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Cytotoxicity due to corrosion of ear piercing studs

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Abstract—It is well known that allergic and/or inflammatory reactions can be elicited from the use of gold-coated studs, particularly the type used for piercing ears, since they are left in contact with body fluids until the puncture heals. Inasmuch as gold is known as a non-toxic element, other elements of the substrate material may be responsible for some allergies. Therefore, characteristics of the coating, such as defects that expose the substrate to the human skin or body fluids, play an important role in the development of skin sensitization. In this study, the cytotoxicity of commercial studs used for ear piercing and laboratory-made studs was determined in a culture of mammalian cells. The corrosion performance of the studs was investigated by means of weight loss measurements and electrochemical impedance spectroscopy. The elements that leached out into the medium were also analysed by instrumental neutron activation analysis. Further, the surfaces of the studs were examined by scanning electron microscopy and analysed by energy dispersive spectroscopy to identify defects and reaction products on the surface, both before and after their exposure to the culture medium. The stud which showed lower corrosion performance resulted in higher cytotoxicity. Ti showed no cytotoxicity and high corrosion resistance, proving to be a potential material for the manufacture of ear piercing studs.

Keywords: cytotoxicity; allergic reactions; corrosion; ear piercing studs.

Abbreviations: PBS–CMF = calcium and magnesium free phosphate saline buffer; EDS = energy dispersive spectroscopy; EIS = electrochemical impedance spectroscopy; INAA = instrumental neutron activation analysis; MEM = minimum Eagle's medium; MEM–FCS = minimum Eagle's medium-foetal calf serum; Pf = gold-coated copper–zinc alloy; SCE = saturated calomel electrode; SEM = scanning electron microscopy; St = stainless-steel; Ti = titanium.

INTRODUCTION

Allergic contact dermatitis to metals is a common skin disease in many countries. Nickel sensitization is the most frequent (Bordji *et al*., 1996; Breitstadt, 1992; Ikarashi *et al*., 1996; Rynänen *et al*., 1997; Yang and Merrit, 1994), and it was initially acknowledged to be induced by ear piercing (Breitstadt, 1992). The prevalence of nickel sensitization has been reported in 0.8–2% of men and 9.5–16% of women, and cobalt sensitization in 3.0–4.9% of men and in 4.5–9.6% of women. Overall, nickel/cobalt sensitization has increased as the habit of piercing earlobes has increased in popularity (Meijer *et al*., 1995).

Stainless-steels, particularly biomedical AISI 316L, are widely used as stud substrates since they have demonstrated good biocompatibility, have adequate physical and mechanical properties and can be moulded into a variety of shapes and sizes. One of the main limitations to their clinical use is their tendency to corrode in the presence of chlorides, considering that it contains significant amounts of chromium (16–18 wt%) and nickel (10–14 wt%), elements well known for their carcinogenic and toxic effects, respectively (Bordji *et al*., 1996). However, they still retain an important place in the manufacture of gold-coated studs. Gold has been generally accepted to have little or no toxicity (Wataha *et al*., 1991), although some gold compounds may be toxic or allergenic to some people.

Therefore, the coating should be as defect-free as possible and its adherence to the substrate adequate, to avoid contact between the substrate elements and body fluids while the pierced earlobes are healing.

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The presence of defects in gold coatings, on studs used for ear piercing, would allow contact between the stud substrate and body fluids. A corrosion reaction could occur as a consequence of this interaction, causing the release of metal ions. These metal ions bind to tissue and interstitial fluid proteins as soon as they are released from the metallic substrate. Nickel is the major cause of allergic contact dermatitis, this being caused by Ni^{2+} ions, which bind to carrier protein and this nickel–protein complex activates immune reactions (Rynänen *et al.*, 1997).

The high incidence of contact allergy to nickel is due not only to the sensitization capacity, but also to the frequency or repeated exposure to various nickel products. It is well known that the release of a certain amount of metal ions from metal products cannot be avoided. Nevertheless, skin reactions will not occur if the amount released does not exceed the elicitation concentration of the chemical in the person (Ikarashi *et al.*, 1996).

To avoid allergic skin reactions, many proposals have been made, such as replacing the nickel or coating it with white bronze (Breitstadt, 1992). Wright and Gallant, studying the corrosion behaviour and cytotoxicity of Au–Cu–Ag alloy, concluded that there is a direct correlation between corrosion properties and cytotoxic response (Wright and Gallant, 1982). The ready dissolution of copper in the culture medium has been acknowledged as a great contributor to the cytotoxicity of alloys containing copper (Craig and Hanks, 1990). In practice, it has been found that substrates containing nickel are still used for making gold-coated studs.

This study reports the results of laboratory investigations carried out to evaluate the cytotoxicity and corrosion performance of both commercial gold-coated ear piercing studs, with a copper–zinc based alloy and stainless-steel as substrates, and titanium studs.

MATERIALS AND METHODS

Materials

Three types of ear piercing studs have been studied: gold coated austenitic stainless-steel (St); gold coated copper–zinc alloy (Pf) and titanium (Ti). Fig. 1 shows the macrograph of one of the three types of studs. The compositions of the materials used as substrates were determined by instrumental neutron activation analysis (INAA) and the results are given in Table 1. In these analyses, the stems and butterfly backs of studs were analysed individually after removing the gold coating.

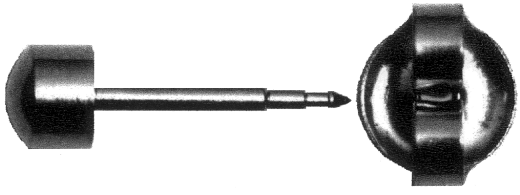

Fig. 1. Macrograph of one of the tested studs.

Preparation of extract

According to the International Standards Organization ISO 10993–part 5 (ISO, 1992), the ratio between the surface area of the material and the volume of extraction vehicle should be between 0.5 cm^2/ml and 6 cm^2/ml. Hence, in this study, the ratio used was 0.53 cm^2/ml. Also, since the extraction conditions should simulate as closely as possible the conditions under which the device is normally used, the extraction time chosen was 10 days, time necessary for healing the puncture caused by the ear piercing studs.

The studs tested were placed in a 120-ml capacity screw-capped glass bottle and sterilised by autoclaving at 120°C for 20 min. Subsequently, 60 ml MEM–FCS (minimum Eagle's medium (MEM) supplemented with 10% foetal calf serum (FCS) and 1% penicillin–streptomycin solution) were added. The bottle was shaken and incubated stationary at 37°C for 10 days. When the time given for extraction was over, part of the culture medium was taken for cytotoxicity assay and another part for chemical analysis.

Weight loss measurements

The studs were weighed before and after 10 days of incubation. At the end of the incubation time, the studs were rinsed with distilled water and dried at 60°C for 12 hr, before weighing.

Corrosion test

The corrosion performance of the three types of studs was investigated by means of Electrochemical Impedance Spectroscopy (EIS). EIS was carried out using a Solartron Model SI 1255 Frequency Response Analyser coupled to a Princeton Applied Research (PARC) Model 273A Potentiostat/Galvanostat, controlled by an Electrochemical Impedance Software model 398. These measurements were carried out at room temperature in a MEM culture medium, at the open circuit potential. The perturbation amplitude of voltage for the EIS test was 10 mV and the frequency range was from 50 kHz to 5 mHz.

A three-electrode cell arrangement was used for the EIS tests, with a graphite rod as the auxiliary electrode, a saturated calomel electrode (SCE) as the reference electrode and cold resin mounted studs as the working electrode. The studs were mounted in an epoxy resin, leaving only their stem exposed to the culture medium. The specimens were immersed in the culture medium for 1 day before the EIS tests.

Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS)

The surface characteristics of the studs were examined in an SEM and analysed by EDS before

Table 1. Elemental composition of ear piercing studs obtained by instrumental neutron activation analysis

Element	Substrate of Pf ear piercing studs		Substrate of St ear piercing studs		Ti ear piercing studs
	Stem	Butterfly back	Stem	Butterfly back	Stem
As μg/g	8.1±0.5	69.6±0.4	57.4±0.8	72.5±1.0	17.7±0.3
Co μg/g	27.4±0.4	722±4	2203±11	737.9±3.7	⩽1
Cr%	0.0070±0.0005	17.6±0.2	16.1±0.2	16.3±0.2	0.0096±0.0001
Cu%	36.5±1.2	0.26±0.01	0.35±0.01	0.226±0.008	⩽0.04
Fe%	9.0±0.2	73.5±0.2	67.9±0.2	71.4± 0.2	⩽0.04
Mn%	2.34±0.07	0.90±0.01	1.81±0.02	1.03±0.02	0.0007±0.0002
Mo%	⩽0.2[a]	0.328±0.002	0.394±0.002	0.321±0.009	⩽0.0003
Ni%	6.80±0.07	8.6±0.1	7.86±0.07	7.11±0.06	0.009±0.001
Ti%	⩽23	⩽23	⩽23	⩽18	97.4±6.4
V μg/g	⩽77	⩽85	987±26	⩽9	33±3
Zn%	36.4±3.3	⩽0.06	⩽0.7	⩽0.7	⩽0.002

[a]For the elements not detected, the detection limit values were evaluated according to Currie (1968).

and after 10 days' immersion in the culture medium. The aim of these analyses was to examine the coatings for defects, which could expose the substrate, and for the presence of reaction products on the surface.

Chemical analysis

The extract, culture medium obtained after immersion of the studs, was analysed by applying INAA, according to procedure described in a previous paper (Saiki *et al*., 1999). 500 μl of each extract solution was pipetted and dried in a clean polyethylene capsule for irradiation in the IEA-R1 nuclear reactor. The samples and elemental synthetic standards were irradiated together for 16 hr under a thermal neutron flux of 10^{13} n. cm^{-2} sec^{-1}. After adequate decay times, the samples and standards were measured in a hyper-pure Ge detector coupled to an EG&G Ortec ADCAM 918A Multichannel Buffer, and this to a microcomputer. The gamma ray spectra were processed using appropriate software and the concentrations of the elements were calculated by a comparative method. Also, the blank of the culture medium was analysed to evaluate the elements from this medium.

Cytotoxicity test

The cytotoxicity assay was carried out according to Nakamura *et al*. (1989) and the International Standards Organization (ISO) (1992). The cell line recommended by ISO is a preferred established cell line obtained from recognised repositories as American Type Culture Collection (ATCC). Chinese hamster ovary cells culture (ATCC CHO K1) was used in this investigation. To a CHO cell culture, serially diluted extracts from the gold-coated studs and Ti studs with MEM–FCS, following the corrosion test were added.

CHO cells were grown in MEM–FCS, in a plastic tissue culture flask, at 37°C in a humidified 5% CO_2 air incubator. After a confluent monolayer propagation, the culture medium was removed and the cells were washed with calcium and magnesium free phosphate saline buffer (PBS–CMF).

The culture was treated with 0.25% trypsin solution to detach the cells from the culture tissue flask. After trypsinization, the cells were transferred to a screw-capped plastic tube, centrifuged and washed twice with PBS–CMF. The cells were resuspended in MEM–FCS and adjusted to give 1×10^2 cells/ml. 2 ml of this cell suspension was seeded to each 60-mm diameter assay culture dish and incubated for about 5 hr for adhesion of the cells. The culture medium was then replaced by 5 ml fresh MEM–FCS, in the control plates, and by undiluted (100%) and successively diluted extracts (50%, 25%, 12.5% and 6.25%), in culture dishes with the adhered cells. All concentrations were tested in triplicate.

The culture dishes were incubated for 7 days in a humidified 5% CO_2 atmosphere at 37°C for the formation of cell colonies. The determination of cytotoxicity, according to ISO, can be either qualitative or quantitative. The qualitative evaluation examines the cells microscopically, to assess for changes in general morphology, vacuolisation, detachment, cell and membrane lysis. Quantitative evaluation measures cell death, inhibition of cell growth, cell proliferation or colony formation. In this study, the determination of cytotoxicity was performed by quantitative evaluation, based on cell viability.

After the incubation time, the medium was removed from the dishes, the colonies were fixed with 10% formalin in 0.9% saline and stained with Giemsa. The number of visible colonies on each dish was counted and compared with the number of colonies in the CHO control dish. Phenol solution (0.02%) and titanium extract (60 cm^2/60 ml MEM–FCS) were used as positive and negative control, respectively.

RESULTS AND DISCUSSION

Weight loss measurements

Immersion in the culture medium produced weight losses in the St, Pf and Ti studs, due to corrosion, as shown in Table 2. The corrosion on the commercial

Table 2. Weight losses of different kinds of ear piercing studs

Ear piercing stud	Weight loss (mg)	Surface area exposed to culture medium (cm^2)
St	10.8	36.2
Pf	11.6	20.9
Ti	11.1	26.6

studs was localised and at defects in the coating, whereas the Ti studs presented a slight but uniform attack on the whole surface. EIS

EIS was used to investigate the electrochemical response of the ear piercing studs immersed in the culture medium and to compare responses. The EIS results for the studs are presented in Fig. 2 (a,b).

The EIS results reveal differences in the response of the three types of studs, mainly at frequencies lower than 1 Hz, indicating that the differences are due to the corrosion process. According to the EIS results, the corrosion resistance of the studs increase in the following order: Pf, Ti and St. This indicates that the coating on the St stud stem was protecting the substrate, since stainless-steels have a lower corrosion resistance than that of titanium (Franco *et al.*, 1998).

Despite of the differences observed in the corrosion resistance of the three types of studs, the corrosion resistance of all the studs was more than 10^5 ohms/cm^2, after 1 day of immersion in the culture medium, indicating that the test duration was not sufficient to cause significant corrosion of the studs. The effect of longer periods of immersion on corrosion resistance is under investigation.

SEM and EDS

Examination of the Pf gold-coated stud surface by SEM, before the immersion test, revealed the presence of defects in the coating, as shown in Fig. 3(a). After the immersion test, corrosion products adhering to the surface of the gold coated Pf stud stem, were observed as shown in Fig. 3(b).

EDS analysis (Fig. 3c) of the region shown in Fig. 3(b) revealed the presence of elements such as phosphorus, calcium, sodium and chloride. These are probably reaction products of the culture medium with elements of the studs' substrate.

The presence of aggressive elements such as chlorides in the corroded area suggests that the chlorides from the culture medium may have initiated corrosion. It is possible that after corrosion initiation at the defects in the coating, leaching of zinc to the culture medium takes place and leads to formation of insoluble phosphates, which adhere to the surface of the steel. In the corroded region of the Pf stud, high concentrations of zinc and phosphorus were detected. It is well known that zinc forms insoluble complexes with phosphates (Sunzel *et al.*, 1997), and this is a component of the culture medium.

Defects in the gold coating on some of the tested commercial ear piercing studs, mainly in the stem areas, which are normally in contact with the pierced ears, allow body fluids to contact the studs' substrate. If the substrate contains elements such as nickel, this could be leached from the substrate by reaction with physiological fluids, leading to allergic reactions. In fact, nickel was detected by chemical analysis (INAA) in the culture medium following the immersion test.

The surfaces of the St studs were also observed by SEM and they revealed fewer defects and less corrosion products on their surface. In the case of St and Ti studs, adherent corrosion products were not observed on their surfaces.

Chemical analysis

Substrate

The stainless-steel was not nickel free as indicated in the package of commercial studs. The copper–zinc alloy contained significant amounts of nickel as shown in Table 1. These analyses indicate the need for strict quality control in the gold coating process. Also, the use of nickel free substrates is recommended.

Metal released in the culture medium

The results of element determination, in the extract of studs in culture medium, and in the blank solution composed of the same culture medium are presented in Table 3. In the chemical analysis by INAA, the concentrations of Cr found in the extracts were close to those obtained in the blank. Results obtained for Ni in the extract indicated that this metal was released from the gold-coated studs. The stem substrate of Pf studs presented a high concentration of Cu, and that of the St studs a high concentration of Fe. Consequently, their respective extracts presented high levels of these elements. In the extract from Ti studs, the elements detected were of the same order of magnitude as that in the blanks. The high zinc content in the extract might have been caused by preferential dissolution from the Cu–Zn alloy used as substrate in the Pf studs. The zinc content in the extract was approximately six times that of the culture medium. Therefore, this element was leached from the alloy used as substrate, due to corrosion.

In this study, copper was not analysed by INAA, due to the interference of Na present in high concentration in these samples. A high activity of ^{24}Na masks the photopeak of ^{64}Cu in the gamma spectrum.

Cytotoxicity test

The cytotoxic potential can be quantitatively expressed as $IC_{50(\%)}$ (cytotoxicity index), which is easily determined by plotting the percentage of the colony number in relation to CHO cell control and the concentration of the extract on a graph (data from Table 4). $IC_{50(\%)}$ is the concentration of the extract necessary to kill half the cell population, or

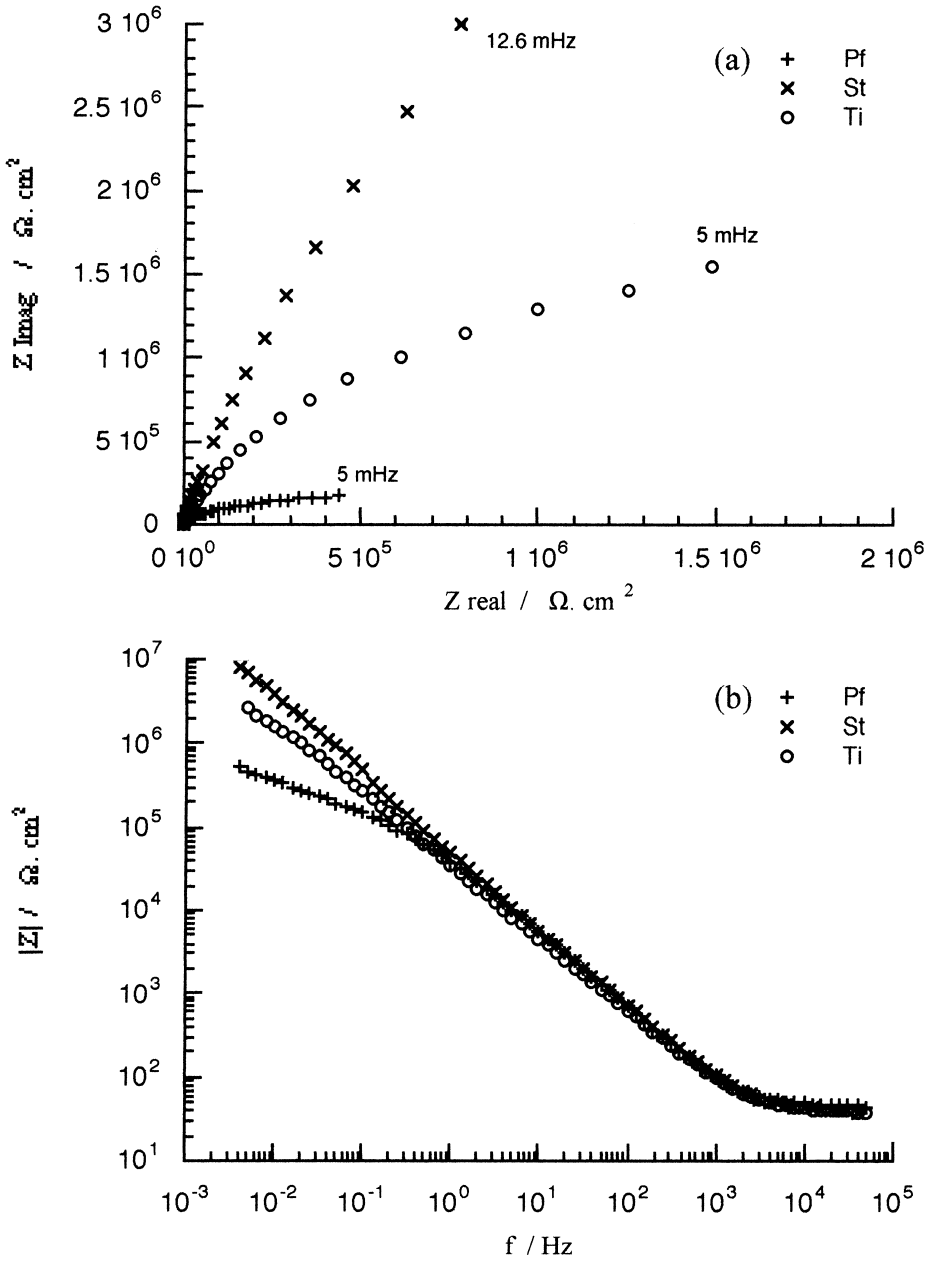

Fig. 2. EIS results of tested studs (a) Nyquist diagram, (b) Bode diagram.

the extract concentration, which suppress colony formation to 50% of the control value. The negative control should not present any effect, as observed with titanium ($IC_{50(\%)} > 100$), and the positive control should present cytotoxic effect, as observed with a phenol solution ($IC_{50(\%)} = 17$), as shown in Fig. 4. St and Pf commercial studs showed cytotoxicity, presenting $IC_{50(\%)} = 78$ and 44, respectively. On the other hand, the extract obtained from Ti studs was not cytotoxic.

The element with the highest concentration in the extract was nickel. A cytotoxicity test was then carried out to investigate whether Ni in the concentration detected in the extract was responsible for the cytotoxicity effect. Even though nickel is known to have toxic effects in cell cultures and in tissues (Bordji *et al.*, 1996), the cytotoxicity test showed that this element was not accountable for the cytotoxicity in the conditions of this study.

The cytotoxic effect observed could be due to copper released from the substrate. The literature reports a direct correlation between the amount of copper found in the culture medium and cytotoxicity (Craig and Hanks, 1990; Wright and Gallant, 1982). According to Tucker and Key (1983), contact dermatitis is an occupational hazard for individuals

working with metallic salts or corroding metals. Metallic implants used increasingly in orthopedic practice currently include fracture fixation plates and rods, bone screws, and joint replacement devices. Most of these devices are made of either stainless-steel, cobalt–chromium, or titanium alloys. Three alloying elements of these commonly used alloy systems are well known contact allergens chromium, cobalt and nickel (Young and Houwing, 1987). Because dissolution, corrosion processes and wear can contribute to the release of alloying elements

Table 3. Elemental concentrations in extract of cell culture media and in the blank

Element	Blank	Extract after immersion		
		Pf	St	Ti
Co ng/ml	12.1±1.2	61.6±0.5	99±10	53.8±2.1
Cr μg/ml	0.72±0.02	0.73±0.04	0.73±0.03	0.75±0.03
Fe μg/ml	0.60±0.09	0.61±0.08	4.03±0.31	0.43±0.11
Ni μg/ml	N.D.[a]	0.96±0.09	0.66±0.06	N.D.
Zn μg/ml	0.58±0.05	3.84±0.41	0.61±0.01	0.75±0.10

[a]N.D. = not detected.

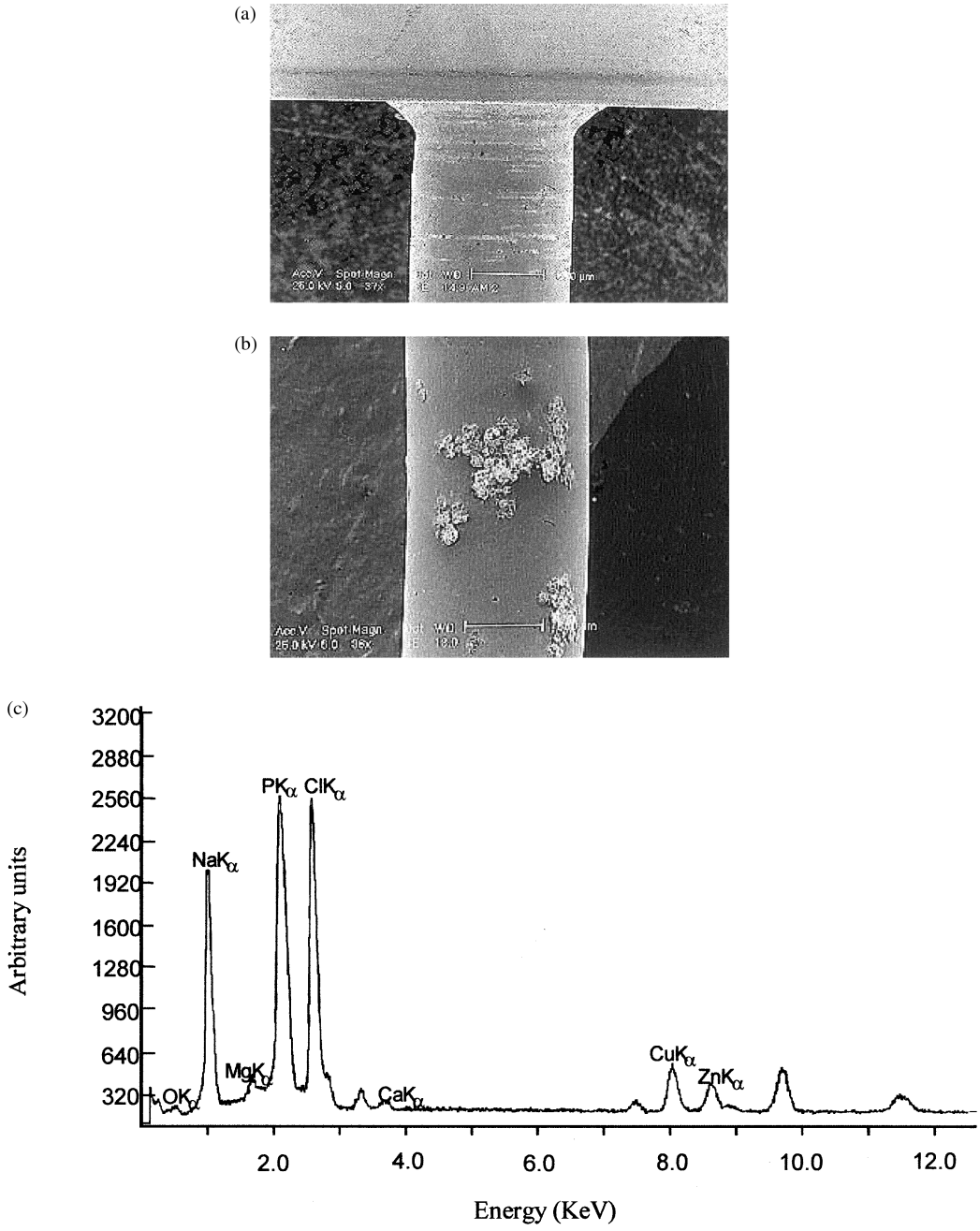

Fig. 3. SEM micrograph of Pf stud stem surface (a) before immersion test and (b) after immersion test showing deposited products on the stem surface, (c) EDS corresponding spectrum of region shown in (b).

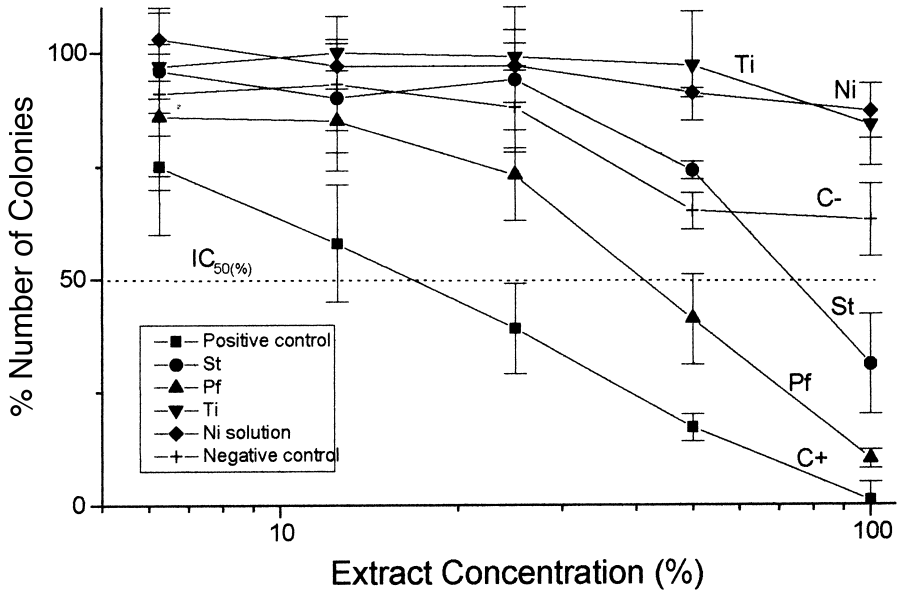

Fig. 4. Colony suppression curve of ear piercing studs.

Table 4. Results obtained from the cytotoxicity assay

Extract Concentration		Negative control	Positive control	St	Pf	Ti	Ni 3.4 μg/ml
100%	X±SD[a]	145±8	3±4	72±11	22±2	191±9	102±6
	% Colony	63	1	31	10	84	87
50%	X±SD	149±4	38±3	171±2	94±10	221±12	107±1
	% Colony	65	17	74	41	97	91
25%	X±SD	202±9	89±10	217±16	167±10	225±3	113±8
	% Colony	88	39	94	73	99	97
12.5%	X±SD	213±10	133±13	206±12	195 ±11	235±8	113±5
	% Colony	93	58	90	85	100	97
6.25%	X±SD	209±18	173±15	220±14	197±16	221±3	120±16
	% Colony	91	75	96	86	97	103

[a]X±SD = mean and standard deviations of three determinations.

from an implant, a possible complication associated with the use of these alloy systems is the development of allergic reactions to the elements in the implant.

The *in vitro* cytotoxicity tests can assess metallic materials released as metal ions and may provide insight into the mechanisms of toxicity exerted by these materials. Metal ions may bind to tissue or bind to tissue intersticial fluid proteins as they get released by the metallic material (De Bruin, 1981). On the basis of immunologic theory, metal ions are too small to provoke an immune response, but they may be immunogenic after their conjugation with protein carriers (Golub and Green, 1992) and the metal part may be an antigen determinant. The allergic contact dermatitis may be evidence that the humoral immunity was developed.

Conclusions

The commercial studs investigated exhibited cytotoxicity and elements from the substrate were detected in the extract. The stud which showed lower corrosion performance, Pf, resulted in higher cytotoxicity. Ti showed no cytotoxicity and higher corrosion resistance, proving to be a potential material for the manufacture of ear piercing studs.

Alloys containing toxic elements should not be used as substrates for ear piercing studs since it is very difficult to produce defect free coatings. The presence of defects in the coating expose the substrate of the studs to body fluids, leading to allergic/toxic reactions.

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