Studies on cytotoxic effect of nickel ions on three cultured fibroblasts

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Cytotoxicity of Ni ions on three fibroblasts such as L929, Balb/3T3 clone A31 and MC3T3–E1 were examined by cell count (CC) and Neutral Red assay (NR). Three cells were incubated for 6 days in 1 ml DME medium containing Ni ions which ranged from 0 to 2 mM/l. The results clarified that Ni ions had dose-dependent cytotoxicity. L929 possessed the largest TC_{50} values (the amount of Ni ion that caused 50% cell death or 50% cell viability) of 0.12 mM/l (CC) and 0.32 mM/l (NR), and Balb/3T3 clone A31 had the least values of 0.05 mM/l (CC) and 0.09 mM/l (NR), whilst MC3T3–E1 had the intermediate values of 0.08 mM/l (CC) and 0.15 mM/l (NR). The dissolution of Ni ions from Ni-containing metallic restorations must be lower than these concentration levels so that body tissues might not be severely damaged.

1. Introduction

Although nickel (Ni) is known to be cytotoxic and carcinogenic to the human body [1], many metallic restorations still include nickel as a constituent such as stainless steel orthodontic wire [2], Ti-Ni super-elastic alloy (Nitinol) orthodontic wire [3] and Ni-Cr-based alloys for crown and bridge [4]. It appears, therefore, still necessary to evaluate cytotoxicity of nickel.

Dissolution of nickel ions into the body fluid from Ni-containing alloys is difficult to control [5], because this phenomenon is complicated, dependent upon corrosion, mechanical damage and wear process [6]. For simplicity, it appeared practical to use a nickel standard solution for atomic absorption spectrophotometer so that data of cytotoxicity of nickel ions released into the liquid medium could be universal and easily reproducible [7]. Selection of a cell for cytotoxicity measurement was also an important factor to consider. Most researchers have utilized a few cells for cytotoxicity tests such as Hela cell [8], L929 cell [9] and Balb/3T3 clone A31 cell [10]. MC3T3-E1 cell has been utilized as osteoblastic-like (fibroblast) cell for bone studies [11].

The purpose of this study was, therefore, to examine the cytotoxicity of nickel ions using three cells such as L929, Balb/3T3 clone A31 and MC3T3–E1 by two experimental procedures, namely cell count (CC) and Neutral Red assay (NR), and to clarify the cytotoxic concentration of nickel ions against these three mammalian fibroblasts (represented by TC_{50}). Hela cell was eliminated because it was derived from a cancerous human tumor cell while the other three selected cells were obtained from normal mouse cells and presumably regarded as normal cell lines.

2. Materials and methods

2.1. Cells used

Three cells were purchased from an authorized cell and gene bank in Japan (Riken Cell Bank, Ibaraki, Japan). The registered cell and lot numbers for the cells were as follows: RCB0081 and Lot No. 012 for L929, RCB0005 and Lot No. 007 for Balb/3T3 clone A31, and RCB1126 and Lot No. 008 for MC3T3–E1.

2.2. Medium preparation with Ni ions

Original medium consisted of Dulbecco's modified Eagle (DME) medium (Cat. No. 11885–084, Life Technologies, NY, USA) supplemented with 10% (V/V) fetal bovine serum (Cat. No. 26140–079, Lot No. 1008629, Life Technologies, NY, USA) and 2% (V/V) antibiotics (penicillin-streptomycin dissolved in 80 ml isotonic saline solution, Cat. No. 15145–014, Life Technologies, NY, USA).

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Ni ion concentration in the DME medium to be checked out were pre-selected to be 0, 0.02, 0.2, 0.5, 1 and 2 mM/l (0 : 1 : 10 : 25 : 50 : 100 ratio). To yield the medium containing the maximum 2 mM/l Ni ion, a Ni standard (1000 mg Ni ion /l, dissolved in 0.1 M/l · HNO_3 solution) (Ni 1000, Cat. No. 147–06461, Wako Jyunyaku, Osaka, Japan) and 8% sodium bicarbonate ($NaHCO_3$) solution were dropwise and stepwise added to the original DME medium and vigorously stirred while maintaining pH of the mixed solution at 7.5 at 37 °C. By diluting the medium containing 2 mM/l Ni ion by original medium, other medium containing four different Ni ion concentrations were also prepared.

2.3. Cell culture

Three cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells were sub-cultured by trypsinization (0.05% trypsin and 0.025% EDTA) before confluence. Prior to cytotoxic studies, cells were seeded into 24-well microplates (Cat. No. 3820, Iwaki Glass, Tokyo, Japan) at a density of 5000 in 1 ml original DME medium and cultured for one day. For each cell count and neutral red staining study, three samples were prepared, respectively.

Next day, the medium were exchanged with original medium (control) and five different Ni ion containing mediums, and three cells examined had been cultured for 6 days.

2.4. Cell count and cell viability by neutral red assay

Six days after medium exchange, cells were trypsinized and the number of each well was counted three times using an inverted phase-contrast light microscope (CK30, Olympus, Tokyo, Japan) and a haemocytometer (Neubauer 03–202–1, Erma, Tokyo, Japan). Cytotoxicity by cell count was judged by the amount of Ni ions that caused 50% cell death (TC_{50}) with respect to the cell number of the control medium without Ni ions.

Cell viability was recorded by means of neutral red assay, which was based on the uptake and lysosomal accumulation of the supravital dye [12]. Briefly, the medium with or without Ni ions were exchanged with 1 ml DME medium containing 50 µl/ml neutral red (Catalog number 15330–079, Life Technologies, NY, USA). After a 3 h incubation period (37 °C, 5% CO_2), the supernatant was removed, and the intracellularly stored neutral red was solubilized in 1 ml dissolving solution consisting of 50% H_2O, 49% C_2H_5OH and 1% CH_3COOH for 1 h at room temperature. Optical density was then measured at 540 nm in an ultraviolet-visible light spectrophotometer (UV-2500PC, Shimadzu Co., Kyoto, Japan). Cell viability (%) was expressed by optical density values derived from the cells in contact with Ni ions, divided by that from control cells without Ni contact (100% represented zero cytotoxicity and lower values indicated cytotoxicity). Cytotoxicity was also expressed by the amount of Ni ions that caused 50% cell viability (TC_{50}).

3. Results

Fig. 1 shows the effect of Ni ion concentration in DME medium on the cell number of three fibroblasts. The cell number was plotted in a log-scale vertical axis. For all three cells, incrementing Ni ion concentration resulted in the decline in the cell number. The cells did not exist in the medium containing more than 1 mM/l Ni ion.

Fig. 2 indicates the effect of Ni ion concentration in DME medium on the cell viability of three fibroblasts. The cell viability was depicted in a normal vertical scale. For all three cells, increasing Ni ion concentration brought about the reduction in the cell viability. The cell viability was not recorded in the medium containing more than 1 mM/l Ni ion.

Fig. 3 illustrates the relationship between the Ni ion concentration and the cell number of L929 cell. There existed a negative linear correlation. The TC_{50} value for L929 was the abscissa corresponding to the intersection between thick data line and (horizontal) 50% cell number line, and estimated as 0.12 mM/l Ni ion. Table I summarizes TC_{50} values of Ni ions in three fibroblasts under two different experimental conditions, such as cell count and cell viability by neutral red assay. TC_{50} values (expressed by mM/l Ni ion) by NR tended to exceed those by CC. L929 had the largest TC_{50} values of 0.12 (CC) and 0.32 (NR), Balb/3T3 clone A31 had the least values of 0.05 (CC) and 0.09 (NR), while MC3T3–E1 had the intermediate values of 0.08 (CC) and 0.15 (NR).

4. Discussion

Three cells examined became sub-confluent in control medium without Ni ions for 6 day incubation following medium exchange. During 6 day incubation, the medium was not exchanged with fresh medium, keeping the culture environment in a closed condition. The color appearance of phenol red added in the medium suggested that pH of all medium did not drop below 6.5 even after 6 day incubation. This pH condition was pre-requisite for physiologically sound cell culture. Beyond 6 days, three cells started to die due to contact inhibition and pH drop.

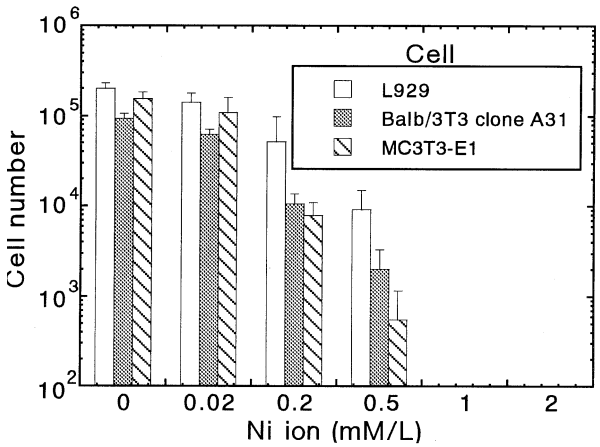

Figure 1 Effect of Ni ion concentrations in DME medium on cell number of three fibroblasts.

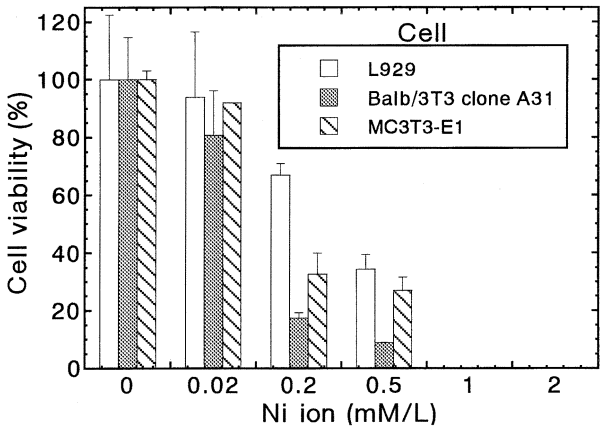

Figure 2 Effect of Ni ion concentrations in DME medium on cell viability (by neutral red assay) of three fibroblasts.

Cytotoxicity of metallic ions has been defined by the amount of metallic elements that caused 50% cell death or 50% cell viability (TC_{50}) [13]. The TC_{50} values of Ni ions has been most widely reported for L929 cells, which ranged roughly from 0.1 to 0.4 mM/l [13–15]. These values agreed well with the results obtained in this study for L929 cell (Table I), proving the validity of this study.

TC_{50} values differed among three cells and between two measuring methods. Such variation must be well considered to interprete *in vitro* cytotoxicity test results into a clinical situation for human patients. For safety reasons, it might be better to adapt the least TC_{50} value (i.e. that obtained for Balb/3T3 clone A31 by cell count) as a standard of cytotoxicity of Ni ions. It was not surprising to see that the TC_{50} values of Ni ions by cell count (CC) were smaller than those by cell viability (NR) [13]. The difference might lie within the allowed level.

Referring to clinics, it could be speculated that the dissolution level of Ni ions from Ni-containing metallic restorations must be lower than the TC_{50} values obtained so that tissue damage such as gingivitis and allergy in the direct vicinity of the restorations [16] could be prevented.

One important aspect to consider is the method to provide Ni ions to the medium. From metal, it was possible to elute Ni ions by simple immersion, mechanical scratch and corrosion testing [6]. This approach was, however, laborious and time-consuming because chemical analyses of the medium must be carried out. Nickel salt such as nickel chloride and nickel sulfate could provide Ni ions when dissolved in the medium [14]. Nickel (II) compound coupled with these anionic counterparts were also known as strong carcinogens [1], and were more cytotoxic than the metal extract [14]. It appeared that Ni ions standard solution might exert a more mild cytotoxic effect while medium preparation with Ni ions was much easier.

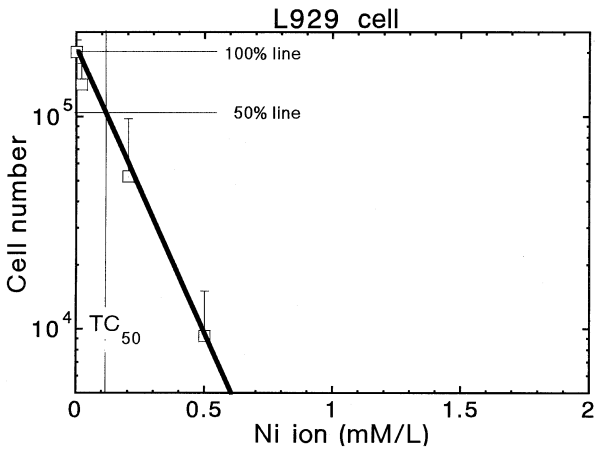

Figure 3 Relationship between Ni ion concentrations and cell number of L929 fibroblast. Note: TC_{50} value was graphically obtained as 0.12 mM/l Ni ions.

TABLE I TC_{50} values of Ni ions in three fibroblasts under two different experimental conditions (mM/l Ni ions)

Cell	Experimental condition	
	Cell count (CC)	Cell viability by neutral red assay (NR)
L929	0.12	0.32
Balb/3T3 clone A31	0.05	0.09
MC3T3-E1	0.08	0.15

The reason of cyotoxicity of Ni ions might be attributed to two major factors. Ni ions may bind to DNA-repair enzymes and hinder the cell mitosis by disturbing DNA repair, replication and recombination [17, 18]. Ni ions also generate oxygen-free radicals to cause protein degradation *in situ* [17]. Such adverse effects might be proportional to Ni ion concentrations in the medium. Research in these areas is highly expected, but beyond the scope of this study.

5. Conclusion

Cytotoxicity of Ni ions on three fibroblasts such as L929, Balb/3T3 clone A31 and MC3T3–E1 was examined by cell count and cell viability (by means of Neutral Red assay) using DME medium containing Ni ions which were supplied from a nickel standard and ranged from 0 to 2 mM/l. Cytotoxicity was evaluated by TC_{50} values. The following conclusions were obtained.

(1) Ni ions had dose-dependent cyctotoxicity for all three cells.

(2) L929 had the largest TC_{50} values, followed by MC3T3–E1, while Balb/3T3 clone A31 had the least.

(3) TC_{50} values by cell viability tended to exceed those by cell count.

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