Effects of Ni^{2+} Ions on Cell Viability and NO Production of Murine Peritoneal Exudate Cells (Macrophages) with and without Lipopolysaccharide Stimulation

Masayuki TAIRA¹, Minoru SASAKI², Chiharu YAMAURA-TANAKA², Setsuo SAITOH¹, Shigenobu KIMURA² and Yoshima ARAKI¹

¹Department of Dental Materials Science and Technology, School of Dentistry, Iwate Medical University, 1-3-27 Chuo-dori, Morioka 020-8505, Japan

²Department of Oral Microbiology, School of Dentistry, Iwate Medical University, 1-3-27 Chuo-dori, Morioka 020-8505, Japan
Corresponding author, E-mail:mtaira@iwate-med.ac.jp

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The purpose of this study was to clarify the cytotoxicity of Ni^{2+} ions against murine peritoneal exudate cells (PEC) (macrophages). First, we examined the cell viability of PEC with and without lipopolysaccharide (LPS) stimulation in culture media containing Ni^{2+} ions up to 1000 $\mu\text{mol/L}$. Results showed that the cytotoxicity of Ni^{2+} ions against PEC was dose-dependent and accelerated by LPS stimulation, especially in media with Ni^{2+} ions exceeding 100 $\mu\text{mol/L}$. Second, we measured the production of nitric oxide (NO) from PEC and found that LPS caused the PEC to produce abundant NO. However, high dose of Ni^{2+} ions at concentration more than 200 $\mu\text{mol/L}$ hindered and inhibited NO production. These results pointed out that the cytotoxicity of Ni^{2+} ions against macrophages depended on both the Ni^{2+} ion concentration and the presence of bacteria with LPS. Further, NO – a killer of bacteria – was lost when LPS-stimulated macrophages were exposed to high dose of Ni^{2+} ions.

Key words: Nickel ions, Peritoneal exudate cells, LPS

INTRODUCTION

Ni^{2+} ions are easily released from Ni-containing dental alloys into the body fluids^{1,2)}. Ni^{2+} ions are absorbed by macrophages which play a central role in the body's self-defense system, and tend to accumulate in their nucleus³⁾. DNA damage by Ni^{2+} ions in nucleus (e.g., through bond between Ni ion and DNA⁴⁾) gives rise to cellular damage, inflammatory reaction, cellular death, immune reaction, and eventually, carcinogenesis^{5,6)}. Although cell viability tests confirmed the dose-dependent cytotoxicity of Ni^{2+} ions against various cultured cell lines^{7–9)}, there have been few studies that thoroughly examined the cytotoxicity of Ni^{2+} ions against macrophages.

Gram-negative bacteria often live in the oral environment, adhere to teeth and metallic restorations, and cause inflammatory *in vivo* reactions leading to periodontitis¹⁰⁾. LPS is a large molecule that contains both lipid and carbohydrate (sugar molecules), and is a biologically highly active amphiphilic component of the outer membrane of Gram-negative bacteria. LPS can act as an endotoxin because it can bind to the CD14 receptor on macrophage, stimulating the macrophage to secrete pro-inflammatory cytokines¹¹⁾ and free radicals such as reactive oxygen species (O_2^- and OH^-)^{12,13)} and nitrogen oxide (NO)¹⁴⁾.

Gaseous NO is an important neural signal transducer if the quantity is subtle, but causes detrimental effect on cell viability if the amount is too excessive¹⁵⁾. To protect the cells against intruding bacte-

ria (e.g., Gram-negative bacteria) with LPS ligand on their outer surface, abundant NO is produced as a result¹⁶⁾. This is because when LPS ligand combines with the receptor (e.g., toll-like receptor 4) of macrophage, signal transduction through NF- κ B pathway is activated and causes intensified mRNA expression of inducible nitric oxide synthetase (iNOS), leading to production of abundant NO¹⁷⁾. Nonetheless, the effect of Ni^{2+} ions on the mechanism of NO production by LPS-stimulated macrophages has not been well studied.

Therefore, the purpose of this study was two-fold. First, to examine the dose-dependent effect of Ni^{2+} ions on the cell viability of PEC (mostly macrophages) with and without LPS. Second, to investigate the dose-dependent effect of Ni^{2+} ions on NO production by PEC with and without LPS stimulation. The mRNA expressions of iNOS and three pro-inflammatory cytokines of PEC with and without LPS and Ni^{2+} ions were also examined in selected culture media by RT-PCR. The mRNA expression of iNOS was further evaluated by real-time RT-PCR. These RT-PCR and real-time PCR analyses were carried out to reveal the underlying mechanisms of cell viability and NO production of PEC, since their details are not well clarified yet. Real-time PCR allows accurate quantitative analysis of mRNA expressions, the application of which was pioneered by Hashimoto *et al.*¹⁸⁾ in dental materials research community.

MATERIALS AND METHODS

Cell culture

The PEC was collected by lavage from five male C57BL/6N mice (eight weeks old, CLEA Japan, Osaka, Japan) that had been given 1 ml of Thioglycolate medium (Nisshui, Tokyo, Japan) by intra-peritoneal injection — the detailed procedure of which has been given in another report¹⁹. The PEC was re-suspended at 1×10^7 cells/ml in sterile (0.20 μ m membrane filtered) RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 50 μ g/ml gentamicin (Sigma, St. Louis, MO, USA) and 10% v/v heat-inactivated fetal calf serum (ICN Biomedical Japan, Osaka, Japan). The PEC was then seeded in 24-well microplates at 1 ml for cell viability and NO measurements, and in 100-mm culture dish at 6 ml for total RNA extraction. This was followed by culturing at 37°C in a humidified 5% CO₂ atmosphere for one hour so that cells could adhere to the dishes. Culture medium was then changed to fresh that with and without LPS and Ni²⁺ ions. For LPS stimulation, a commercial LPS (*Escherichia coli*. O26, DIFCO Lab., Detroit, MI, USA) dissolved in sterile phosphate buffered saline solution was added to the RPMI medium at a final concentration of 10 μ g/ml. This high-concentration LPS was selected to stimulate the PEC to actively produce pro-inflammatory cytokines such as TNF α , IL-1 β , and IL-6²⁰. Nickel (II) chloride anhydrous (98% pure, Jyunsei Chemical Co., Tokyo, Japan) was dissolved in super-pure water at a concentration of 0.2 mol/L (Ni²⁺ stock solution) and sterilized by membrane filtration. By diluting this stock solution, RPMI media containing Ni²⁺ ions with concentrations of 0, 2, 20, 50, 100, 200, 500, and 1000 μ mol/L were prepared with and without LPS. Nickel (II) chloride was used here as the source of Ni²⁺ ions because it was widely employed for media containing Ni²⁺ ions in cell viability tests^{18,20}. The pH of the culture media examined was almost constant, lying in the range of 7 and 7.5.

Cell viability tests and NO measurements

For each medium, cell viability tests of the PEC were

carried out (sample number = 6) by the method described below. Briefly, one day after culturing, the media with and without LPS and with Ni²⁺ ions in 24-well microplates were collected as culture supernatant, and replaced with a mixture of 900 μ l fresh original RPMI medium and 100 μ l dye solution (Cell Counting Kit-8, Doujin Chemical Lab., Kumamoto, Japan). The cells in the microplates were incubated for three hours at 37°C in a CO₂ incubator, followed by adding 100 μ l stop solution (0.1 mol/L HCl solution). The stopped 300 μ l solutions were transferred to 96-well microplates, and their optical densities were measured at 450 nm in a microplate reader (Model 680, Biorad Co., Hercules, CA, USA). The viable cell number ratio (%) of the cells in each medium was expressed as their optical density divided by that of the control cells cultured in LPS (–) 0 μ mol/L Ni²⁺ medium.

The NO produced by PEC and secreted into the culture supernatant was measured by the Griess method. Briefly, 100 μ l supernatant was transferred into 96-well microplates, and 100 μ l Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H₃PO₄) (Sigma Aldrich Japan, Tokyo, Japan) was added, thus converting nitrite into a deep purple azo compound. Absorptions were measured at 550 nm using a microplate reader to determine the NO level, using a pre-determined calibration line between NO concentration and its optical absorption²¹.

RT-PCR and real-time quantitative PCR

Total RNA was extracted from the PEC cultured for four hours in four selected media — namely LPS (–) 0 μ mol/L Ni²⁺ ion, LPS (–) 50 μ mol/L Ni²⁺ ion, LPS (+) 0 μ mol/L Ni²⁺ ion, and LPS (+) 50 μ mol/L Ni²⁺ ion — using a reagent (TRIZOL, Invitrogen, Carlsbad, CA, USA). The mRNA expressions of iNOS, TNF α , IL-1 β , IL-6, and β -actin in the PEC were examined by reverse transcriptase (RT)-PCR. RT-PCR was performed as reported in detail in other literature²¹. Briefly, the RNA was reverse transcribed into cDNA using a reverse transcriptase (Superscript II, Invitrogen, Carlsbad, CA, USA). RT

Table 1 Primers for PCR

Primers for		Sequence	Amplicon size (bp)
		5' 3'	
iNOS	F	CCCTTCCGAAGTTCTGGCAGC	494
	R	GGCTGTCAGAGCCCTCGTGGCTTTGG	
TNF α	F	TCTCATCAGTTVTATTGGCCC	212
	R	GGGAGTAGACAAGGTACAAC	
IL-1 β	F	TTGACGGACCCCAAAAGATG	204
	R	AGAAGGTGCTCATGTCTCTCA	
IL-6	F	GTTCTCGGGAAATCGTGGA	208
	R	TGTACTCCAGGTAGCTATGG	
β -actin	F	TGCTGGGCCGCTCTAGGCAC	243
	R	TGGCCTTAGGGTTCAGGGGG	

products were mixed with DNA polymerase (Ampli-Taq Gold, Applied Biosystems, Foster City, CA, USA), self-designed sense and anti-sense primers of iNOS, TNF α , IL-1 β , IL-6, and β -actin (Table 1), $MgCl_2$, and dNTP. Amplicon size of iNOS primer was relatively large at about 500 bp because it was highly specific for PCR. PCR amplification was then performed in sequential cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds using a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA). All samples were amplified for 25 cycles, and the PCR samples were electrophoresed on 2% agarose gels. Bands in the gel were visualized with ethidium bromide by ultra-violet irradiation.

Real-time quantitative PCR (sample number = 3) was further performed to quantitatively identify the amount of iNOS and β -actin mRNAs in the PEC cultured for four hours in the selected media – namely LPS (–) 0 μ mol/L Ni^{2+} ion, LPS (+) 0 μ mol/L Ni^{2+} ion, LPS (+) 50 μ mol/L Ni^{2+} ion, LPS (+) 200 μ mol/L Ni^{2+} ion, and LPS (+) 1000 μ mol/L Ni^{2+} ion – using a machine (Light Cycler, Roche Diagnostics, Tokyo, Japan) and primer/probe sets (484651 for iNOS mRNA and 410933 for β -actin mRNA, Nippon Gene Research Lab., Sendai, Miyagi, Japan).

Statistical analysis

Statistical analysis was done among the means with pooled Student's t-test.

RESULTS

Fig. 1 shows the cell viability of PEC cultured for one day in the media with LPS (black column) and without LPS (white column) and with varying Ni^{2+} ion concentrations (in horizontal axes). It became apparent that Ni^{2+} ions had dose-dependent cytotoxicity, and that LPS (LPS (+)) accelerated the cytotoxic effect of Ni^{2+} ions especially in the concentration range of more than 100 μ mol/L.

Fig. 2 indicates the NO production by PEC cultured for one day in the media with LPS (black column) and without LPS (white column) and with varying Ni^{2+} ion concentrations (in horizontal axes). It became evident that LPS (LPS (+)) induced significant amount of NO from the PEC, in comparison with LPS (–) PEC. The NO production of LPS (+) PEC was high at 0 μ mol/L Ni^{2+} ion, increased with the addition of Ni^{2+} ion concentration from 0 to 50 μ mol/L, peaked at 50 μ mol/L Ni^{2+} ion, then gradually declined as Ni^{2+} ion concentration increased from 50 to 200 μ mol/L, and virtually vanished when Ni^{2+} ion concentration was more than 500 μ mol/L.

Fig. 3 shows the RT-PCR results of PEC cultured

Fig. 1 Cell viability of murine peritoneal exudate cells (PEC) cultured for 1 day in media with LPS (black column) and without LPS (white column) and with varying Ni^{2+} ion concentrations (in horizontal axes). a indicates significant difference between LPS (–) 0 μ mol/L Ni^{2+} ion (*) and each LPS (–) at varying Ni^{2+} ion concentrations ($p < 0.01$). b indicates significant difference between LPS (+) 0 μ mol/L Ni^{2+} ion (#) and each LPS (+) at varying Ni^{2+} ion concentrations ($p < 0.01$).

Fig. 2 NO production of murine peritoneal exudate cells (PEC) cultured for 1 day in media with LPS (black column) and without LPS (white column) and with varying Ni^{2+} ion concentrations (in horizontal axes). c indicates significant difference between LPS (+) 0 μ mol/L Ni^{2+} ion (\$) and each LPS (+) at varying Ni^{2+} ion concentrations ($p < 0.01$).

Fig. 3 RT-PCR results of murine peritoneal exudate cells (PEC) cultured in media with and without LPS and with Ni²⁺ ion concentrations of 0 and 50 μmol/L, indicating the five mRNA expressions of β-actin, iNOS, TNF α, IL-1 β, and IL-6.

in the media with and without LPS and with Ni²⁺ ion concentrations of 0 and 50 μmol/L. The four peak band intensities of β-actin virtually coincided, implying that the total RNA collected from PEC cultured in the four culture media – namely LPS (–) 0 μmol/L Ni²⁺ ion, LPS (–) 50 μmol/L Ni²⁺ ion, LPS (+) 0 μmol/L Ni²⁺ ion, and LPS (+) 50 μmol/L Ni²⁺ ion – had similar concentration. The mRNA expression of iNOS was facilitated solely by LPS stimulation, and LPS (+) 50 μmol/L Ni²⁺ ion produced a stronger iNOS mRNA expression than that of LPS (+) 0 μmol/L Ni²⁺ ion. No iNOS mRNA expressions were found in the PEC cultured in LPS (–) 0 μmol/L Ni²⁺ ion and LPS (–) 50 μmol/L Ni²⁺ ion. The mRNA expression of TNF α was significantly up-regulated by LPS stimulation (LPS (+)) (i.e., LPS (+) 0 μmol/L Ni²⁺ ion and LPS (+) 50 μmol/L Ni²⁺ ion), but weakly indicated in LPS (–) 0 μmol/L Ni²⁺ ion and LPS (–) 50 μmol/L Ni²⁺ ion. The mRNA expressions of IL-1 β and IL-6 were also considerably up-regulated by mere LPS stimulation (LPS (+)) with Ni²⁺ ion concentration at 0 or 50 μmol/L, but distinctly absent from the PEC cultured in LPS (–) media with Ni²⁺ ion concentrations of 0 and 50 μmol/L. It should be noted here that the dose dependency of Ni²⁺ ions was observed only on iNOS mRNA of LPS (+) PEC, and not so for the other three pro-inflammatory cytokines (TNF α, IL-1 β, and IL-6) of LPS (+) PEC. Therefore, we displayed five mRNA expressions of the PEC with and without LPS stimulation and with Ni²⁺ ion concentrations of 0 and 50 μmol/L representatively (Fig. 3), while pointing out that LPS (+) 50 μmol/L Ni²⁺ ion produced a larger mRNA expression of iNOS than LPS (+) 0 μmol/L Ni²⁺ ion.

Fig. 4 shows the results of real-time quantitative RT-PCR, displaying the copy number ratios of iNOS mRNA/β-actin mRNA of the PEC cultured for four hours in five media: LPS (–) 0 μmol/L Ni²⁺ ion, LPS (+) 0 μmol/L Ni²⁺ ion, LPS (+) 50 μmol/L Ni²⁺ ion, LPS (+) 200 μmol/L Ni²⁺ ion, and LPS (+) 1000

Fig. 4 Results of real-time quantitative RT-PCR, indicating the copy number ratios of iNOS mRNA/β-actin mRNA of murine peritoneal exudate cells (PEC) cultured for 4 hours in LPS (–) 0 μmol/L Ni²⁺ ion medium, LPS (+) 0 μmol/L Ni²⁺ ion medium, LPS (+) 50 μmol/L Ni²⁺ ion medium, LPS (+) 200 μmol/L Ni²⁺ ion medium, and LPS (+) 1000 μmol/L Ni²⁺ ion medium. d indicates significant difference between LPS(+) 0 μmol/L Ni²⁺ ion (@) and each LPS (+) at varying Ni²⁺ ion concentrations (p<0.01).

μmol/L Ni²⁺ ion. It was confirmed that iNOS mRNAs were induced only when PEC was stimulated by LPS, and that LPS (+) 50 μmol/L Ni²⁺ ion exhibited the highest iNOS mRNA expression, followed by LPS (+) 0 μmol/L Ni²⁺ ion and LPS (+) 200 μmol/L Ni²⁺ ion in this order, whilst LPS (+) 1000 μmol/L Ni²⁺ ion exhibited the lowest. These iNOS mRNA expressions of PEC cultured for four hours in the five media (Fig. 4) coincided relatively well with the NO produced by PEC cultured for one day in the

same five media (Fig. 2).

DISCUSSION

The results obtained here clearly demonstrated that (i) Ni^{2+} ions had dose-dependent cytotoxicity on PEC with and without LPS stimulation; and (ii) this cytotoxicity was accelerated by LPS stimulation when Ni^{2+} ion concentration was more than 100 $\mu\text{mol/L}$. The mechanism of the accelerated cytotoxicity of Ni^{2+} ions by LPS was not clearly revealed in this study. We, however, speculated that this accelerated cytotoxicity might occur due to an interaction between the dual phenomena of DNA damage (caused by Ni^{2+} ion's accumulation in the nucleus³⁾) and cellular inflammatory response (represented by mRNA expressions of pro-inflammatory cytokines such as $\text{TNF } \alpha$, $\text{IL-1 } \beta$, and IL-6 ¹⁹⁾ (Fig. 3)), which are associated with cell cycle arrest¹⁶⁾, apoptosis, and necrosis⁶⁾.

It should be also noted here that NO did not directly cause the accelerated cytotoxicity of Ni^{2+} ions on LPS (+) PEC, because NO production disappeared when PEC was cultured in media with LPS and with Ni ion concentrations exceeding 500 $\mu\text{mol/L}$ (Figs. 1 and 2). Other free radicals such as O_2^- and OH^- and their derivatives^{12,13)}, however, might have been much generated from the PEC cultured in media with LPS and with Ni ion concentrations of more than 500 $\mu\text{mol/L}$, thus causing the accelerated cytotoxicity. When combined with superoxide (O_2^-), NO changes into a derivative such as peroxynitrite, which causes severe damage of the cells¹⁴⁾. NO alone is less toxic¹⁴⁾. Superoxide dismutase (SOD) is known as a scavenger that erases superoxides (e.g., O_2^-)²²⁾. In the body's natural self-defense reaction against bacteria, macrophages not only release free radicals (e.g. O_2^- , OH^- , and NO), but also secrete SOD so that the interior of the cell could be protected while attempting to kill the bacteria²²⁾. We speculated that SOD secretion and its enzyme activity might be reduced in the PEC cultured in LPS (+) media with Ni^{2+} ion concentrations of more than 100 $\mu\text{mol/L}$. Such adverse effect seemed to intensify when Ni^{2+} ion concentration was increased. If SOD activity were to decrease and hence superoxide activity increased, apoptosis via activation of caspase cascade might occur, thereby reducing cell viability²³⁾. On the other hand, macrophage without LPS could tolerate the cytotoxicity of Ni^{2+} ions up to the concentration of 500 $\mu\text{mol/L}$ (Fig. 1) with the aid of SOD whose activity might not be hindered by inflammatory cytokines including $\text{IL-1 } \beta$ and NO²⁴⁾. On this note, it is strongly expected that these aspects of the PEC will be further evaluated in the near future.

With LPS stimulation, it was confirmed that much NO was released from the PEC, as indicated by the intensified iNOS mRNA expression¹⁵⁾ (Figs. 2 and

4). At 24 hours after PEC was cultured in media with LPS and with Ni^{2+} ion concentrations from 0 to 50 $\mu\text{mol/L}$, NO production was increased (Fig. 2). The addition of 50 $\mu\text{mol/L}$ Ni^{2+} ion predominantly increased the iNOS mRNA expression (Figs. 3 and 4). We preclude that Ni^{2+} ions in the concentration range of less than 50 $\mu\text{mol/L}$ might up-regulate a multi-step NF- κ B signaling pathway²⁵⁾ for LPS (+) PEC. This detailed mechanism is, however, not well understood, and needs to be further uncovered.

We extracted total RNA from the PEC after four hours of culturing, and examined the NO amount secreted from the PEC after one day of culturing. This was so done because the peak mRNA expression periods of iNOS, $\text{TNF } \alpha$, $\text{IL-1 } \beta$, and IL-6 usually appear four hours later following the onset of LPS stimulation. As for actual NO and cytokine secretions, they usually peak one day after the start of LPS stimulation²¹⁾.

In the oral cavity, many bacteria with LPS live in dental plaque. Macrophages are, therefore, easily activated *in vivo*¹⁵⁾. Ni^{2+} ions, once much released from Ni-containing alloys into the oral fluid, have an adverse effect on LPS-stimulated macrophages by reducing cell viability and NO production. Caution must be exercised when clinically treating patients suffering from periodontitis with Ni-containing alloys, because Ni^{2+} ions cause the self-sterilizing power with NO to be weakened or lost.

The PEC usually consists of about 90% macrophage and 10% other cells (T-cells, natural killer T-cells, etc.)²⁶⁾. The PEC's biological reaction against LPS and Ni^{2+} ions does not represent pure macrophage reaction, but appears to simulate more of *in vivo* systematic macrophage reaction than that of mouse macrophage cell line (e.g., RAW264)²⁷⁾.

When testing the cytotoxicity of dental materials and their elutes in the future, it is strongly recommended to use LPS-stimulated PEC or macrophage cell line so that the roles of inflammatory reaction and oxidative stress can be better considered. Wataha *et al.*^{7,28)} studied the cytotoxicity of Ni ions on LPS-stimulated monocyte, a precursor of the macrophage. Alternatively, LPS stimulation can be substituted by interferon- γ stimulation²⁹⁾ for macrophage and monocyte. These studies are becoming increasingly important in assessing the inflammatory reaction, allergy, and carcinogenesis caused by Ni^{2+} ions.

CONCLUSION

We examined the dose-dependent effect of Ni^{2+} ions up to 1000 $\mu\text{mol/L}$ on the cell viability of and production of NO by murine peritoneal exudate cells (macrophages) with and without LPS stimulation. The following results were obtained, and related speculations were drawn.

Without LPS stimulation, the dose-dependent cytotoxicity of Ni^{2+} ions was low and NO was not produced. Superoxide produced by intake of Ni^{2+} ions could have been effectively erased by superoxide dismutase (SOD).

With LPS stimulation, the dose-dependent cytotoxicity of Ni^{2+} ions was high especially when Ni^{2+} ion concentration was more than 100 $\mu\text{mol/L}$. In addition, high amounts of NO were produced in Ni^{2+} ion concentrations of less than 100 $\mu\text{mol/L}$, and mRNAs of three pro-inflammatory cytokines ($\text{TNF}\alpha$, $\text{IL-1}\beta$, and IL-6) were strongly expressed. NO production was confirmed to be regulated by iNOS mRNA expression. When exposed to both LPS and a high dose of Ni^{2+} ions, macrophage will lack gaseous NO and lose the power to kill invading bacteria, thus causing the inflammatory condition in the patient to exacerbate. It was hinted that absorption of Ni^{2+} ions might decrease the SOD activity of LPS (+) macrophage by DNA damage and inflammatory cytokines, leading to reduced cell viability.

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REFERENCES

- Jensen CS, Lisby S, Baadsgaard O, Byrjalsen K, Menne T. Release of nickel ions from stainless steel alloys in dental braces and their patch test reactivity in nickel-sensitive individuals. *Contact Dermatitis* 2003; 172: 1926-1934.
- Yonekura Y, Endo K, Iijima M, Ohno H, Mizoguchi I. *In vitro* corrosion characteristics of commercially available orthodontic wires. *Dent Mater J* 2004; 23: 197-202.
- Edwards DL, Wataha JC, Hanks CT. Uptake and reversibility of uptake of nickel by human macrophages. *J Oral Rehabil* 1998; 25: 2-7.
- Hartzell B, McCord B. Effect of divalent metal ions on DNA studied by capillary electrophoresis. *Electrophoresis* 2005; 26: 1046-1056.
- Thierse HJ, Moulon C, Allespach Y, Zimmermann B, Doetze A, Kuppig S, Wild D, Herberg F, Weltzien HU. Metal-protein complex-mediated transport and delivery of Ni^{2+} to TCR/MHC contact sites in nickel-specific human T cell activation. *J Immunol* 2004; 172: 1926-1934.
- Kawanishi S, Oikawa S, Inoue S, Nishino K. Distinct mechanisms of oxidative DNA damage induced by carcinogenic nickel sulfide and nickel oxides. *Environ Health Perspect* 2002; 110: 789-791.
- Wataha JC, Hanks CT, Craig RG. The *in vitro* effects of metal cations on eukaryotic cell metabolism. *J Biomed Mater Res* 1991; 25: 1133-1149.
- Yamamoto A, Honma R, Sumita M. Cytotoxicity evaluation of 43 metal salts using murine fibroblasts and osteoblastic cells. *J Biomed Mater Res* 1998; 39: 331-340.
- Taira M, Toguchi MS, Hamada Y, Takahashi J, Itou R, Toyosawa S, Ijyuin N, Okazaki M. Studies on cytotoxic effect of nickel ions on three cultured fibroblasts. *J Mater Sci Mater Med* 2001; 12: 373-376.
- Dumitrescu AL, Abd-El-Aleem S, Morales-Aza B, Donaldson LF. A model of periodontitis in the rat: effect of lipopolysaccharide on bone resorption, osteoclast activity, and local peptidergic innervation. *J Clin Periodontol* 2004; 31: 596-603.
- Wang PL, Ohura K, Fujii T, Oido-Mori M, Kowashi Y, Kikuchi M, Suetsugu Y, Tanaka J. DNA microarray analysis of human gingival fibroblasts from healthy and inflammatory gingival tissues. *Biochem Biophys Res Commun* 2003; 305: 970-973.
- Kasprzak KS, Sunderman FW, Salnikow K. Nickel carcinogenesis. *Mutat Res* 2003; 533: 67-97.
- Cavallo D, Ursini CL, Setini A, Chianese C, Piegari P, Perniconi B, Iavicoli S. Evaluation of oxidative damage and inhibition of DNA repair in an *in vitro* study of nickel exposure. *Toxicol In Vitro* 2003; 17: 603-607.
- Joshi S, Hasan SK, Chandra R, Husain MM, Srivastava RC. Scavenging action of zinc and green tea polyphenol on cisplatin and nickel induced nitric oxide generation and lipid peroxidation in rats. *Biomed Environ Sci* 2004; 17: 402-409.
- Brennan PA, Thomas GJ, Langdon JD. The role of nitric oxide in oral disease. *Arch Oral Biol* 2003; 48: 93-100.
- Vadiveloo PK, Keramidaris E, Morrison WA, Stewart AG. Lipopolysaccharide-induced cell cycle arrest in macrophages occurs independently of nitric oxide synthetase II induction. *Biochem Biophys Acta* 2001; 1539: 140-146.
- Royle MC, Totemeyer S, Alldridge LC, Maskell DJ, Bryant CE. Stimulation of toll-like receptor 4 by lipopolysaccharide during cellular invasion by live *Salmonella typhimurium* is critical but not exclusive event leading to macrophage responses. *J Immunol* 2003; 170: 5445-5454.
- Hashimoto Y, Ueda A, Nakamura M. Evidence that HSP70 gene expression may be useful for assessing the cytocompatibility of dental biomaterials. *Dent Mater J* 2004; 23: 184-189.
- Takada H, Kimura S, Hamada S. Induction of inflammatory cytokines by a soluble moiety prepared from an enzyme lysate of *Actinomyces viscosus* cell walls. *J Med Microbiol* 1993; 38: 395-400.
- Wataha JC, Ratanasathien S, Hanks CT, Sun Z. *In vitro* $\text{IL-1}\beta$ and $\text{TNF}\alpha$ release from THP-1 monocytes in response to metal ions. *Dent Mater* 1996; 12: 322-327.
- Sasaki M, Ohara-Nemoto Y, Tajika S, Kobayashi M, Yamaura C, Kimura S. Antigenic characterization of a novel *Streptococcus agalactiae* antigen that induces

- nitric oxide synthesis by murine peritoneal exudates cells. *J Med Microbiol* 2001; 50: 952-958.
- 22) Brockhaus F, Brune B. Overexpression of Cu/Zn superoxide dismutase protects RAW264.7 macrophages against nitric oxide cytotoxicity. *Biochem J* 1999; 338: 295-303.
- 23) Kim JS, Kwon HY, Choi WH, Jeon CY, Kim JI, Kim J, Lee JY, Kim YS, Park JB. Phagocytosis of serum- and IgG-opsonized zymosan particles induces apoptosis through superoxide but not nitric oxide in macrophage J774A1.1. *Exp Mol Med* 2003; 35: 211-221.
- 24) Fakhrzadeh L, Laskin JD, Gardner CR, Laskin DL. Superoxide dismutase-overexpressing mice are resistant to ozone-induced tissue injury and increases in nitric oxide and tumor necrosis factor- α . *Am J Respir Cell Mol Biol* 2004; 30: 280-287.
- 25) Leiro J, Arranz JA, Yanez M, Ubeira FM, Sanmartin ML, Orallo F. Expression profiles of genes involved in the mouse nuclear factor-kappa B signal transduction pathway are modulated by mangiferin. *Int Immunopharmacol* 2004; 4: 763-778.
- 26) Tominaga K, Saito S, Matsuura M, Funatogawa K, Matsumura H, Nakano M. Role of IFN- γ on dissociation between nitric oxide and TNF/IL-6 production by murine peritoneal cells after re-stimulation with bacterial lipopolysaccharide. *J Leukoc Biol* 1999; 66: 974-980.
- 27) Virca GD, Kim SY, Glaser KB, Ulevitch RJ. Lipopolysaccharide induces hyporesponsiveness to its own action in RAW 264.7 cells. *J Biol Chem* 1989; 264: 21951-21956.
- 28) Wataha JC, Lewis JB, Volkmann KR, Lockwood PE, Messer RLW, Bouillaguet S. Sublethal concentration of Au (III), Pd (II), and Ni (II) differentially alter inflammatory cytokine secretion from activated monocytes. *J Biomed Mater Res Part B Appl Biomater* 2004; 69B: 11-17.
- 29) Flesch IE, Schwamberger G, Kaufmann SH. Fungicidal activity of IFN- γ -activated macrophages. Extracellular killing of *Cryptococcus neoformans*. *J Immunol* 1989; 142: 3219-3224.