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Dose-dependent effects of Ni (II) ions on production of three inflammatory cytokines (TNF-α, IL-1β and IL-6), superoxide dismutase (SOD) and free radical NO by murine macrophage-like RAW264 cells with or without LPS-stimulation

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Abstract The effect of Ni (II) ions on macrophages is not well understood. The purpose of this study was to examine the dose-dependent effects of Ni (II) ions up to 1,000 μmol/L on production of three inflammatory cytokines (TNF-α, IL-1β and IL-6), superoxide dismutase (SOD) and nitric oxide (NO) by murine macrophage-like RAW264 cells with (+) or without (−) lipopolysaccharide (LPS) -stimulation. Ni (II) ions caused LPS (−) RAW264 cells to slightly increase production of TNF-α and IL-6, proportionally to the Ni (II) ion concentration while IL-1β was not produced, and to slightly increase production of SOD and NO. It can be concluded that Ni (II) ions dose-dependently increased the inflammatory and oxidative stress conditions of LPS (−) RAW264 cells. LPS-stimulation caused RAW264 cells to produce in abundance the three inflammatory cytokines, SOD and NO. Ni (II) ions dose-dependently reduced the three cytokine quantities and NO amounts in LPS (+) RAW264 cells, while dose-independently increasing SOD amounts. It was noted that Ni (II) ions dose-dependently reduce the resistance power against bacteria of LPS (+) macrophages, because the production of volatile NO—bacteria killer is diminished proportionally to the Ni (II) ion concentration.

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1 Introduction

Ni (II) ions are easily released from medical and dental alloys containing Ni in body fluids [1, 2]. Ni (II) ions and Ni (II) ion-bound proteins are easily absorbed by macrophages, the key to the body's self-defense, and Ni (II) ions tend to accumulate in their nuclei [3] and damage DNA (e.g. through bonds between Ni (II) ions and DNA) [4]. As a result, signaling pathways such as Nrf2 [5] are activated so that inflammatory cytokines [5] and free radicals [6] are generated, giving rise to cellular damage, inflammatory reaction, cellular death, immune reaction and eventually carcinogenesis [7]. The production of inflammatory cytokines and free radicals by macrophages exposed to Ni (II) ions is not, however, well understood [8, 9].

When stimulated with LPS of gram-negative bacteria present in dental plaque, macrophages are activated through the NF-kappaB signaling pathway via Toll-like receptor 4 and produce abundant inflammatory cytokines and free radicals [10]. Few researchers have examined the effects of Ni (II) ions on inflammatory cytokines and free radicals produced by LPS-stimulated macrophages [11].

As for inflammatory cytokines, TNF-α, IL-1β and IL-6 are well known to cause systematic pathological symptoms (e.g. periodontitis) [12]. Oxidative stress can be evaluated by superoxide dismutase (SOD) and nitric oxide (NO). When superoxide (O_2^-) is generated to attack or dissociate xenobiotic matters (e.g. bacteria), SOD, a scavenger of superoxide, is concomitantly produced [13]. In this study, the amount of SOD production was regarded as a secretion scale of superoxide because enzyme SOD was easily measured while superoxide, with a short life, is very difficult to accurately quantify [14]. Another free radical, volatile NO, is produced to protect cells against intruding

bacteria with LPS, but its excessive production has a detrimental effect on the viability of cells [15].

The purpose of this study was, therefore, to examine the dose-dependent effects of Ni (II) ions up to 1,000 μmol/L on the production of three inflammatory cytokines (TNF-α, IL-1β and IL-6) and two oxidative stress scales (SOD and NO) by murine macrophage-like RAW264 cells with or without LPS-stimulation, and to evaluate their clinical meaning.

2 Materials and methods

2.1 Culture of RAW264 cells

RAW264 cells (RCB0535, Riken Biosource Center Cell Bank, Tsukuba, Ibaraki, Japan) were routinely cultured in α-minimum essential medium (Cat. No. 10099, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Cat. No. 10099-141, Invitrogen, Carlsbad, CA, USA), 2% antibiotics (penicillin–streptomycin–amphotericin, Cat. No. 15240-096, Invitrogen, Carlsbad, CA, USA) and 0.1 mM non-essential amino acid solution (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2 incubator at 37 °C. After confluence, cells were collected by trypsinization with phosphate buffered saline solution (PBS(−)) containing 0.08% trypsin (Cat. No. 15090-046, Invitrogen, Carlsbad, CA, USA) and 0.14% EDTA (Cat No. 15576-010, Invitrogen, Carlsbad, CA, USA) and sub-cultured by 1:3 ratios for an additional 14 days while the medium was exchanged twice a week.

2.2 Preparation of culture media with Ni (II) ions and LPS

Nickel (II) chloride anhydrous (Code No. 19035-0401, Jyunsei Chemical Co., Tokyo, Japan) was dissolved in super-pure water at the concentration of 0.2 mol/L (Ni (II) stock solution) and sterilized by membrane filtration. By diluting this stock solution, media containing Ni (II) ions with concentrations of 0, 50, 200, 500 and 1,000 μmol/L were prepared. For LPS-stimulation, a commercial LPS (*Escherichia coli* O26, DIFCO Lab., Detroit, MI, USA) dissolved in sterile PBS (−) solution was added to the media at a final concentration of 1 μg/mL. This high concentration LPS was selected to cause RAW264 cells to actively produce inflammatory cytokines such as TNFα, IL-1β and IL-6 [16].

2.3 Cell culture with or without Ni (II) ions and LPS

RAW264 cells (1×10^5) were first seeded on the 24-well microplates filled with 1 mL LPS (−) control medium without Ni (II) ions, followed by culturing for 1 h so that cells adhered to the wells. The medium was then exchanged for ten test media with or without LPS and Ni (II) ions. There were ten kinds of test culture media: LPS (−) 0 μmol/L Ni (II) ion, LPS (−) 50 μmol/L Ni (II) ions, LPS (−) 200 μmol/L Ni (II) ions, LPS (−) 500 μmol/L Ni (II) ions, LPS (−) 1,000 μmol/L Ni (II) ions, LPS (+) 0 μmol/L Ni (II) ion, LPS (+) 50 μmol/L Ni (II) ions, LPS (+) 200 μmol/L Ni (II) ions, LPS (+) 500 μmol/L Ni (II) ions, LPS (+) 1,000 μmol/L Ni (II) ions ($n = 6$ for each culture condition); and cells were cultured for one day.

2.4 Measurements of DNA contents

Cells cultured for one day were washed with 1 mL PBS (−) solution twice, collected by cell scrapers, centrifuged, resuspended in 200 μL PBS (−) solution with 1 wt% Triton X100 and homogenized for 30 s (Physcotoron NS-310E, Microtec Nition, Funabashi, Chiba, Japan). The DNA contents in cell lysate were then measured with a Fluorescent DNA quantitation kit (Biorad, Hercules, CA, USA) and a fluorometer (Versafluor, Biorad, Hercules, CA, USA).

2.5 Cytokine assays

Three cytokines' (TNF-α, IL-1β and IL-6) amounts in the culture supernatant were examined with Mouse TNF-α, IL-1β and IL-6 ELISA Kits (EMTNFA, EMILB and EMIL6, respectively; Pierce Endogen, Rockford, Ill., USA) and a microplate reader (Model 680, Biorad Co., Hercules, CA, USA).

2.6 SOD measurements

SOD production of the cells, thawed in a −30 °C freezer, was measured using a SOD Assay Kit (S311, Doujin Chemical, Kumamoto, Japan) and a plate reader.

2.7 Nitrite assay

The NO production of RAW264 cells secreted into the culture supernatant was measured by the Griess method. Briefly, 100 μL supernatants were transferred into the 96-well microplates, and 100 μL Griess reagents (1% sulfanilamide/0.1% naphtylethylenediamine dihydrochroride/2.5% H_2PO_4) (Sigma Aldrich Japan, Tokyo, Japan) were added, which converted nitrite into a deep purple azo compound. Absorptions were then measured at 550 nm

using a microplate reader to determine the NO level, using the pre-determined calibration line between NO concentrations and their optical absorption [17].

2.8 Statistics

Statistical analysis was performed by computer software (StatView-J 5, SAS Institute, Cary, N.C., USA) with the unpaired *t*-test.

3 Results

3.1 Effects of Ni (II) ions on DNA contents of RAW264 cells with or without LPS-stimulation

Figure 1 shows the effects of the Ni (II) ion concentrations on DNA contents of LPS (−) and LPS (+) RAW264 cells. DNA amounts of LPS (−) RAW 264 cells (white columns) declined with increasing the Ni (II) ion concentration. The DNA content of LPS (−) RAW264 cells cultured in the media with 500 μmol/L Ni (II) ion declined to about half (IC_{50}) that cultured in control medium (LPS (−) without Ni (II) ions). On the other hand, those of LPS (+) RAW264 cells (black columns) remained almost constant, irrespective of the Ni (II) ion concentration.

3.2 Effects of Ni (II) ions on three inflammatory cytokine productions of RAW264 cells with or without LPS-stimulation

Figure 2 indicates the effects of the Ni (II) ion concentrations on production of three inflammatory cytokines (TNF-α, IL-1β and IL-6) by LPS (−) RAW264 cells. LPS (−) RAW264 cells always produced tiny amounts of TNF-α whose secretions increased dose-dependently proportional to the Ni (II) ion concentration, did not generate any IL-1β, and formed minute quantities of IL-6 dose-dependently when the Ni (II) ion concentration was more than 500 μmol/L. Figure 3 shows the effects of the Ni (II) ion concentrations on production of three inflammatory cytokines (TNF-α, IL-1β and IL-6) by LPS (+) RAW264 cells. LPS (+) RAW264 cells produced abundant TNF-α whose amounts were quasi-constantly abundant for Ni (II) ion concentrations less than 200 μmol/L and declined with further increasing the Ni (II) ion concentration from 200 to 1,000 μmol/L; small amounts of IL-β which increased dose-dependently in the Ni (II) ion concentration range from 0 to 500 μmol/L but declined at the Ni (II) ion concentration of 1,000 μmol/L; and generated high IL-6 which was also quasi-constant when the Ni (II) ion concentration was less than 500 μmol/L but declined at the Ni (II) ion concentration of 1,000 μmol/L.

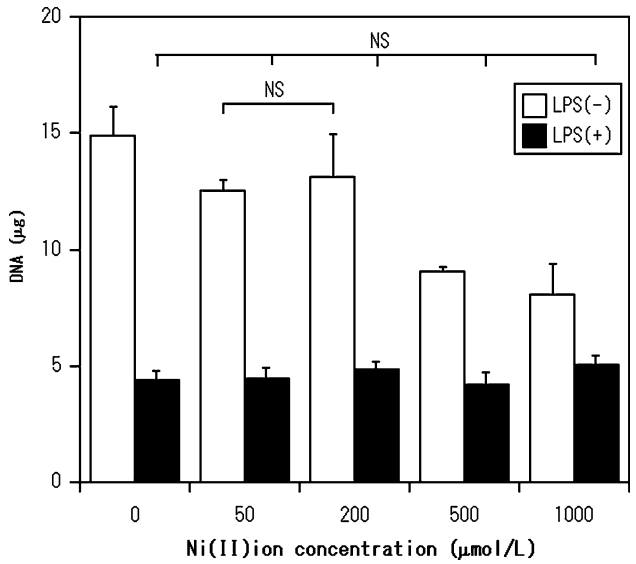

Fig. 1 The effects of the Ni (II) ion concentrations on DNA contents of LPS (−) and LPS (+) RAW264 cells, expressed as mean values with standard deviations (bar). Note: $n = 6$ for each condition. NS = statistically not different ($P > 0.05$). All other pairs of mean values in the LPS (−) group had statistically significant difference ($P < 0.05$)

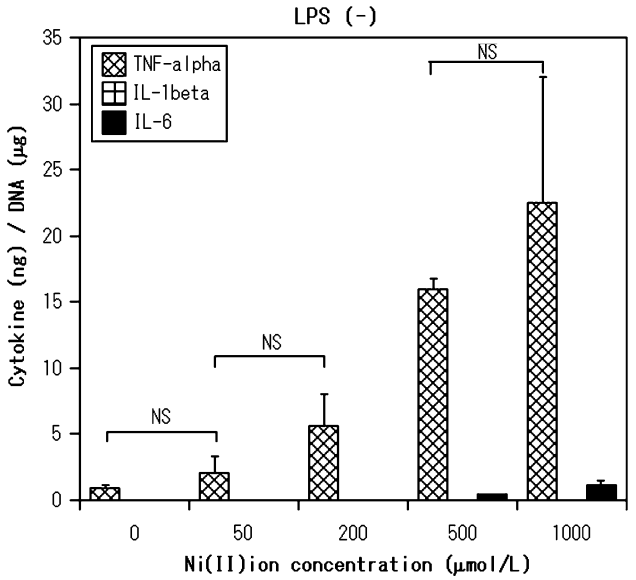

Fig. 2 The effects of the Ni (II) ion concentrations on production of three inflammatory cytokines (TNF-α, IL-1β and IL-6) by LPS (−) RAW264 cells, expressed as mean values with standard deviations (bar). Note: $n = 6$ for each condition. NS = statistically not different ($P > 0.05$). All other pairs of mean values of two cytokines (TNF-α and IL-6) had statistically significant difference ($P < 0.05$)

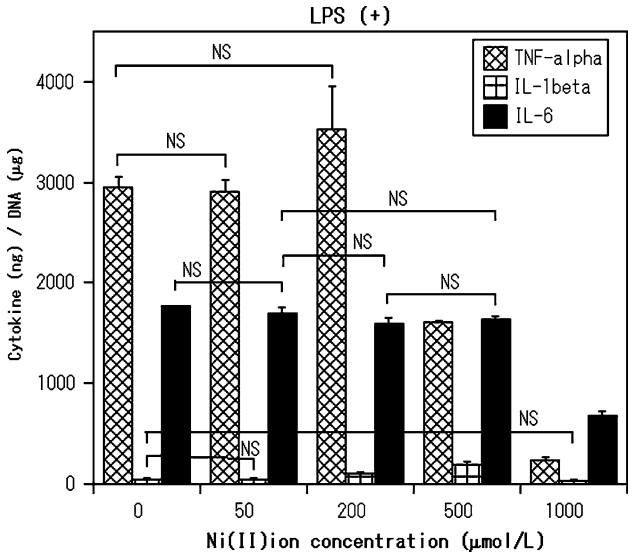

Fig. 3 The effects of the Ni (II) ion concentrations on production of three inflammatory cytokines (TNF-α, IL-1β and IL-6) by LPS (+) RAW264 cells, expressed as mean values with standard deviations (bar). Note: $n = 6$ for each condition. NS = statistically not different ($P > 0.05$). All other pairs of mean values of each cytokine had statistically significant difference ($P < 0.05$)

3.3 Effects of Ni (II) ions on SOD production of RAW264 cells with or without LPS-stimulation

Figure 4 shows the effects of the Ni (II) ion concentrations on SOD production of LPS (−) and LPS (+) RAW264 cells. LPS (−) RAW 264 cells always secreted SOD (white columns) with its amount increased by Ni (II) ions. SOD amounts of LPS (−) RAW264 cells cultured in the media containing 50 and 200 μmol/L Ni (II) ions exceeded about 20% that of LPS (−) control cells without Ni (II) ion while those cultured in the media containing 500 and 1,000 μmol/L Ni (II) ions doubled or tripled that of LPS (−) control cells without Ni (II) ions. On the other hand, SOD amounts of LPS (+) RAW264 cells (black columns) cultured in 50, 200, 500 and 1,000 μmol/L Ni (II) ions were similar to each other, and about 50% greater than that of LPS (+) control RAW264 cells without Ni (II) ions.

3.4 Effects of Ni (II) ions on NO secretion of RAW264 cells with or without LPS-stimulation

Figure 5 indicates the effects of the Ni (II) ion concentrations on NO production of LPS (−) and LPS (+) RAW264 cells. LPS (−) RAW 264 cells also always produced NO (white columns) with their amounts increased about 20% by Ni (II) ions, irrespective of the Ni (II) ion concentration. On the other hand, LPS stimulation significantly increased NO production (black columns) from RAW264 cells (LPS (+) 0 μmol/L Ni (II) ion), compared with control cells (LPS (−) 0 μmol/L Ni (II) ion). The NO production of LPS (+) RAW264 cells, however, dramatically declined stepwise with increasing the Ni (II) ion concentration up to 1,000 μmol/L.

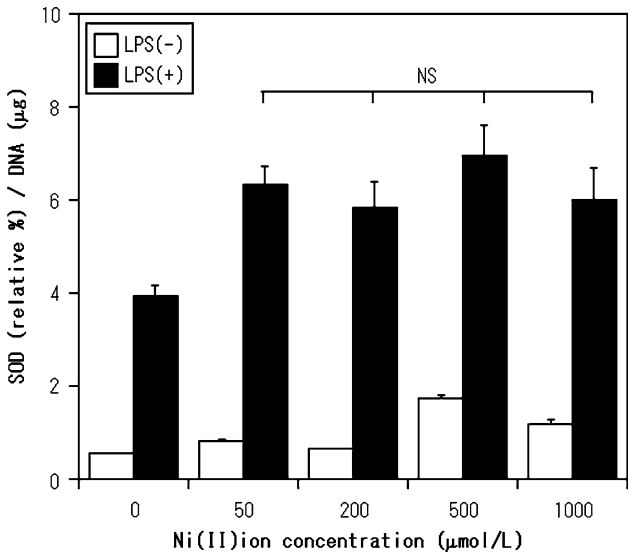

Fig. 4 The effects of the Ni (II) ion concentrations on SOD production of LPS (−) and LPS (+) RAW264 cells, expressed as mean values with standard deviations (bar). Note: $n = 6$ for each condition. NS = statistically not different ($P > 0.05$). All other pairs of mean values in the LPS (−) group and the LPS (+) group had statistically significant difference ($P < 0.05$)

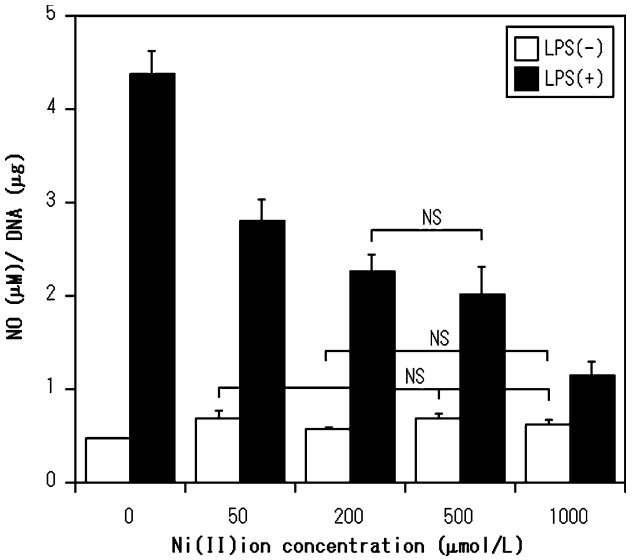

Fig. 5 The effects of the Ni (II) ion concentrations on NO production of LPS (−) and LPS (+) RAW264 cells, expressed as mean values with standard deviations (bar). Note: $n = 6$ for each condition. NS = statistically not different ($P > 0.05$). Any other pairs of mean values in the LPS (−) group and the LPS (+) group had statistically significant difference ($P < 0.05$)

4 Discussion

The elution of Ni ions from dental Ni-containing alloys is literarily considered first. Costa et al. [18] reported that immersion of type 304 stainless steel in neutral (pH = 6.75) artificial saliva for 42 days resulted in the elution of Ni (II) ions in the range of about 0.1 μmol/L. Wataha et al. [19] reported that when Ni-containing alloys are placed in a highly acidic environment with pH ranging from 1 to 7, which simulates sites of carious dentin infected by bacteria, elution of Ni (II) ions was intensified by more than ten to hundred fold, possibly reaching the concentration range of about 1–10 μmol/L. The cell culture test period in this study was 1 day (24 h) while the tissue-irritating phenomenon by eluted Ni (II) ions often occurs after long-term contact between Ni (II) ions and cells, often in the range of months or years. The cell culture experiment conducted here is a somewhat accelerated test, increasing the Ni (II) ion concentration based on the idea that a higher Ni (II) ion dose experiment for short period (one day) could simulate lower-dose Ni (II) ion cytotoxicity over a long period [18, 20]. For these reasons, the concentration levels from 0 to 200 μmol/L, which were sublethal and less than IC_{50}, might be of scientific and clinical significance. This way of thinking seemed to be supported by the report of Bergman et al. [21] indicating that nickel–chromium dental alloys implanted in mice released over 100 ppm (1,700 μmol/L) Ni (II) ions into the tissue capsule around the implant and elevated Ni (II) ion levels in tissues distant from the implants.

The effect of LPS is secondly considered. The in vitro cell culture condition is very clean and unrealistic, without bacteria. In the actual oral environment (i.e., in the clinical condition), however, gram-negative oral bacteria with LPS are always present in dental plaque, and, if oral care is neglected, significantly multiply and attack the teeth and surrounding tissues through hard tissue erosion by acid and activation of the NF-kappaB signaling pathway leading to an inflammation reaction by host cells (e.g. macrophages), causing caries and periodontitis, respectively [12]. Ni-containing alloys are undoubtedly attacked by acid [19, 22] and inflammation reaction containing superoxide so that Ni (II) ions are eluted in vivo. The effect of LPS is, therefore, an important consideration in the evaluation of Ni (II) ion cytotoxicity, but little has been clarified to date [16, 21].

Experimental results obtained are reviewed here separately, dependent on the absence or presence of LPS, as follows.

For LPS (−) RAW264 cells, Ni (II) ions dose-dependently reduced DNA contents, reflecting the situation that Ni (II) ions dose-dependently accumulated in nuclei [3] and damaged DNA, thereby decreasing the cell number (i.e., DNA contents). Ni (II) ions also dose-dependently increased two cytokine (TNF-α and IL-6) secretions while IL-1β was not produced. TNF-α appeared to be a very sensitive cytokine, followed by IL-6 whilst IL-1β was most sluggish. The reasons for the difference in production of these three cytokines by Ni (II) ions and their clinical meaning are not yet well-understood [20] are still under evaluation. The increase in SOD (i.e., SOD1 (Cu, Zn)) production [13] with the addition of Ni (II) ions might reflect active SOD production to dissociate superoxides that phagocytize Ni (II) ion-containing-protein-complex. The free Ni (II) ions generated might then combine with intracellular de-toxication proteins such as metallothionein [23], but excessive Ni (II) ions might diffuse into the nucleus and damage DNA. The increase in NO production by Ni (II) ions was small (about 20%, irrespective of the Ni (II) ion concentration) and might reflect a stress reaction [24].

For LPS (+) RAW264 cells, the DNA contents remained quasi-constant irrespective of the Ni (II) ion concentration, possibly due to cell cycle arrest [15], so that duplicating DNA was protected from superoxide and volatile NO, killers of invading bacteria with LPS [25]. LPS (+) RAW 264 cells produced TNF-α, about 3,000 times more than that by control LPS (−) RAW264 cells. Activation of the NF-kappaB signaling pathway by LPS [10] accounted for the high production of the three cytokines (TNF-α, IL-1β and IL-6) by LPS (+) RAW264 cells. The decline of the three cytokine production by Ni (II) ions might stem from the dose-dependent interference (blockage) in the signaling pathway by Ni (II) ions [26]. The mechanism underlying this phenomenon needs to be examined in detail in the future. The increment in two cytokines (TNF-α and IL-6) by LPS (−) RAW264 cells exposed to Ni (II) ions in the concentration range from 0 to 1,000 μmol/L was extremely small compared with those produced by counterpart LPS (+) RAW264 cells that declined with the addition of Ni (II) ion concentration. With LPS stimulation, it was reported that in addition to SOD1 high amounts of SOD2 (Mn) were produced as a stress reaction product against LPS [27]. Although SOD1 and SOD2 were not separately measured in this study, it appeared that Ni (II) ions and LPS increased the SOD1 and SOD2 amounts, respectively, constituting the total SOD amounts (= SOD1 + SOD2). High NO production by LPS (+) RAW264 cells is regulated by the expression of inducible nitric oxide synthetase (iNOS) mRNA, which is present downstream of the NF-kappaB signaling pathway [27]. The Ni (II) ion-dose-dependent decline of NO by LPS (+) RAW264 cells might also be attributable to the dose-dependent interference (block) by Ni (II) ions in the signaling pathway [28] and resultant reduction in the expression of (iNOS) mRNA. The exact mechanism relating to NO production under the influence of both Ni (II) ions and LPS-stimulation is still

unclear and needs to be studied in the future as well. It should be noted at this point that the power of bacteria-disinfection by NO is reduced for LPS (+) RAW264 cells when exposed to Ni (II) ions, dose-dependently. It was postulated that low-dose Ni (II) ions eluted from Ni-containing dental alloys might worsen the clinical condition of patients who suffer from periodontitis, because the disinfectant power of NO against bacteria rapidly declines in a highly Ni(II)-ion-dose-dependent and dose-sensitive manner, and bacteria attack might be accelerated.

In summary: (i) Ni (II) ions dose-dependently slightly increased inflammatory and oxidative stress conditions in the minimum states of LPS (−) RAW264 cells; (ii) LPS-stimulation alone caused cell cycle arrest, and considerably intensified productions of three inflammatory cytokines, SOD and NO for RAW264 cells; and (iii) Ni (II) ions dose-dependently reduced production of three cytokines, increased SOD secretion but decreased the disinfectant power of NO of LPS (+) RAW264 cells.

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