi group outperformed the Ti group as it had a longer period preceding first lump detection (394 vs 364, and 365 in intact group). In the Ni group, the first lump was detected on 119 day after implantation. By the end of 30 months the only rats surviving were in the NiTi (6%) and intact (15%) groups. These results could not be understood based on the assumption of better biocompatibility of Ti. However, they can be explained based on the present study that indicates an immuno-stimulatory effect induced by Nitinol *in vitro*. Another piece of evidence supporting this hypothesis comes from the long term clinical observations indicating that no cancerous growth is observed at the sites of Nitinol implants, though cancer can be detected at the sites remote from the implants[54]

CONCLUSIONS

In summary, human peripheral blood lymphocytes responded to Nitinol similarly to that observed in Lewis rats. Nitinol surfaces prepared in the study did not cause a toxic effect on cells. Stronger stimulation was revealed in the case of Nitinol samples with higher Ni surface concentration that caused higher Ni release on nano-scale, and samples sterilized with EtO (~ zero Ni release). The 15-32% stimulation effect of Con A activated lymphocytes is interpreted as a positive sign of enhanced immune response that can be associated with reduced infection and tumor development *in vivo*. To explore further the nature of PBL stimulation, the production of cytokine (IL-1, IL-2, IFN-gamma), activators of macrophages and T cells must be evaluated; the effects of specific antigens, effects of tumor cytotoxic cells (NK cells, T-cytotoxic cells), and antibody responses (B cell responses) examined. Ethylene oxide sterilization altered Nitinol surface chemistry and induced uniform surface coating whose nature must be explored to find out whether it is beneficial for the fatigue life performance.

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Table 1. Ni concentrations obtained using XPS analysis for a 20^{o} electron escape angle (at. %) and Ti/Ni ratios on the surfaces of Nitinol samples, and Ni concentrations found in medium after exposure to metal samples with various surface treatments (per ~1 cm^2 sample surface area).

Sample	Mp	Ce	CeWb	CeWb Ht	Ep1	Ep2	EtO	Ep1 Wb	Pure Ni	medium
Ni, at.%	1.4	6.7	2.2	0.9	1.9	3.3	1.5	0.7	25.4	
Ti/Ni	4	2	7	24	9	4	10	23		
Ni, ng/ml	6 (1)	11 (3)	0 (0)	1 (1)	6 (3)	7 (1)	0 (0)	0 (1)	1080 (20)	61 (5)

Figure captions.

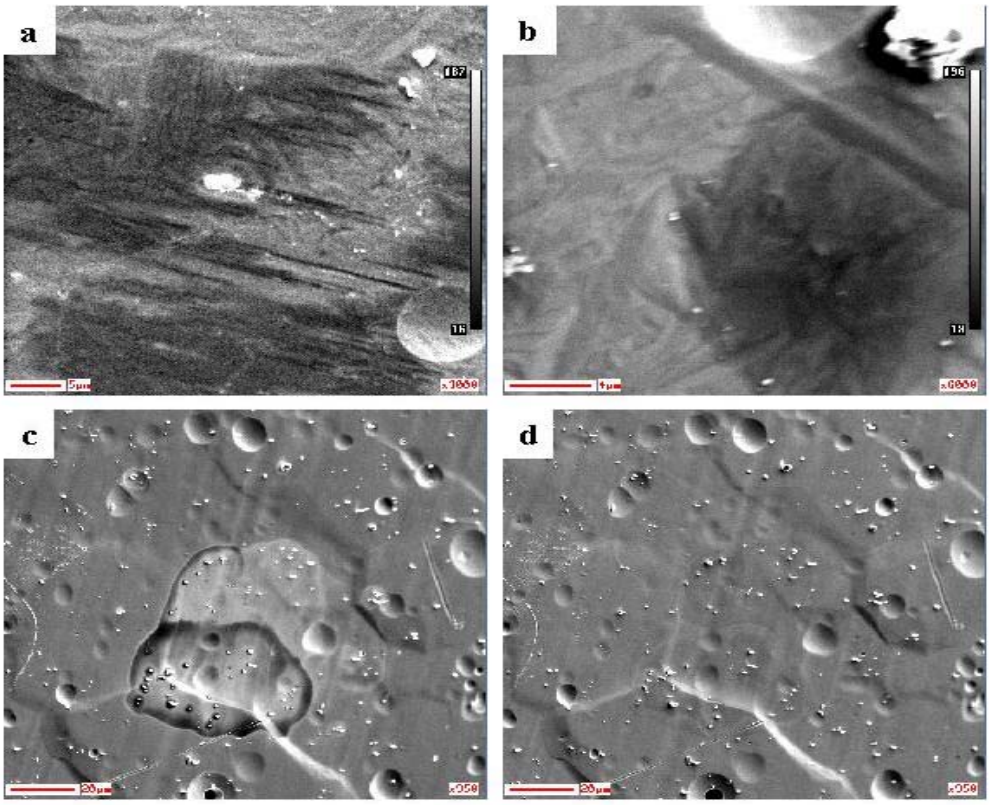

Fig. 1

Fig. 1. SEM images of Nitinol samples after ethylene oxide sterilization in rebuild autoclave (a, b) and commercial EtO sterilizers (c, d). The images c and d present a surface fragment before (c) and after exposure to electron beam (d) during acquisition of Auger spectrum. Faded stain indicates organic nature of surface deposits induced by EtO sterilization.

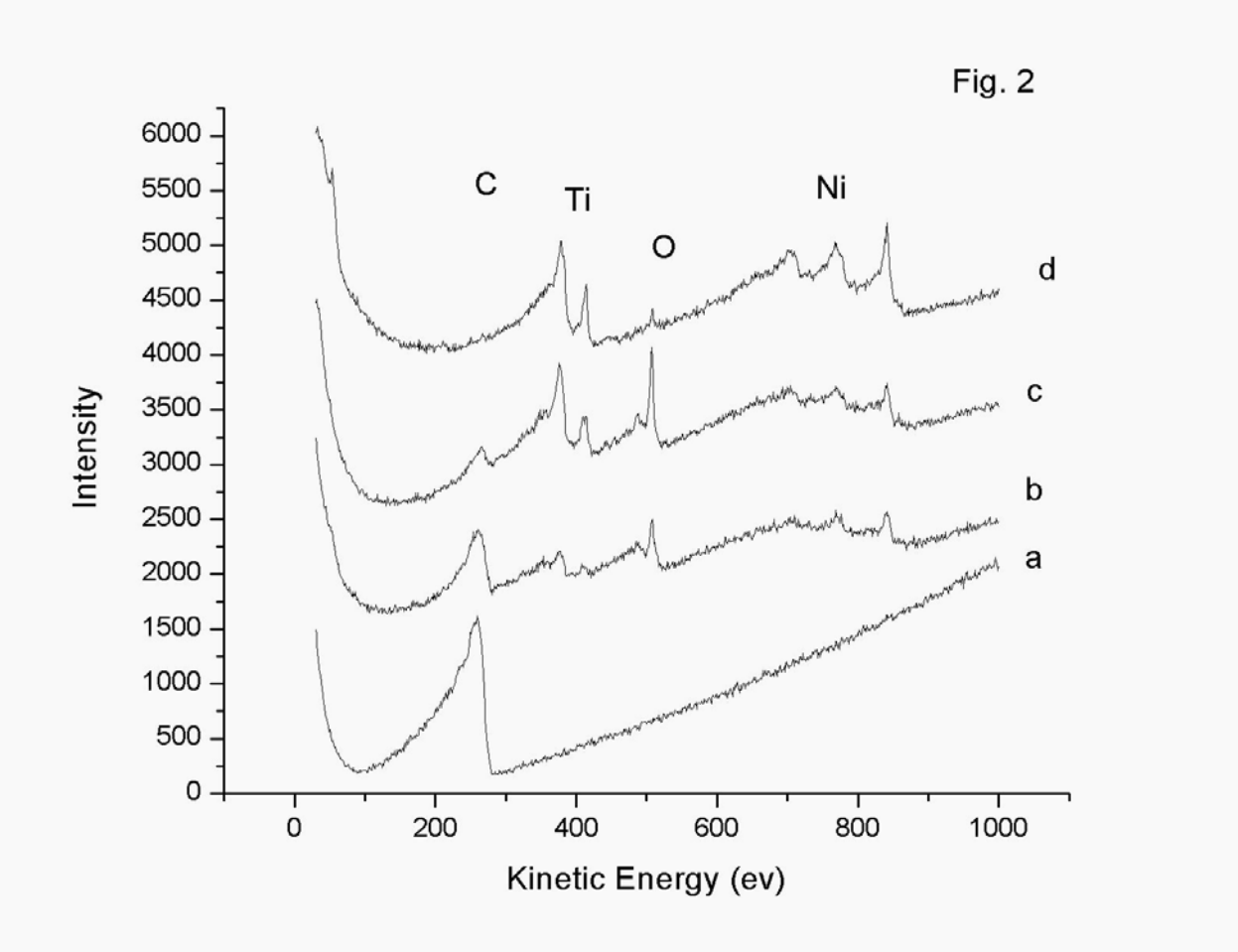

Fig. 2.Auger spectra obtained from the surface fragment exhibited in Fig. 1 c, d.

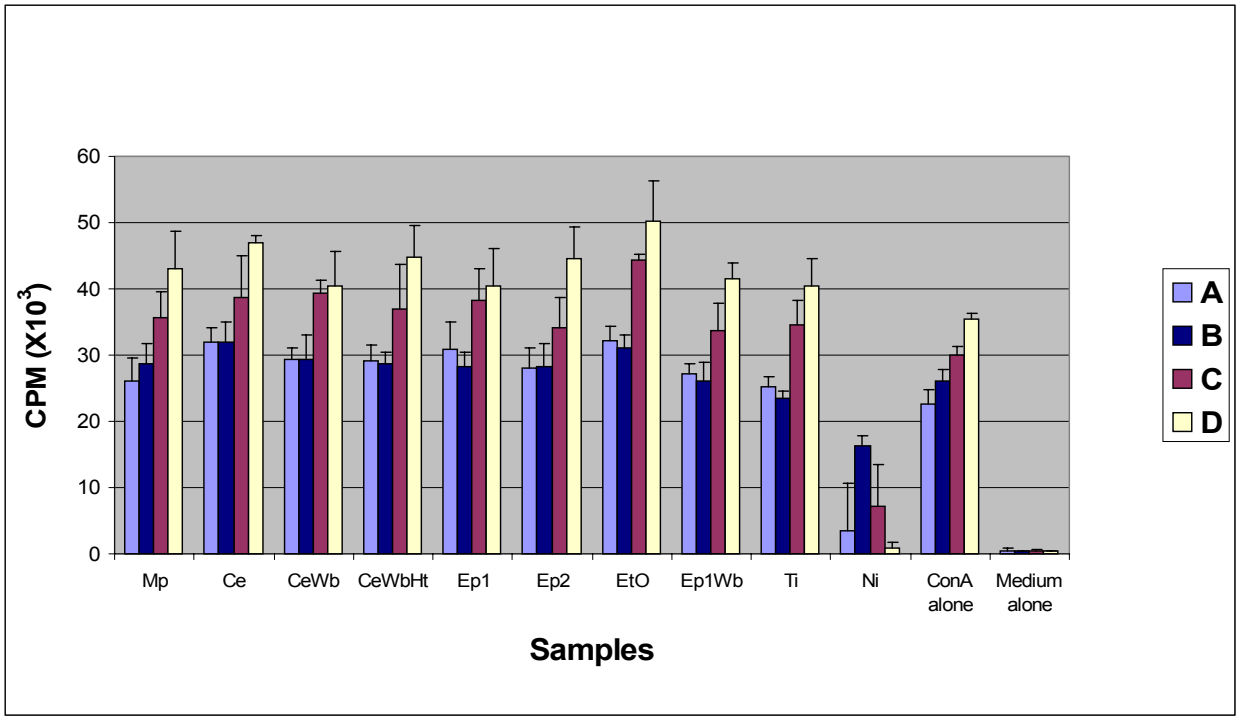

Figure 4. Proliferation of peripheral blood lymphocytes (from four individuals) incubated with Con A and samples of Nitinol, Ti and Ni.

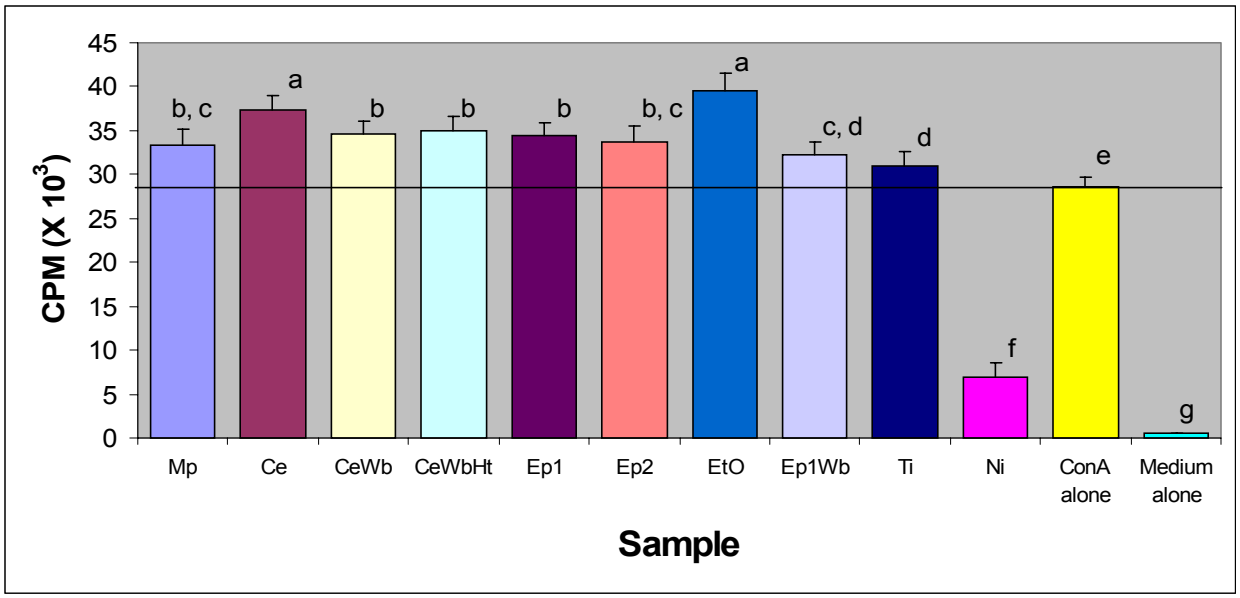

Figure 5. Mean proliferation of peripheral blood lymphocytes incubated with ConA and samples of Nitinol, Ti, and Ni. Bars with different lettered superscripts are significantly different ($p < 0.05$).

Figure 3 –see separate pdf file.

Fig.3. SEM images (a, e) and elemental Auger maps demonstrating heterogeneous surface conditions resulting from ethylene oxide sterilization. The Auger maps presented in the lower raw correspond to the surface fragment displayed in Fig. 1b. The bars in the upper and lower rows represent a 4 : m scale, and in the middle row- 40 : m.

Fig 3