J Mater Sci: Mater Med (2006) 17: 267–275
DOI 10.1007/s10856-006-7313-3

Promising *in vitro* performances of a new nickel-free stainless steel

Lucio Montanaro · Marina Cervellati · Davide Campoccia · Carla Renata Arciola

Received: 15 June 2004 / Accepted: 13 July 2005

Abstract Stainless steel is a metallic alloy largely employed in orthopaedics. However, the presence in its composition of a high quantity of nickel, an agent known to trigger toxic and allergic responses, is cause for concern. In this study, we have investigated the *in vitro* biocompatibility of a new nickel-reduced stainless steel, namely Böhler P558, in comparison to the conventional stainless steel AISI 316L. The neutral red (NR) uptake and the amido black (AB) tests were performed on L929 fibroblasts and MG63 osteoblasts to assess the cytotoxicity, while cytogenetic effects were evaluated on CHOK1 cells by studying the frequency of Sister Chromatid Exchanges (SCE) and chromosomal aberrations. Ames test was used to detect the mutagenic activity. The expression of selected markers typical of differentiated osteoblasts, such as alkaline phosphatase activity (ALP), type I collagen (CICP) and osteocalcin (OC) production, were also monitored in MG63 cells cultured on the tested materials. Our results indicate the absence of significant cytotoxicity and genotoxicity for both test alloys. ALP, CICP and OC analyses confirmed that both materials support the expression of these phenotypic markers. Overall, these data show that this Ni-free alloy possesses good *in vitro* biocompatibility and could have a potential for orthopaedic applications.

L. Montanaro · M. Cervellati · D. Campoccia · C. R. Arciola
Research Unit on Implant Infections, Rizzoli Orthopaedic Institute, Bologna, Italy

L. Montanaro (✉) · C. R. Arciola (✉)
Experimental Pathology Department, University of Bologna, Italy
e-mail: lucio.montanaro@unibo.it; carlarenata.arciola@ior.it

Introduction

Stainless steel (AISI 316L ASTM F55 in prevalence) is a metallic alloy largely used in orthopaedics, where it is employed in the manufacture of fracture fixation devices to a great extent [1]. The main advantages of the use of this material are its low cost, good mechanical properties, and ease of handling [2]. Nevertheless, the tendency of this metallic alloy (above all in variants with low contents of nitrogen and molybdenum) to corrode, with consequent release of metal ions, is well known [3].

Some of the released metal ions such as iron can easily enter in its specific metabolic pool and thus be utilised by physiologic mechanisms, while others (especially chromium, nickel and cobalt) tend to accumulate in target organs (e.g. lungs, liver, kidneys and spleen), as indicated by several systemic toxicity studies [4, 5]. Although all these elements are essential in the human diet, when their quantity increases over physiological levels, the biochemical balance is altered, with consequent toxicity [6–8]. Clinical experience has revealed that severe and prolonged corrosion processes can lead to toxic reactions and tissue alterations with resultant clinical failure of the implant [9].

In this context, of particular concern is the presence in nickel content. It is indeed known that this element can cause allergic sensitisation especially in females. In fact, it is estimated that 30% of women have skin allergy to objects containing nickel [10, 11]. Furthermore, stainless steel has been proved to induce lung cancer in welders inhaling aerosols containing nickel as elemental compound [12, 13].

All this evidence shows how important it is to develop and use steels that do not corrode in the human body and whose nickel content is as low as possible. Recently, modern metallurgic processes have enabled the production of stainless

steels with controlled addition of nitrogen, which, besides its role in stabilizing non-magnetic austenite, imparts higher resistance to pitting corrosion in the biological environment [14].

Böhler P558 is a new type of austenitic and amagnetic stainless steel alloyed to nitrogen in protective gas at high pressure with negligible amounts of nickel. Although this steel appears to be very interesting because it has good mechanical properties, elasticity, and resistance to general and local corrosion [15, 16], at present, there are not exhaustive confirmative biocompatibility data available on it.

The purpose of this study was to assess the *in vitro* biocompatibility of Böhler P558 and to compare it to conventional stainless steel AISI 316L. Two different cytotoxicity tests, respectively the neutral red (NR) uptake and the amido black (AB) staining techniques, were performed to assess the influence of the material extracts on the integrity of cell membrane and on the cell proliferation [17–19]. These tests have been performed using mouse L929 fibroblasts and human MG63 osteoblasts; the L929 cell line is an established cell line recommended in cytotoxicity determinations [20], while the MG63 cell line exhibits many osteoblastic traits which are characteristic of bone forming cells and is frequently utilized in *in vitro* experimental models aimed at exploring the interactions between osteoblast-like cells and biomaterials [21, 22].

Furthermore, some specific functional characteristics of MG63 osteoblasts cultured directly onto the materials have been determined. In fact, for the success of an orthopaedic implant, beside being chemically atoxic, the material should be able to support proliferation and differentiation of the osteoblastic cells in the surrounding tissue during the phases of repair and regeneration after implantation. The expression of selected biochemical markers typical of differentiated osteoblasts, such as alkaline phosphatase activity (ALP), type I collagen (CICP) and osteocalcin (OC) production , were monitored.

Finally, possible cytogenetic effects were evaluated by studying the frequency of Sister Chromatid Exchanges (SCE) and chromosomal aberrations, while the Ames test was used to detect the mutagenic activity [23–26]. SCE and the evaluation of chromosomal aberrations were performed on Chinese hamster epithelial-like CHO K1 cells which are a secondary cell line that is widely used for genotoxicity screenings [27].

Materials and methods

Materials

Stainless steel Böhler P558 (Böhler Edelstahl Gmbh & CO KG through Böhler Sales Division of Böhler Uddeholm Italia S.p.A) and stainless steel AISI 316L (Goodfellow Cambridge Limited, Huntingdon, England), cut into discs 33 mm in diameter, were the test materials (see Table 1). Surface roughness of the discs was determined by a Mahr Perthometer PGK120. The disks were measured to obtain an average roughness value (Ra) that was always $\leq 0.2\mu$m. Ra was not determined for the tissue culture plastic surfaces used as controls. However, in previous studies they were found to have Ra values of less than 0.2 μm. Samples were sterilised by γ-rays.

Table 1 Percentage of elements in the analyzed alloys

Chemical composition (%) of AISI 316L and P558		
Element	AISI 316L	P558
C	0.002	0.18
Mn	1.31	9.85
P	0.035	<0.005
S	0.007	0.007
Si	0.39	0.47
Cr	17.00	16.50
Ni	11.00	<0.02
Mo	2.01	3.24
Fe	Balance	Balance

For cytotoxicity and genotoxicity testing, the material was prepared as an aqueous extract in Minimum Essential Medium (MEM) with Earle's salts (Gibco Laboratories, Grand Island, N.Y.), (3 cm^2/ml of medium). The extraction was performed at 37 ± 1 °C for 72 ± 2 hours. At the end of extraction, the extracts were aliquoted and frozen at -80 ± 15 °C until tested.

Cytotoxicity

Human osteoblast-like MG63 cells (ATCC, USA) and mouse fibroblast L 929 cell line (Sandoz Institute, Vienna) were used. The cells, cultured in MEM supplemented with 10% heat inactivated foetal calf serum (FCS, Sigma), 20 mM glutamine 1% (Sigma), vitamins (BME vitamins, Gibco), non-essential amino acids (Gibco), penicillin 100 U/ml and streptomycin 100 μg/ml (Sigma), were plated $1 \times 10^4/200\,\mu$l/well into 96 multiwell plates (Costar, Cambridge, MA,USA) and incubated for 24 hours. Then, the medium was replaced with 200 μl/well of extracts.

Prior to adding to the cells, all the extracts were supplemented with the above mentioned additive for MEM. The negative control of the extract was obtained by pouring an amount of MEM into a cell culture-treated polystyrene flask and the positive control was obtained by dissolving at each run phenol (MW 94.11) 0.64% in polystyrene-extracted MEM.

The plates were reincubated for 24 hours and then assayed.

NR uptake assay: NR is a vital dye, which is actively engulfed into the lysosomes of viable cells and provides an

index of cell viability. A stock solution of 0.4% NR dye (C.I.50040, cell culture grade) in distilled water was prepared and was diluted 50 μg/ml in culture medium when used (NR solution).

In this study, after treatment with the extracts, the medium was replaced with 100 μl/well of NR solution and the plates were reincubated for 2 hours at 37 °C. At the end of incubation time, the cells were rinsed with 100 μl/well of Dulbecco's Phosphate Buffered Solution (D-PBS) and 100 μl/well of lysing solution (1% glacial acetic acid, 50% ethanol, 49% distilled water) were added. After gently shaking the plates for 1 min, the colour intensity of each well was read in absorbance at 540 nm wavelength by a spectrophotometer Multiskan-Ex (Labsystems). Mean and standard deviation of the optical density (O.D.) values obtained for 3 to 5 replicates of controls and samples, respectively, were calculated.

AB staining assay: AB is considered a rapid and sensitive marker of the number of cultured cells and can be used as a marker of cell growth after contact with biomaterials *in vitro*. AB staining assay is based on the binding of an acid solution of the AB dye to the cellular proteins after fixation of cells. The bond is relatively weak, so the dye can be eluted by alkaline solution.

After treatment with the extracts, the cells were washed with 100 μl/well of D-PBS and fixed with 1% glutaraldehyde in D-PBS for 15 min. The fixative was replaced by 100 μl/well of AB 10B dye (C.I.20470) diluted 0.1% in sodium acetate buffer (pH 3.5). The plates were incubated for 30 min. The protein-bound AB was released by treating cells with a lysing solution (50 nM NaOH) and the colour intensity was read at 620 nm and 405 nm wavelengths (the maximum and minimum absorbance of AB, respectively). Results were expressed as the difference between the optical densities recorded at two wavelengths. Mean and standard deviation of the O.D. values obtained for 3 to 5 replicates of controls and samples, respectively, were calculated. The medium was regularly replaced twice a week.

Data management: the viability and growth of cells using NR uptake and AB staining were calculated as indexes: % viability or % growth = (A/B) $\times$ 100, where A is the O.D. of cells challenged with the material and B is the O.D. of the negative control cells, i.e. cultured without material in polystyrene-treated MEM. The sample was considered as cytotoxic if the index was $\leq$ 70. Final results were expressed as the arithmetic mean $\pm$ standard deviation of three separate experiments.

Genotoxicity

Chinese hamster epithelial-like CHO K1 cells (ATCC, USA) were used. The cells were routinely cultured in medium HAM F12 (Sigma) with the addition of 10% FCS and penicillin 100 U/ml and streptomycin 100 μg/ml (Sigma), under standard culture conditions. The medium was regularly replaced twice a week.

SCE: SCE are the cytologic manifestation of the breaks and repair of DNA at the homologous sites of two chromatids of a single chromosome which occur during replication. In this work, CHO K1 cells at confluence were detached with trypsin/EDTA, seeded in 2 ml of medium in 12-well tissue culture plates at a density of 3×10^5 cells/well (2 replicates) and maintained at 37 $\pm$ 1 °C. After 24 h, 500 μl of sample extracts for each ml of culture medium were added, with or without 200 μl/ml of LS-9 mix. In fact, to highlight also the damage induced by pre-mutagenic substances, a metabolic activator is added to the system (Mol Tox LS-9 mix), made up of enzymes extracted from rat liver treated with Aroclor 1254. Using LS-9 mix, the metabolic transformations that the examined substance could undergo in the mammalian body are simulated.

The negative control was the extraction medium (MEM). The positive control for the tests performed without metabolic activation was a solution of mitomycin C (30.0 μg/ml of cell suspension). Positive controls used in the tests with metabolic activation were prepared with a 10.0 μg/ml solution of cyclophosphamide. After 90 min, the medium was discarded and the cells were cultured for a further 22 h with 12.5 μg/ml of Bromodeoxyuridine (BrdU), an analogue of thymidine, which is incorporated into replicating DNA. Cells were then treated with 2 μg/ml colchicine in order to stop mitosis at metaphase. Slides with chromosomes on plate were prepared according to the traditional methods for the study of the metaphases.

Then a solution of 15 mg/100 ml H_2O of bis-benzimide (Hoechst 33258) was added: this compound specifically binds cell DNA with great affinity for A-T site and polyA sequences. The slides were then exposed to ultra-violet light so that a photolysis reaction could take place. Finally, Giemsa staining was performed; one of the sister chromatids which were coupled during the second metaphase was blue and the other one was not coloured.

Microscope observation was carried out with an oil objective at 100 $\times$ magnification. On each slide a longitudinal scanning was performed, recording each visible metaphase and classifying it as first, second or third metaphase, based on the dye affinity of the chromatids.

Only the metaphases of type 2° were counted, and the number of exchanges among sister chromatids occurring on the total chromosomes was evaluated. The observation was performed to score at least 20 metaphase plates of type 2° whenever possible.

Chromosomal aberrations: the evaluation of chromosomal aberrations permits demonstration of the *in vitro* effect of genotoxic substances on chromosomes. A certain number of chromosomal aberrations occur spontaneously in culture,

but exposure to mutagenic agents *in vitro* markedly increases this number. The ratio between spontaneous aberrations and the induced ones provides the genotoxicity index.

CHO K1 cells were seeded in 2 ml of medium in 12-well tissue culture plates at a density of 300.000 cells/well (2 replicates) and maintained at 37 ± 1°C. After 24 hours, 500 μl of sample extract for each ml of culture medium were added, with or without 200 μl/ml of S9 fraction. The negative and the positive controls were prepared as in SCE assay.

After 90 min the medium was discarded, the cells were cultured for further 22 hours and then treated with 2 μg/ml colchicine. Slides with chromosomes on plate were prepared and stained with Giemsa. Microscope observation was carried out with an oil objective at 100 × magnification; each slide was scanned longitudinally, recording each metaphase with good chromosome distribution. Whenever possible, the observation was performed to score at least 20 mitotic cells; chromatidic and chromosomal gaps as well as breaks followed by deletions and inversions were recorded. To each aberration the score assigned was: 1 for single and double gaps, chromatidic breakage and fragments; 2 for isochromatidic breakage, dicentric and centric translocation; 4 for complex figures.

Data management: in SCE evaluation, the average number of exchanges among sister chromatids in twenty-two chromosomes was calculated. The test was considered positive if, by Student's *t*-test, a statistically significant increase of the number of SCE vs the negative control was found ($p < 0.05$).

In the evaluation of chromosomal aberrations the average value of aberrations in twenty-two chromosomes was calculated. The test was considered positive if, by Student's *t*-test, a statistically significant increase of the value of chromosomal aberrations vs the negative control was found ($p < 0.05$).

Ames test

This test is a microbial assay for the detection of chemically induced gene mutations. In this test, special apathogenic strains of Salmonella (histidine defect mutants) were used, which under the influence of mutagens will undergo a mutation back to the wild strain, which is capable of synthesising histidine.

The following Salmonella strains were used in the test: TA 98 and TA 1538 (which primarily respond to frameshift mutagens), TA 100 and TA 1535 (which respond to base-pair substitution mutagens). All strains were uvr B- (they had lost DNA excision repair system) and rfa- (they had partially lost their lipopolysaccharide barrier so they were more permeable to macromolecules).

TA 98 and TA 100 strains also have an incorporated plasmid (pKM101) that causes an increased error-prone DNA repair. The genetic identity of the strains, their sensitivity to UV radiation (uvr B) and crystal violet (rfa-), and their resistance to ampicillin (pKM 101) were regularly checked.

Test strains were thawed from a frozen stock kept at –80°C in nutrient broth containing 9% dimethyl sulfoxide (DMSO) and overnight cultures were grown in incubator for 16 h at 37 ± 1°C in Oxoid 2 nutrient broth. Final cell density was approximately 10^8–10^9 cells/ml.

In this study, upon a layer of histidine-free agar (minimal glucose agar), a second layer of top agar containing 0.6% Noble Agar (Difco), 0.5% NaCl, 0.5 mM L-histidine, 0.5 mM biotin, 0.5 ml of phosphate buffer, 0.1 ml of Salmonella suspension and 0.1 ml of test substances or control (positive or negative) was placed.

The negative control used was MEM from the same batch used for the extraction. Positive controls used in the tests without metabolic activation were 4 nitro *o*-phenylendiamine (20.0 μg per plate) for strains TA 1538 and TA 98, sodium azide (1.5 μg per plate) for strain TA 1535 and sodium azide (1.0 μg per plate) for strain TA 100. Positive controls used in the tests with metabolic activation were 2-anthramine (1.0 μg per plate) for all strains.

The trace of histidine present in the top agar allowed the logarithmic division of the histidine-requiring Salmonellae; this period of bacteria proliferation is essential for the mutagenic lesions of DNA to take place and results in the formation of a lawn of histidine-requiring bacteria whose further division is prevented by exhaustion of histidine. Only the fraction of bacteria which have reverted to histidine- independence (either spontaneously or by the action of the test substance) will continue to divide and will form visible colonies.

In the assays with metabolic activation, 0.5 ml of phosphate buffer were replaced by 0.5 ml of Mol Tox LS-9 mix. After 48 hours of incubation, the revertant colonies were counted. Each experiment was repeated twice, with and without metabolic activation. The test resulted positive, that is the assayed material was considered genotoxic, if both the following conditions were met: the number of revertant colonies was at least double than negative control for strains TA 98 and TA 100 or at least three times for strains TA 1535 and TA 1538.

Cytocompatibility studies

For the cytocompatibility studies, MG63 cells were plated at a density of 1×10^4 cells/cm^2 on material samples placed in 6-well polystyrene plates (Costar Corp., Cambridge, MA). A volume of 500 μl of cell suspension was seeded on the entire top surface of each test sample (8.55 cm^2). For the controls, the cell suspension was distributed on an equivalent area in empty wells of 6-well plates. Once the cells had adhered, 5 hours following seeding, 3 ml of fresh medium were

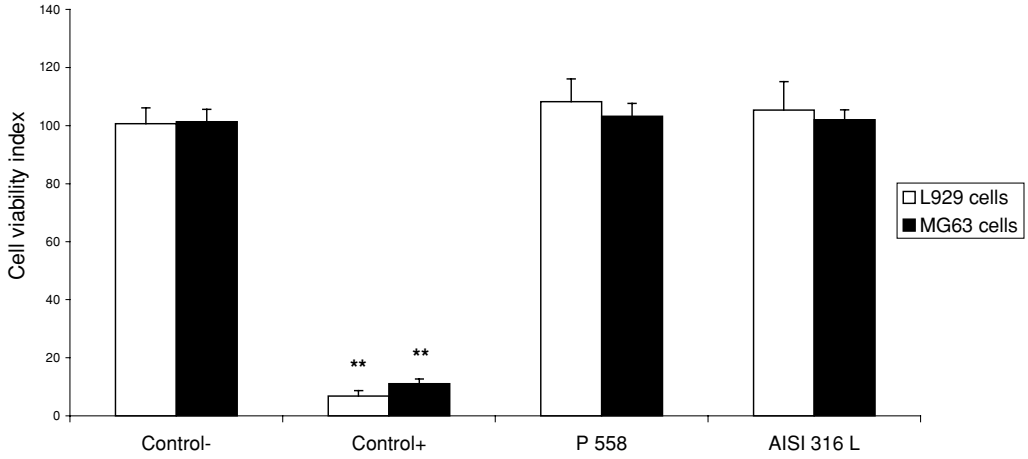

Fig. 1 Viability evaluation of MG63 and L929 cells challenged with the P558 and AISI 316L extracts using NR assay. The negative control (control−) is represented by the extraction vehicle with no material and the positive (control+) by a 0.64% phenol solution of the same medium. The data mean valuse at least of three replicates ± standard deviation of three independent experiments; $^{**}p < 0,01$.

carefully added to each well. At day 10, the supernatants were collected and stored frozen at –80 °C until assayed for osteocalcin and type I collagen measurements. At the same time point, cells were harvested from control wells and from test samples for alkaline phosphatase activity and protein quantification.

Alkaline phosphatase activity and protein determination: the analyses were performed on cell lysates using a freeze-thaw technique. At harvest, cell layers were washed twice with phosphate buffered saline solution (PBS, Gibco). A volume of 950 μl of distilled water was added to each well. 50 μl of 1% Triton X-100 were added to each well and the cell layers were carefully scraped and stored at -80°C. Just before assay for alkaline phosphatase activity, cell lysates were thawed and treated by sonication on ice for 5 min. Complete cell lysis was checked under light microscopy and, eventually, the treatment was repeated. Alkaline phosphatase activity of cell lysates was assayed using a commercially available kit (Metra^{TM} BAP EIA kit, Quidel Corporation, USA). The measurements were performed using 20 μl of lysates and the enzymatic activity was finally expressed as μmoles of Pi released per minute per mg of cellular proteins. Protein determination was performed by the commercially available Micro BCA^{TM} Protein Assay Reagent kit (Pierce Chemical Co, Rockford Illinois, USA) and the content was expressed as mg/well.

Type I collagen determination: the assay was performed measuring type I C terminal propeptide released in the medium by the MG63 cells.

Collagen is synthesized as procollagen that consists of mature collagen with extension peptides at both the amino and carboxy termini. These extension propeptides are cleaved from the collagen molecule by specific proteases prior to incorporation of collagen into a growing collagen fibril; the release of these peptides into the medium provides a stoichiometric representation of the production of collagen. The assay was performed using the Metra^{TM} CICP EIA kit, Quidel Corporation, USA, according to the directions of the manufacturer. The collagen concentration was expressed in ng/ml.

Osteocalcin assay: the free osteocalcin released in the medium by the MG63 cells was measured using the Metra^{TM} Osteocalcin kit, Quidel Corporation, USA. Each single standard and sample was assayed in duplicate. The osteocalcin concentration was expressed in ng/ml. Cytocompatibility results were expressed as mean values ± standard deviation. Statistical significance was considered at $p < 0.05$.

Results

Cytotoxicity tests

The results of NR and AB tests are shown in Figs. 1 and 2. The cell viability and growth for Böhler P558 and AISI 316 L extracts were comparable to those of the negative control (polystyrene extract) and significantly higher ($p < 0.01$) than positive controls (solution 0.64% phenol). The extracts, which were tested undiluted, did not cause any reduction in viability or inhibition of cell growth. Therefore, they had no toxic effects. The results from the cytotoxicity tests carried out on both cell types were also in good agreement with each other.

Genotoxicity tests

No significant increase in the average number of SCE in twenty-two chromosomes and no significant increase in

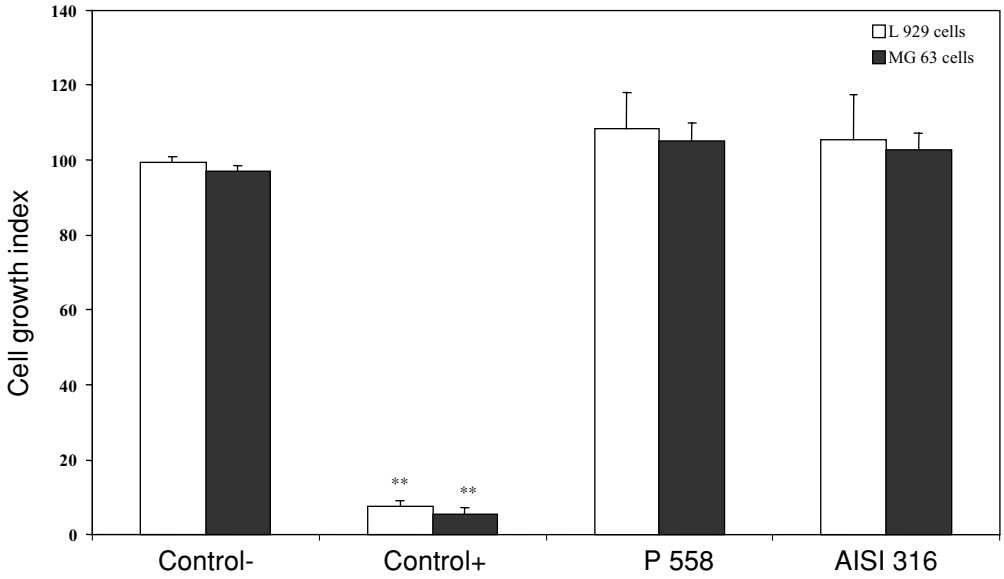

Fig. 2 Growth evalution of MG63 and L929 cells challenged with the P558 and AISI 316L extracts using AB assay. The negative control (control−) is represented by the extraction vehicle with no material and the positive control (control+) by a 0.64% phenol solution of the same medium. The data are mean values of at least three replicates ± standard deviation of three independent experiments; $^{**}p < 0,01$.

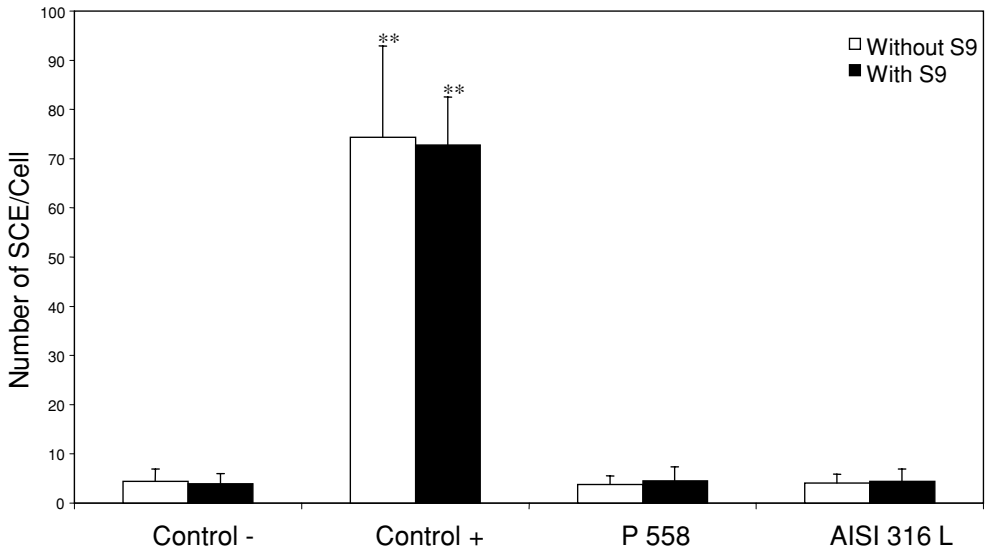

Fig. 3 Number of sister chromatid exchange (SCE) per cell in the CHO-k1 cells treated with varying dilutions of amalgam extracts prepared using complete culture medium. The negative control (control−) is represented by the extraction medium with no material while he positive control (control+) used for the system with or without S9 mix were a 10.0 μg/mL solution of cyclohosphamide and a 30.0 μmL solution of mitomycin C, respectively. The data are mean values of at least 40 observations of metaphase plates of type 2° ± standard deviation of two independent experiments; $^{**}P < 0,01$.

chromosomal aberrations were observed after exposure of the Böhler P558 and AISI 316L extracts as compared to the solvent control, either with or without metabolic activator (Figs. 3 and 4). None of the assays (with or without metabolic activation) of the Ames test showed a mutation factor that was significantly higher in any of the four Salmonella strains. The Böhler P558 P558 and the AISI 316L extracts and the negative controls showed similar results (Table 2).

Cytocompatibility studies

Table 3 shows the results concerning the biochemical parameters of osteoblastic phenotype. The amount of osteocalcin released into the medium by the osteoblasts seeded on Böhler P558 was significantly higher than on plastic ($p < 0.01$) and on AISI 316L ($p < 0.05$). Moreover, the cells grown on the nickel-free stainless steel showed an increase, albeit not

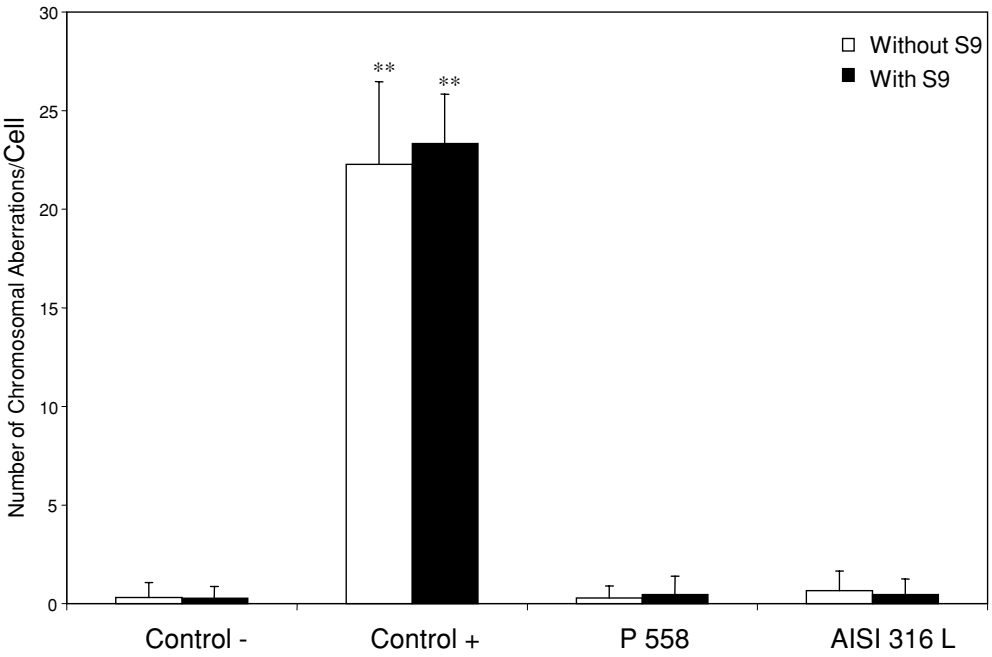

Fig. 4 Number of chromosomal aberrations per cell in the CHO-K1 cells treated with varying dilution of amalgam extracts using complete culture medium. The negative control (control−) is represented by the extraction medium with no material while the positive control (control+) used for the system with or without S9 mix were a 10.0 μg/mL solution of cyclophosphamide and a 30.0 μg/ mL solution of mitomycin C, respectively. The data are mean values of at least 40 observation of metaphase plates of type 2° ± standard deviation of two independent experiments; $^{**}p < 0,01$.

significant, in alkaline phosphatase activity and type I collagen production.

Results from the total protein content, an indirect reference parameter of the number of cells in culture, indicated that at 10 days the cells grown on P558 appeared to be significantly fewer with respect to AISI 316L and the polystyrene control.

Discussion

The most important pathologic aspects of nickel-containing medical biomaterials are the hypersensitive reactions [28]. In fact, the slow dissolution of nickel from prosthetic implants may act as sensitising agent and previously sensitised persons may react to the introduction of such metal into the body.

Table 2 Ames test results.

Test sample	Strain TA 98		Strain TA 100		Strain TA 1535		Strain TA 1538	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Negative control	13.5±1.9	15.3±1.5	107.8±5.5	111.3±8.8	12.8±2	13±2.4	12±2.6	11.3±2.6
Positive control	>1000	>100	>400	>400	>500	73±5.5	>1000	85.3±13.1
AISI 316L	15±2.8	15.7±2.2	105.7±8.9	107.7±9.1	12.7±3.1	11.3±2.6	10.8±3.1	12±2.7
P558	14.8±1.7	16.2±2.1	104.5±7	108±6.9	13.2±3.7	13.3±3.4	11.3±2.8	12.3±1.7

The results are expressed as colony forming units (CFU). Values are means ± standard deviation of 6 measurements from two independent experiments performed with and without metabolic activation (S9).

Table 3 (Expression of phenotypical markers in MG63 cells).

MG63 osteoblasts were cultured on Böhler P558, on AISI 316L and on polystyrene control for 10 days. Significance level with respect to polystyrene: * $p < 0.005$; ** $p < 0.01$. Cross-comparision of Böhler P558 and AISI 316L: †p, 0.05.

Biochemical markers	Polystyrene	Böhler P558	AISI 316L
ALP (μmol pNPP/min/mg protein)	3.15± 0.29	3.62 ±0.20	3.33± 0.71
Osteocalcin (ng/mg protein)	72.25± 8.38	91.83± 6.27	77.47± 8.32
Type I collagen (μg mg protein)	1.12± 0.13	1.18± 0.09	1.13± 0.17
Totla protein content (mg/well)	1.41± 0.14	0.89± 0.07	1.09± 0.14

Both urticarial and eczematous nickel dermatitis have been associated to implanted prostheses and it is a controversial question whether the aseptic loosening of the implants among nickel-sensitive patients is caused by sensitisation or vice versa [29, 30].

Despite the large number of studies investigating the biological behaviour of stainless steel and the other nickel containing alloys, current literature shows there is a lack of data available with respect to the biocompatibility and the possible implant applications of the new nickel-free stainless steels recently developed. These materials, in spite of the absence of nickel, maintain their austenitic structure and thus avoid to nickel sensitivity [31].

In this study, the in vitro biocompatibility of the nickel-free stainless steel Böhler P558 was investigated *in vitro* by means of cytotoxicity, genotoxicity and cytocompatibility tests and compared to conventional AISI 316L.

In cytotoxicity studies, the results showed that the extracts obtained by both the metallic alloys did not affect cell viability (NR assay) and proliferation (AB assay); moreover, the findings of the two tests were in agreement.

Genotoxicity was tested in 3 ways, two of which used mammal cells as a target. The tests chosen covered three levels of genotoxic effects: genic mutations (Ames test), damage and repair of DNA (SCE) and chromosomal aberrations.

Böhler P558 and AISI 316L were not genotoxic in any test, so it can be concluded that they did not induce any damage to the DNA.

In the present study, we also assessed the effect of the test materials on MG63 cells monitoring the expression of some differentiation markers of osteoblastic phenotype, such as alkaline phosphatase activity, type I collagen production and osteocalcin release.

During the development of their completely differentiated phenotype, the osteoblastic cells undergo a temporal sequence of phases: proliferation, differentiation and mineralization [32]. Briefly, cells initially increase their number and produce extracellular matrix. The phase of differentiation follows, which is characterised by the production of high levels of alkaline phosphatase and modifications of the matrix that lead to the deposition of hydroxyapatite crystals. This phase of mineralization is also characterised by the synthesis of osteocalcin, a calcium-binding polypeptide that plays a fundamental role in bone remodelling and represents the specific marker of final differentiation of osteoblasts.

When the osteoblastic cells were seeded directly onto Böhler P558 and AISI 316L, cell proliferation occurred differentially on all substrates, revealing after 10 days significant differences between the two metallic alloys, as shown by the total protein content. Differences in cell behaviour were also evident considering the biochemical parameters of cell differentiation. Cells grown on Böhler P558 showed a higher but statistically non-significant activity of alkaline phosphatase and collagen production, in comparison with cultures grown on AISI 316L and polystyrene. Both the metallic test materials exhibited the same cell behaviour stimulating the osteocalcin production, but for osteoblastic cells on Böhler P558 the increase of this marker of final differentiation was more pronounced and significantly greater than that observed for AISI 316L.

In the literature, it is well known that the behaviour of osteoblasts is influenced not only by the chemical nature but also by the physical characteristics of the substrate on which the cells are cultured. A number of studies using MG63 cells suggest that cell adherence, attachment, spreading, growth and differentiation are influenced by surface roughness [33–35]. In general, when compared to MG63 cells cultured on smoother surfaces, cells grown on rougher surfaces exhibit decreased levels of cellular proliferation and attributes of more differentiated osteoblasts.

However, the optimal surface roughness, that is to say where proliferation is reduced but not blocked and phenotype differentiation is enhanced, appears to be that with Ra values around 4 μm and surfaces with Ra $\leq$ 0.2 μm are considered, as far as their effect on cell behaviour is concerned, smooth surfaces, comparable to tissue culture plastic [36]. In this study, all the samples examined had an average roughness value (Ra) that was always $\leq$ 0.2 μm; for this reason the different performance of Böhler P558 with respect to conventional stainless steel and polystyrene, as far as osteocalcin production was concerned, was unlikely to be related to the topology of the steel specimens, but rather to their chemical composition.

In conclusion, our results show that Böhler P558 is an interesting material, which does not cause direct cytoxicity and genotoxicity, allows osteoblast adhesion and growth, and supports the expression of the typical phenotypic markers, to the same extent or even better than AISI 316L. These results should stimulate further studies *in vivo* considering systemic toxicology, tissue reaction and osseointegration, necessary to confirm that this new nickel-free steel could represent a valid or even better alternative to other conventional steel alloys.

References

1. D. WILLIAMS, R. W. CAHN and M. B. BEVER, in "Concise encyclopedia of medical & dental materials" (Pergamon Press, Oxford, New York, Beijing Frankfurt, Sào Paulo, Sidney, Tokyo, Toronto, 1990) p. 4.
2. A. CIGADA, G. DE SANCTIS, A. M. GATTI, A. ROOS and D. ZAFFE, *J. Appl. Biomater*. **4** (1993) 39.
3. J. J. JACOBS, J. L. GILBERT and R. M. URBAN, *J. Bone. Jt. Surg*. **80** (1998) 268.

4. M. E. FERREIRA, M. DE LOURDES PEREIRA, F. GARCIA E COSTA, J. P. SOUSA and G. S. DE CARVALHO, *J. Trace Elem. Med. Biol.* **17** (2003) 45.
5. M. C. PEREIRA, M. L. PEREIRA and J. P. SOUSA, *J. Biomed. Mater. Res.* **40** (1998) 40.
6. D. B. LOURIA, M. M. JOSELOW and A. A. BROWDER, *Ann. Intern. Med.* **76** (1972) 307.
7. P. D. WHANGER, *Toxicol. Appl. Pharmacol.* **25** (1973) 323.
8. G. ROSTOKER, J. ROBIN, O. BINET, J. BLAMOUTIER, J. PAUPE, M. LESSANA-LEIBOWITCH, J. BEDOUELLE, J. M. SONNECK, J. B. GARREL and P. MILLET, *J. Bone Joint. Surg.* **69A** (1987) 1408.
9. J. V. LINDEN, S. M. HOPLER, H. R. GOSSLING and F. W. SUNDERMAN, *Ann. Clin. Lab. Sci.* **15** (1985) 459.
10. M. CRAMERS and U. LUCHT, *Acta Orthop. Scand.* **48** (1977) 245.
11. S. M. PERREN, M. ALLGOWER and H. BRUNNER, *Injury* **22** (1991) 1.
12. J. P. GENNART, C. BALEUX, C. VERELLEN DUMOULIN, J. P. BUCHET and R. DE MEYER, *Mutat. Res.* **299** (1993) 55.
13. B. SJOGREN, K. S. HANSEN, H. KJUUS and P. G. PERSSON, *Occup. Env. Med.* **51** (1994) 335.
14. C. ÖRNHAGEN, J. O. NILSSON and H. VANNEVIK, *J. Biomed. Mater. Res.* **31** (1996) 97.
15. J. BERNAUER and G. HOCHÖRTLER, in Proceedings of the International Congress Stainless Steel '99 Science and Market / 3-rd European Congress, Chia Laguna Sardinia, June 1999, (Associazione Italiana di Metallurgia, Milano, 1999) p. 473.
16. G. HOCHÖRTLER, J. BERNAUER and K. KRISZT in "Materials for Medical Engineering" (Wiley-VCH, Weinheim:, 2000) p. 204.
17. E. BORENFREUND and J. A. PUERNER, *Toxicol. Letters* **24** (1985) 119.
18. M.B. HANSEN, S.E. NIELSEN and K. BERG, *J. Immunol. Methods* **119** (1989) 203.
19. J. SCHULZ, S. DETTLAFF, U. FRIZSCHE, U. HARMS, H. SCHIEBEL, W. DERER, N. E. FUSENING, A. HULSEN and M. BOHM, *J. Immunol. Meth.* **167** (1994) 1.
20. ISO 10993-5: 1999 Biological evaluation of medical devices – Part 5: Tests for in vitro cytotoxicity.
21. C. J. KIRKPATRICK, C. MITTERMAYER, *J. Mater. Sci. Mater. Med.* **1** (1990) 9.
22. J. CLOVER and M. GOWEN, *Bone* **15** (1994) 585.
23. R. S. VERAM and A. BABU, in "Human Chromosome – Manual of Basic Techniques" (Pergamon Press, Oxford, 1989) p. 47.
24. H. G. SCHWARZACHER and U. WOLF, in "Metodi nella Citogenetica Umana" (Piccin Editore, Padova, 1978).
25. B. N. AMES, J. MC CANN and E. YAMASAKI, *Mutation. Res.* **31** (1975) 347.
26. D. M. MARON and B. N. AMES, *Mutation. Res.* **113** (1983) 173.
27. J. BOMSER and J. E. CASIDA, *J. Biochem. Mol. Toxicol.* **14** (2000) 346.
28. N. JACOBSEN, A. HENSTEN-PETTERSEN and H. HOFSOY, in "Systemic Aspects of Biocompatibility" (CRC Press, Boca Raton, Florida, 1981) p.125.
29. F. CANCILLERI, P. DE GIORGIS, C. VERDOIA, L. PARRINI, A. LODI and C. CROASTI, *Ital. J. Orthop. Traumatol.* **18** (1992) 407.
30. V. MILAVEC-PURETIC, D. ORLIC and A. MARUSIC, *Arch. Orthop. Trauma Surg.* **117** (1998) 383.
31. J. A. DISEGI and L. ESCHBACH, *Injury* **31** (2000) 2.
32. J. B. LIAN, *J. Oral. Implant* **19** (1993) 5.
33. Z. SCHWARTZ, C. H. LOHMANN, J. OEFINGER, L. F. BONEWALD, D. D. DEAN and B. D. BOYAN, *Adv. Dent. Res.* **13** (1999) 38.
34. B. D. BOYAN, V. L. SYLVIA, Y. LIU, R. SAGUN, D. L. COCHRAN, C. H. LOHMANN, D. D. DEAN and Z. SCHWARTZ, *Biomaterials* **20** (1999) 2305.
35. Z. SCHWARTZ, C. H. LOHMANN, A. K. VOCKE, V. L. SYLVIA, D. L. COCHRAN, D. D. DEAN and B. D. BOYAN, *J. Biomed. Mater. Res.* **56** (2001) 417.
36. J. LINCKS, B. D. BOYAN, C. R. BLANCHARD, C. H. LOHMANN, Y. LIU, D. L. COCHRAN, D. D. DEAN and Z. SCHWARTZ, *Biomaterials* **19** (1998) 2219.