Metallomics

Gene Expression in Mouse Muscle over Time after Nickel Pellet Implantation

Journal:	*Metallomics*
Manuscript ID	MT-ART-11-2019-000289.R1
Article Type:	Paper
Date Submitted by the Author:	24-Jan-2020
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Significance to Metallomics: This work provides describes the long term global gene expression response of muscle tissue to nickel pellet implants in mice. The immune system was strongly stimulated, while mitochondria were negatively impacted. Several other pathways relevant to nickel toxicology were impacted. High upregulation of matrix metallopeptidases, prolactins, and chemokines indicate a diverse impact on cellular processes which persisted up to 46 weeks, indicating an ongoing toxic effect of nickel. The work increases our understanding of the long term toxicology of nickel in tissue at locally high doses.

Metallomics

ARTICLE

Gene Expression in Mouse Muscle over Time after Nickel Pellet Implantation

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Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

The transition metal nickel is used in a wide variety of alloys and medical devices. Nickel can cause a range of toxicities from allergy in humans to tumors when implanted in animals. Several microarray studies have examined nickel toxicity, but so far none have comprehensively profiled expression over an extended period. In this work, male mice were implanted with a single nickel pellet in the muscle of the right leg with the left leg used as a control. At 3-week intervals up to 12 months, nickel concentrations in bioflulids and microarrays of surrounding tissue were used to track gene expression patterns. Pellet biocorrosion resulted in varying levels of systemic nickel over time, with peaks of 600 µg/L in serum, while global gene expression was cyclical in nature with immune related genes topping the list of overexpressed genes. IPA and KEGG pathway analyses was used to attribute overall biological function to changes in gene expression levels, supported by GO enrichment analysis. IPA pathways identified sirtuin, mitochondria, and oxidative phosphorylation as top pathways, based predominantly on downregulated genes, whereas immune processes were associated with upregulated genes. Top KEGG pathways identified were lysosome, osteoclast differentiation, and phasgosome. Both pathway approaches identified common immune responses, as well as hypoxia, toll like receptor, and matrix metalloprotinases. Overall, pathway analysis identified a negative impact on energy metabolism, and a positive impact on immune function, in particular the acute phase response. Inside the cell the impacts were on mitochondria and lysosome. New pathways and genes responsive to nickel were identified from the large dataset in this study which represents the first long-term analysis of the effects of chronic nickel exposure on global gene expression.

Introduction

Transition metals with essential functions in biology include iron, copper, zinc, manganese, cobalt, and nickel[1]. Optimal use of such metals must be balanced with potential toxicity, as excess levels can overwhelm defence mechanisms, injure tissues, and result in disease[2]. Homeostatic mechanisms maintain a balance between metal deficiency, sufficiency, and excess (potential toxicity) using a combination of controlled absorption and transport, storage capacity, and excretion of metals[2]. The best examples of metal homeostasis are iron[3] and copper[4] where elaborate binding, transport, and storage mechanisms are well conserved. Inside cells, metal buffering proteins[5, 6] maintain extremely low levels of free cations[7-9] while anti-oxidant defences act to quench metal-generated free radicals. Potential toxicity advances when homeostasis is disrupted and/or antioxidant defences are overwhelmed[2, 10].

Nickel has widespread applications in alloys for both medical and industrial uses. While nickel toxicity is well defined[11-13], evidence for deficiency is unclear in humans[14], partly due to widespread nickel availability in the diet, though nickel does appear to have critical biological functions in non-mammalian species[10]. While a nickel-deficient phenotype has not been experimentally demonstrated in humans[10, 14], nickel has been shown to affect glucose metabolism in rodents, promoting hyperglycemia at high doses[15] or altering lipid metabolism in laboratory-based dietary nickel deficiency[16]. Nickel toxicity, on the other hand, is well documented for both human and animal immune and respiratory systems[13]. Nickel-induced allergic contact dermatitis is the most common cause of contact hypersensitivity in industrialised countries[11], affecting up to twenty percent of the population, while nasal irritation[13] and carcinogenesis has historically been associated with occupational airborne exposure from industrial processes such as nickel refining[12]. In the European Union, regulation of nickel concentrations in jewellery through REACH has reportedly reduced the prevalence of nickel allergies[17, 18].

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† Electronic Supplementary Information (ESI) available.
See DOI: 10.1039/x0xx00000x

Some of the underlying causes of nickel toxicity have been identified. Research into the molecular mechanisms underlying nickel immune hypersensitivity have identified binding to human Toll-like receptor 4 as the initiating mechanism for the allergic response in humans[11, 19]. The situation with carcinogenesis is more complex. *In vitro* genotoxicity tests for nickel are predominantly negative[20] indicating a non-mutagenic mode of action, so molecular studies have focused on alternative mechanisms of carcinogenicity. Proposed mechanisms include nickel insolubility and tissue reaction[21], oxidative stress[22, 23], hypermethylation and gene silencing[24], depletion of intracellular ascorbate[25], and tumour suppressors and oncogenes,[26, 27] suggesting an interplay of complex factors.

Internal metal exposure from implanted devices of alloys in dentistry[28], orthopaedics[29], and cardiology[30] has raised new questions about potential effects of low levels of solubilised nickel[31], including potentiation of allergy[29]. In addition, metal fragments lodged in tissue from bullets[32], explosions, or accidents may also contain nickel or nickel-based fragments[33]. In some cases, fragments cannot safely be surgically removed either due to small size or proximity to critical organs; therefore, the long-term molecular response to such foreign bodies is worth examining in detail.

Studies of nickel toxicity using RNA expression microarrays have been carried out in a broad range of species, including bacteria[34], yeast[35], fibroblasts[36], liver cells[37], and fish[38], as well as blood samples from refinery workers[39], though none to date have examined the molecular response over an extended period of time. Previously, this group looked at the gene expression of implanted tungsten-nickel-cobalt alloy in F344 rats using a small subgroup of tumours[40]. As part of a follow-up study with tungsten alloy and nickel, we examined the timeline of gene expression in muscle tissue surrounding a small nickel pellet in the hind leg of mice. The results show that implanted pellets elicited a co-ordinated cyclical response of gene expression over time which primarily included immune and mitochondrial components. The observed responses at the site of implantation persisted both qualitatively and quantitatively for up to 1 year.

Experimental

Animals: Male C3H mice (Charles River), which have previously been used to examine nickel toxicology[41] were used for this study. All experiments were performed in compliance with relevant laws and institutional guidelines of the Army Public Health Centre and the animal protocol was approved by the Army Public Health Center's Institutional Animal Care and Use Committee (IACUC). Using a small needle with a single pure nickel pellet (1x2 mm) in the bore, anesthetised mice (n=80) were intramuscularly injected via a shaved and sterilised area of the right leg only (gastrocnemius muscle) while the left leg was sham injected. A few treatments were implanted with tantalum (1x2 mm, Ta) or untreated (Sham) as a negative control (n=5). All mice received additional pain relief during recovery from anaesthesia and implantation of pellets. Groups of five mice were humanely euthanized at 3-week intervals up to 1 year, and a small piece of tissue was recovered from around the implant (~20 mg) for RNA extraction[40]; at necropsy, urine (pooled for n=5) and individual blood samples (cardiac) were also taken. Precautions were taken to avoid introducing confounders or systematic error, including randomization and consistency for times of necropsy. Expression data was carried out for animals up to 46 weeks, while chemical analysis was completed for animals up to 54 weeks. A Supplementary Animal Data file contains additional observational data, but it was not possible with this study design to divide samples (~20 mg) for both microarray and histopathology analysis.

Expression Analysis: To preserve mRNA, tissue was flash frozen in liquid N_2 and stored at -80°C prior to shipment to Expression Analysis (Durham, NC) (now Q2 Solutions) for microarray analysis. Total RNA extraction was carried out using Qiagen RNeasy® columns and was of high quality (260/280 ≈ 2; RIN ≥ 6); cRNA was transcribed from extracted RNA and hybridized to Illumina® Sentrix Illumina MouseWG-6 v2.0 Expression BeadChips. Samples were randomized to Beadchips to avoid downstream bias.

Bioinformatics: Microarray analysis was carried out up to 46 weeks. After Beadstudio processing and cubic spline normalization, data was imported into JMP® Genomics 5.0 (SAS, Inc) and statistical analysis statistical analysis carried out using a mixed-model ANOVA with fixed effects for treatment, leg, and week and random effects for completion date (RNA extraction), plate, and animal; results included fold-change (mean expression for right – left leg), t-value, log of the p-values for each gene (Supplementary Data Table 1). Additional analysis of significant gene lists for biological interpretation used Enrichr[42, 43]. Data was also analyzed with IPA (QIAGEN Inc.,https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis.

Chemical Analysis: Analytical measurements of metals was carried out at the Fort Detrick Center for Environmental Health Research Department of Chemistry using acid-digestion followed by analysis on an Agilent 7500ce Inductively Coupled Plasma-Mass Spectrometer. For serum and kidney analysis, biological replicates were used (n=5) for urine pooled samples for each time point. Because of sample volume limitations, creatinine measurements were not undertaken, and final concentrations were therefore expressed as µg/L of sample.

Statistics: ANOVA analysis was followed by FDR (Benjamini and Hockberg) at α=0.05. Genes that were 1.5-fold differentially expressed and exceeded the FDR value at α = 0.05 were used as the final dataset in subsequent analysis. Statistical analysis of nickel concentrations in serum and kidney was carried out using JMP Genomics 5.0 (SAS, Inc). Urine samples were not analysed for significance as they were pooled into a single sample at each time point. Statistical analysis are shown in Supplementary Statistics for Serum and Kidney.

Results

The time profiles of nickel concentrations in both serum and urine mirrored each other, with peak concentrations occurring within the first 15 weeks, followed by decreasing levels that were still high compared with background nickel (Figure 1) at time 0 (62 vs 6 µg/L). Nickel levels in serum at each time point were not all significantly different from the control after 15 weeks (Supplementary Statistics for Serum and Kidney), but the levels at the site of implantation were high enough to impact global gene expression. While background nickel levels were low in serum and urine, background levels in kidneys were relatively high (~600 µg/g), further increasing after implantation to levels approaching 900 µg/g to 15 weeks, and then dropping quickly over a 3-week period, with a subsequent

slow rise to 54 weeks. Background kidney levels have been shown to be similarly high in some animal studies[44] and in livestock[45] but not in unexposed humans[20]; however, these kidney levels were not expected to affect the local expression levels in muscle responding to the nickel pellet. At 54 weeks, nickel levels remained elevated in all three biological samples, indicating persistent internal exposure due to nickel corrosion from the implanted pellet.

High systemic nickel levels are assumed to indicate even greater local concentrations in tissue at the site of the corroding pellet, so we examined the global gene expression response due to nickel exposure in the right and left legs. Unsupervised clustering of the gene expression data (Figure 2A) showed separation into two main clusters composed of either right (implanted) or left (unimplanted) legs. Non-toxic tantalum controls clustered with the unimplanted legs (not shown in dendrogram); indicating that the effect on surrounding tissue due to the presence of implant alone (foreign body effect) was similar to control legs. For one time point (37 weeks), both the left and right legs separated into the same cluster, indicating a potential outlier. This time point was left in place to see how gene expression would compare to other time points.

At necropsy, gross examination of tissues did not indicate any significant changes in individual organs/tissues. The tissue surrounding the pellet implant sites was variable; sometimes a solid tissue or mass, sometimes a semi-solid fluid, in rare cases, an apparent local solid tumour. Pathology analysis showed one clear tumour to be a fibrosarcoma (Supplementary Animal Data), but there was no observed metastasis. For continuity with other tissues, it was decided to include the tumour tissues in the overall analysis. By the end of the study at 46 weeks, there was no uniform response of muscle tissue to implants and no consistent fibrosis across all samples.

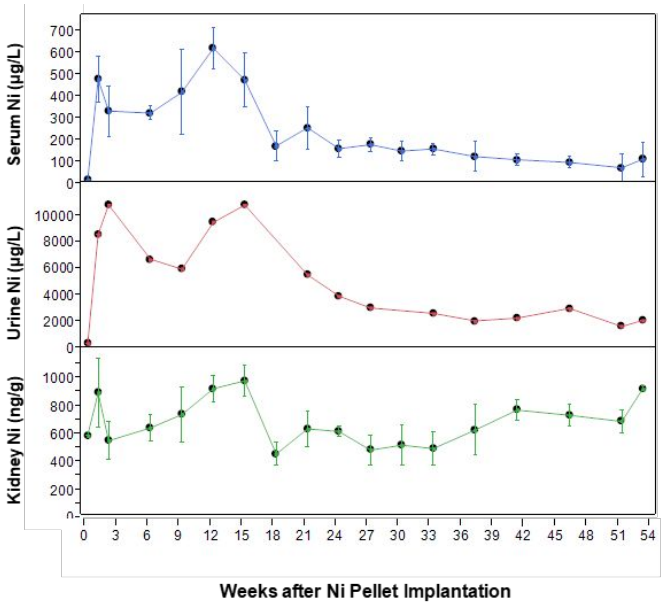

Figure 1. Profile of Nickel Concentrations over Time in Mice after Ni Pellet implantation. A single pure Ni pellet measuring 2x1 mm was implanted in the gastrocnemius muscle of male C3H mice, and Ni metal was measured by ICP-MS in serum, urine, and kidney. Samples were taken at time of necropsy and include mean (±sd) of up to five animals (serum, kidney) or a single composite sample (urine). Significant differences varied depending on time points; detailed statistical analysis is shown in the Supplementary Statistics. Serum different to control up to 15 weeks at α=0.05. Samples at time 0 were from unimplanted controls.

The approach to calculating the relative fold-change in expression over time between left legs (control) and right legs (implanted) is shown for two selected genes, prolactin and Nramp1 (Prl2c2 and Slc11a1) in Figure 2B and 2C. The right leg (black circles) clearly had higher mean overall expression levels over time than the left leg (black square), with the difference between them (red triangles) showing the average relative fold-change (log2) in tissue surrounding the pellet compared to tissue from the unimplanted left leg (5 biological replicates). Prl2c2 (prolactin) has been correlated with nickel in urine of nickel-exposed workers[46] and Slc11a1 (Nramp1) expression is associated with increasing oxidative stress [47] and metal withholding during infection[48]. Note that the difference between right and left leg for Tantalum controls (purple diamonds) or for Sham controls at 9 and 21 weeks was at or below that of the unimplanted left leg controls, indicating that the effect due to implant of a same size pellet (1x2 mm) made of non-toxic metal alone (foreign body effect) was relatively negligible compared to nickel treatments. While nickel levels varied between 100 and 600 µg/L in serum, gene expression did not seem to track these changing circulating concentrations of metal (compare nickel levels over time in Figure 1 to expression profiles in Figure 2B and 2C).

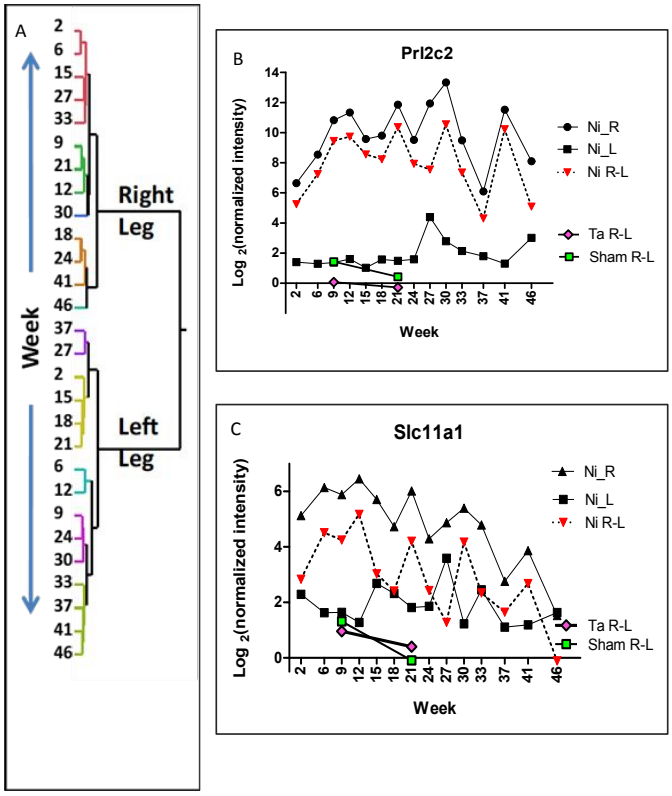

Figure 2. Gene expression data. The dendrogram (A) shows all of the expression data from the study. The left and right legs (with the exception of week 37) were separated into two main clusters. Time profiles for relative expression data for the genes Prl2c2 (prolactin) and Slc11a1 (Nramp1) are shown in (B) and (C), respectively. R=right leg, L=left leg. R-L = expression corrected for left leg control is shown as red triangles. Tantalum (Ta, purple diamonds) and unimplanted controls (Sham, green squares), also corrected for left leg, are shown at 9 and 21 weeks.

Overall, genes were either upregulated or downregulated (compared to the control legs) across all 14 time points; only 5 genes crossed over from up- to downregulated over 46 weeks.

(Figure 3A) and these few crossover genes did not indicate any significant biological effect. The number of significant genes over time appeared cyclical (Figure 3B), and the magnitude of gene expression over time was also cyclical, at least for the highly expressed genes (Figure 3A, note that scale is log2), with apparent peaks at 9, 21, 30, and 41 weeks. The variation in expression for control legs over time was notably less than that of treated legs, and the peaks in expression were driven by the effect of right leg treatment. A list of the top 20 genes (averaged over time) that were consistently upregulated is shown in Table 1 (see also Supplementary Top 20 Data Table) with the genes sorted by fold-expression. Immune function and tissue remodelling dominated these top upregulated genes, including acute phase proteins Mmp13[49], Saa3 and Spp1[50], and cytokines (Il1b, Ccl4), as well as tissue inhibitor of matrix metalloproteinases Timp1[51]. The chemokine Ccl4 has been shown to be associated with wound healing in mice[52], while the immunoglobulin receptor Fcgr4 is expressed on mouse innate cells such as monocytes and neutrophils[53]. Prolactin (Prl2c2), which has roles in modulation of the immune system[54], has a high affinity[55] for and is inhibited by nickel[56]; several isoforms of this gene were in the top upregulated genes. The proinflammatory cytokine Il1b, which is involved in both acute and chronic immune responses[57], was also highly expressed. The top 20 downregulated genes are shown in Supplementary Top 20 Data Table; the magnitude of the fold-change was not as great as upregulated genes, but several genes (Syne1, Actb, Cd209b) were repeatedly decreased in expression across some time points. Syne1 is known to be expressed in skeletal and smooth muscle.

The list of top genes at each time point remained qualitatively similar across all time points (Table 1 and Supplementary Data Table 1), differing for the most part in quantitative expression. Note that week 37, for which treated and untreated legs are clustered together in Figure 2, appears to be an outlier based on the list of genes expressed which were inconsistent with all other time points. For the top downregulated genes, the cyclical trend was not clear for fold-change, and the expression level changes between up- and downregulated genes (Supplementary Data Table 1) differed by about 8-fold (log2 value of ~3 difference). Biologically, downregulated genes were less informative than upregulated genes and there was comparatively higher upregulation of genes across time (Figure 3A). Top downregulated genes included Syne1, which is involved in muscle, while beta actin is involved in cell structure and integrity.

Gene ID	Mean FC	Description	Main Function
Mmp13	1234	matrix metallopeptidase 13	breakdown of extracellular matrix; tissue remodelling
Saa3	722	serum amyloid A3	acute phase protein; toll-like receptor 4 binding; chemoattractant
Prl2c2	485	prolactin family 2, subfamily c, member 2	lactogen and growth hormone; may have role in wound healing
Fcgr4	361	Fc receptor, IgG, low affinity IV	neutrophil activation; positive regulation of bone resorption
Ccl4	257	chemokine (C-C motif) ligand 4	chemoattractand for monocytes and other immune cells
Mmp10	214	matrix metallopeptidase 10	breakdown of extracellular matrix; tissue remodelling
Cxcl1	208	chemokine (C-X-C motif) ligand 1	inflammation; chemoattractant for neutrophils
Prl2c3	202	prolactin family 2, subfamily c, member 3	lactogen and growth hormone; may have role in wound healing
Clec4d	166	C-type lectin domain family 4, member d	inflammation and immune response
Prl2c4	159	prolactin family 2, subfamily c, member 4	lactogen and growth hormone; may have role in wound healing
Spp1	159	secreted phosphoprotein 1	attachment of osteoclasts to the mineralized bone matrix
Il1b	158	interleukin 1 beta	produced by activated macrophages; mediator of the inflammatory response,
Cd72	143	CD72 antigen	B-cell differentiation and proliferation
Timp1	137	tissue inhibitor of metallopeptidase	metalloproteinase inhibitor; functions as a growth factor
Ccl7	113	chemokine (C-C motif) ligand 7	Chemotactic factor that attracts monocytes and eosinophils
Serpina3g	96	serine peptidase inhibitor, clade A, member 3G	innate immune system
Mmp3	86	matrix metallopeptidase 3	breakdown of extracellular matrix; tissue remodelling
AA467197	81	expressed sequence AA467197	unknown
Fpr2	78	formyl peptide receptor 2	receptor for N-formyl-methionyl peptides, neutrophil chemotactic factors
Slc15a3	71	solute carrier family 15, member 3	proton oligopeptide cotransporter; expressed in macrophages

Table 1. Top 20 upregulated genes over time. Mean values were taken for each gene over all time periods and genes were then sorted from highest to lowest.

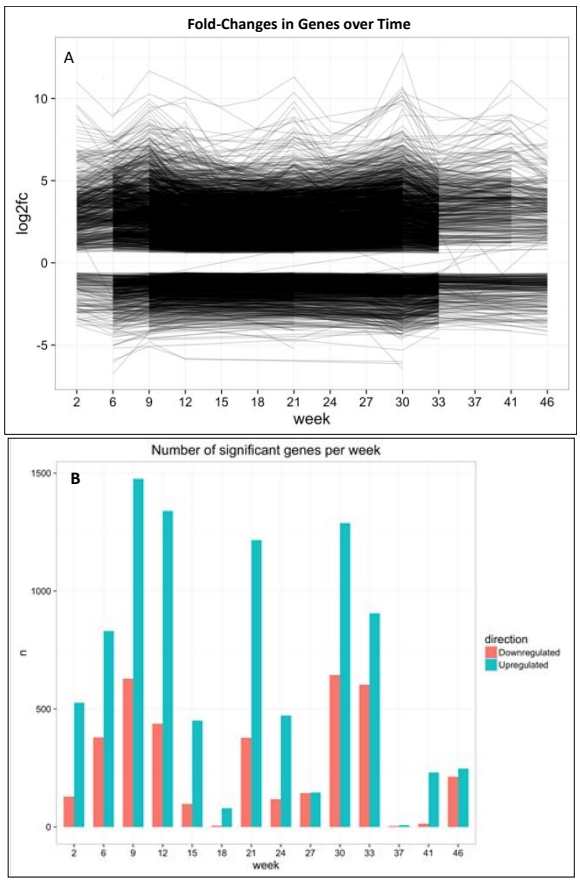

Figure 3. Gene expression over time. Top graph (A) shows the number of significant genes (up- or downregulated) at each time point. Bottom graph (B) shows the fold change over time (note that the Y axis values are log2 fold change). The cut-off value was 1.5-fold, and the FDR value was 0.05. The majority of genes were either up- or downregulated with only a few genes crossing over from up- to downregulated over time.

Biological meaning of the expression data over time was examined using IPA pathway analysis. After initially carrying out pathway analysis at each time point, the IPA Comparison Analysis tool was used to profile the top pathways over time; and the top pathways were further clustered based on similarity over time. Pathways for sirtuin signalling, mitochondrial dysfunction, and oxidative phosphorylation pathways clustered together (Figure 4A); notably all three pathways were dominated by downregulated genes as shown by the example data for week 21 (Figure 4B). These three pathways followed similar expression profiles over time that were distinct in their profile from the other listed pathways (Figure 4A). Individually, the mitochondrial genes Sirt 2, Sirt3 and Sirt5 were downregulated over time (See Supplementary Data Table 1) whereas the nuclear located Sirt 7 (deacetylation) was upregulated. Sirt 3 and Sirt 5 are localized to the mitochondrion while Sirt 2 and Sirt 7 are found in the nucleus or cytoplasm. Overall, both sirtuin signalling and energy metabolism pathways were downregulated.

Other top clusters (Figure 4A) consisted of several immune related pathways. Granulocyte (neutrophils, eosinophils, basophils) and agranulocyte (monocytes and lymphocytes) adhesion/diapedesis pathways are key events in the process of inflammation. Neutrophils and macrophages seem to be the primary immune cell types involved in this response. Pro and anti-inflammatory chemokines, IL8 and IL10 respectively were upregulated. Proinflamatory IL 8 induces chemotaxis, particularly for neutrophils. Anti-inflammatory IL-10, mainly produced by monocytes, acts on many immune cells to inhibit the negative impacts effects of inflammation. IL-6 is a strong inducer of the acute phase response - the acute phase response signalling pathway was also upregulated. The other pathways clustered in this group were Nf-ķB, which is an inducible transcription factor regulating a number of genes involved in inflammation, and TREM-1 (triggering receptor expression on myeloid cells), which stimulates the response of neutrophils and monocytes serving to amplify inflammation. These pathways are all involved in acute phase response, and individually, acute phase genes (Saa3) were some of the most highly expressed genes in the dataset. Inhibition of matrix metalloproteinases may be a response to the high expression of several Mmp genes (see Table 1) found in the tissues.

We looked at KEGG pathways for the same dataset, compiling the scores for each time point into an overall score. The list of top KEGG pathways is shown in Table 2 for both up and downregulated genes. The top three KEGG pathways involve two pathways related to engulfment or sequestration while osteoclast differentiation is related to the formation of foreign body giant cells. Pathways found in common with the IPA analysis included Apoptosis, Nf-kappa B signalling. Oxidative stress has consistently been shown to result from nickel exposures[23, 36] and the HIF-1 signalling pathway, also involved in oxidative stress, was significant. KEGG pathways for metabolism in the mitochondria were consistently downregulated (see Supplementary Data Table 2). Other KEGG pathways included Toll-like receptor[11], which has a significant role in nickel contact allergy. Toll-like receptors are membrane receptors that are expressed on innate immune cells, such as macrophages and dendritic cells. Toll-like receptor pathway included several Tlr genes including TLR4? While primarily responding to bacteria, these receptors have also been shown to underpin contact hypersensitivity to nickel[19] and have been proposed as transition metal sensors[19]. Phagasome is a central mechanism of tissue remodelling and inflammation and formed phagasomes have regulated interaction with other organelles including lysosomes. Collectively, these pathways indicate a range of processes responding to the presence of nickel pellets over time.

We also used GO analysis (using EnrichR) to contrast biological interpretation using all the data with the pathway analysis – selection bias could skew pathway results because of genes that were not utilized in IPA or KEGG pathway analysis. Supplementary Table 3 shows a summary of the three GO processes (biological process, cellular component, and molecular function) with the list of top significant GO terms at each time point; the full table of significant GO terms for all time points is shown in Supplementary Data Table 3. For upregulated genes, there was a strong immune component for biological processes, which was topped by an acute phase response (neutrophils, cytokines). In the cellular compartment process, granules, vacuoles, the ER, and lysosomes featured while actin binding was top in the molecular function. The processes for downregulated genes were dominated by terms associated with mitochondria and energy. While these GO results show a more comprehensive overview of the effects of nickel on tissue, the overall responses show inflammatory immune reactions to implants accompanied by downregulation of mitochondrial functions which is overall consistent with the pathway analysis.

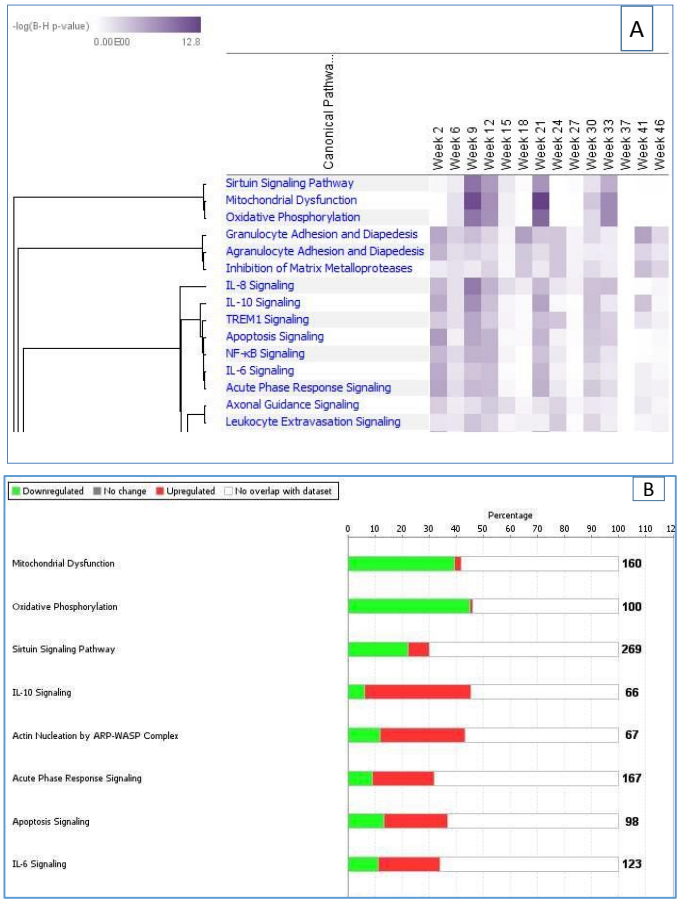

Figure 4. Top graph (A) shows the comparison analysis for all times points with a hierarchical cluster analysis by row. IPA Pathway Comparison Analysis over time. Bottom graph (B) shows typical individual time point analysis at 21 weeks. X-axis shows percentage of genes in pathway changed. Note that the majority of genes for the top 3 pathways are downregulated.

KEGG Pathway (Upreg. Genes)	Score	KEGG Pathway (Downreg. Genes)	Score
Lysosome	981	Citrate Cycle	218
Osteoclast differentiation	523	Valine, leucine and isoleucine degradation	132
Phagosome	452	Propanoate metabolism	113
TNF signaling pathway	448	Peroxisome	96
Apoptosis	384	Glyoxylate and dicarboxylate metabolism	79
Toll-like receptor signaling pathway	367	Ubiquitin mediated proteolysis	76
Other glycan degradation	312	Ubiquinone and other terpenoid-quinone biosynthesis	73
Proteoglycans in cancer	313	GnRH signaling pathway	69
NF-kappa B signaling pathway	312	Oxidative phosphorylation	54
HIF-1 signaling pathway	254	Starch and sucrose metabolism	49
		Fatty acid degradation	38

Table 2. Top KEGG Pathways over all time points. KEGG pathways were accessed using EnrichR. Significant pathways at each time point are shown in Supplementary Data Table 2. Score values are from combined scores from each time point. The full dataset showing KEGG results at each time point is shown in Supplementary Table 2.

Discussion

Biocorrosion of implants in gastrocnemius muscle of mice produced high systemic levels of nickel, a surrogate for even higher local exposures of tissues surrounding the pellets. Locally high tissue concentrations of nickel have been shown to induce severe inflammation compared to non-corrosive nickel-chromium alloy or plastic[58]. In our work, tissue around implants had highly upregulated gene expression profiles that were associated with inflammation, while systemic nickel levels had minimal impact on gene expression in control muscle (unimplanted left legs), possibly because nickel was bound to serum proteins[59] and unavailable. Furthermore, gene expression around non-toxic tantalum implants was more similar to the non-implanted control legs in nickel treatments (Figure 2), indicating that the observed molecular response was due to nickel levels around pellets and not a foreign body response to the pellet alone.

Viewed over months, the variation in nickel levels was not tracked by gene expression profiles; instead, there was an apparent cyclical response in gene expression over time. This response was for both the number of genes and the magnitude of gene expression, with regular peaks at about 10-week intervals. Transient gene expression has been observed in short term nickel studies, but over longer periods the immune system has also been demonstrated to undergo cyclical oscillations[60], particularly in disease states[61, 62]. Circadian responses have been demonstrated in murine skeletal muscle, but these changes are small (1-2 fold over 24 hours)[63, 64] compared to our study, which showed large oscillations occurring for genes associated with the immune system (>100 fold over weeks), as well as other genes. While a feedback loop drives the circadian rhythm in gene expression[65], the underlying reason for our dramatic changes in gene expression is not clear but is likely related to dynamic cellular processes occurring around the implant; one possibility being waves of acute phase immune cell recruitment to the affected implant area[66]. Neutrophils and macrophages are both involved in immune responses to implants[67], though macrophages alone have been shown to be necessary and sufficient for fibrosis and encapsulation of implants[67]. The acute phase gene serum amyloid (Saa3; found at very high levels in our study) has been correlated with the number of neutrophils, which themselves can increase up to 500-fold in an implant microenvironment[66]. Oscillations have been identified in particularly for the immune system[60, 62] which is most likely driving the effects observed here.

The clustering of three pathways; sirtuin, mitochondrial dysfunction, and oxidative phosphorylation and the overall downregulation of component genes in these pathways indicates that energy production was decreased due to nickel treatments. Sirtuins play a critical role in aging, metabolism, and restoring homeostasis during stress via mitochondrial protein acetylation but they also have other diverse functions in inflammation and apoptosis. In the sirtuin pathway, four of the seven Sirt genes were significant and all but one (Sirt 7) was downregulated; Sirt 5, Sirt 3, and Sirt 2 were downregulated. The fact that upregulated Sirt 7 is primarily a nucleolar protein, but Sirt3 and Sirt5 are downregulated in mitochondrial suggests that mitochondria themselves may be targets for nickel. Sirt3 downregulation inhibits cell proliferation and induces cell apoptosis while Sirt5 is involved in respiration, electron transport chain, and fatty acid oxidation[68]. While the impact of nickel on energy metabolism in mitochondria has been noted[69], evidence for nickel effect on sirtuins has not been published.

Three KEGG pathways predominated in this study; osteoclast differentiation, lysosome, and oxidative phosphorylation, the latter due almost entirely to downregulated genes (Supplementary Data Table 2), while other pathways were related to the immune system (Toll-like receptor signalling, chemokine signalling), phagosome, apoptosis. Aside from its role in absorption and removal of bone, osteoclast differentiation involves the fusion of macrophages to form multinucleate giant cells that envelop foreign bodies known as foreign body giant cells (FBGC)[70], especially when such bodies are too large for macrophage engulfment and elimination. The normal

process of wound healing[71] and reaction to foreign body fragments[72] or surgical implants[73] involves early innate immune response, tissue repair, formation of fused giant cells[73] around the foreign body, and, ultimately, walling off of the foreign body by fibrosis. Tactically situated macrophages can undergo activation, adhesion, and fusion to form FBGCs; these FBGCs (also known as Multi-Nucleated Giant Cells) can further act on the biomaterial surface[73], resulting in accelerated biocorrosion. Several highly expressed genes in our study are known markers of fused giant cells including CD44 and ICAM-1[74], as well as CD68 and MMP9[75] (See Supplementary Data Table 1). The upregulated chemokine CCL2 is critical for the recruitment of monocytes to implant materials, participating in both fusion of macrophages and giant cell formation[76] while CD68, the surface marker for FBGCs, was highly expressed in this and other studies[75]. We did not see upregulation of colony stimulating factor 1 receptor (Csf1r), which is considered critical for macrophage progression to FBGCs[67]; rather, there were increases in Csf3r, which is involved in neutrophil activation.

Activation of Toll-like receptor signalling pathway in this work is consistent with observations that nickel activates dendritic cells by ligating with Toll-like receptor 4 (TLR4)[11]. This pathway has also been shown to be significant in tungsten alloy fragments containing nickel but only in human skeletal muscle cells and not in a rat cell line[77]. TLR4 has been proposed as a transition metal-sensitive receptor with a particular affinity for nickel[19] and was highly over-expressed up to 30 weeks (Supplementary Data Table 1) in our work. However, though critical in the development of human nickel sensitivity, mouse TLR4 does not promote sensitisation to nickel due to a lack of critical non-conserved histidine residues[11] found in humans, so the Toll-like receptor signalling pathway may be modulating other immunotoxicological responses. We did carry out follow-up work to examine sensitisation or modulation of the immune system.

Any transition from normal skeletal muscle physiology to fibrotic, necrotic or even pre-carcinogenic tissue around implants will be underpinned by changes in gene expression, as in this case with oxidative phosphorylation. Similar to our work, a burn model of mouse skeletal injury has shown upregulation of inflammation and downregulation of oxidative phosphorylation, including downregulation of a wide range of electron transport components [78]. This indicates that local trauma to cells (produced by the force of implantation in our study), rather than nickel alone, might be a key initiating event in downregulation of oxidative phosphorylation. However, soluble nickel has independently been shown to reduce ATP synthesis without injury[69], so both local injury and soluble nickel ions from pellets may be interacting in the downregulation of oxidative phosphorylation. The overall effect would be of tissue surrounding pellets becoming more reliant on glycolysis for energy production rather than oxidative phosphorylation, a local environment more likely to be associated with necrosis or carcinogenesis. Other factors will also be at play, since few tumours were identified around pellets, and the majority of mice tumour free after one year (it is not known whether an additional year would have resulted in increased tumours). But the fact that tissue injury and soluble nickel ions independently inhibit ATP production, thereby altering metabolic demands, may be an important determinant in the toxicological outcome of metal implants.

In other microarray studies of nickel, terms associated with oxidative damage were identified in mouse fibroblasts and other cell types[36, 37]. Oxidative stress has been proposed to underpin nickel toxicity[79] via Fenton reaction chemistry and generation of free radicals. Furthermore, Il1b, which was increased over the course of our study, has been shown to be induced by exposure to nickel via mitochondrial reactive oxygen species[80, 81]. In addition, several individual genes involved in oxidative stress responses were overexpressed in our work. Heme oxygenase 1 (HMOX1) was increased over time, and extracellular superoxide dismutase (SOD3) increased in the early weeks of the study while glutathione peroxidase (GPX1) was consistently overexpressed (Supplementary Data Table 1). The master regulator of oxidative stress Nrf2 was not increased, but evidence exists of an Nrf2-independent inflammatory pathway activated by nickel[82]. Nfkb1, which can regulate the inflammatory response as well as the response to oxidative stress, was increased over the course of the study. While not directly involved in oxidative stress, several isoforms from the prolactin family (Prl2c2, Prl2c3, Prl2c4) were highly upregulated; prolactin has been identified as a biomarker of occupational nickel exposure[46] while nickel is also a potent inhibitor of prolactin secretion[56]. More work is needed to identify the role of nickel in the prolactin response.

Pathway analysis results depended on which of two tools was used but there were overlapping effects observed. Both IPA and KEGG pathways showed that mitochondria were strongly impacted by downregulated genes while for upregulated genes different aspects of the immune system were upregulated, with the highest gene expression associated with acute phase response. Immune pathways dominated for upregulated genes, including the acute phase response, pro and anti-inflammatory cytokines (IL 10 IL8), neutrophils and macrophages, apoptosis, and others. When the results for pathway analysis were combined with GO analysis, it was evident that the cellular response involves neutrophils and macrophages while the intracellular response involved mitochondria and lysosomes, both of which has been reported to be involved in metal sequestration of homeostasis.

Expression data from a nickel implant study in muscle of mice revealed patterns that were qualitatively similar over time but varied in strength in a cyclical fashion. An overarching inflammatory response was accompanied by downregulation of energy metabolism; other processes upregulated were cytokine signalling, apoptosis, toll like receptor, and apoptosis. It should be noted that nickel levels in this study are much higher than would be expected from occupational exposures. Our dataset provides a list of genes in tissue surrounding nickel implants over time that can be used by other researchers as a point of departure for studies investigating molecular aspects of long-term nickel exposure.

Conclusions

This work examined the local molecular response of muscle tissue to a single nickel-implanted pellet. Released nickel caused high upregulation of genes associated with immunity and downregulation of genes associated with energy metabolism. The gene expression response over time was consistent but oscillated in strength. In summary, we generated a first compendium gene expression and pathway changes in response to long term exposure to nickel. These data provide potential pathways for future research into nickel toxicity.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

Disclaimers:
The views expressed in this document are those of the author(s) and do not necessarily reflect the official policy of the U.S. Department of Defense, U.S. Department of the Army, U.S. Army Medical Department, or the U.S. Government.
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Funding:
This work was funded by the U.S. Department of Defense Army Environmental Center and the U.S. Army Corps of Engineers Environmental Research and Development Center.

Acknowledgment:
We acknowledge the invaluable assistance of Dr. Emily Lent and Dr. Craig McFarland during necropsy, as well as the helpful technical review of the MS by Dr. Mark Williams and Dr. Lindsay Holden. Our long-time friend and colleague, Dr. Will McCain passed away (10/9/2018) before completion of this MS.

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