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Inhibition and stimulation of enzymatic activities of human fibroblasts by corrosion products and metal salts

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Fibroblasts from normal human skin were cultured for a period of 21 days in the absence or in the presence of metal ions. The effects of stainless steel (SS) corrosion products were compared to the effects of iron, chromium and nickel ions used either separately (Fe, Cr, or Ni solutions) or combined (Fe + Cr + Ni solution). At several periods of time (4, 7, 14 and 21 days) the cell cultures were analysed for the following parameters: (a) metal ion accumulation by atomic absorption spectrometry; (b) cell morphology and viability by the neutral red assay; (c) cell proliferation by DNA assessment, and enzyme activity by both (d) MTT reduction and (e) acid phosphatase activity. Results showed that SS-corrosion products and the corresponding metal ions combined at the same concentrations, Fe + Cr + Ni solution, had opposite effects on fibroblast cultures. In fact, SS-corrosion products caused no apparent effects on cell morphology nor on cell proliferation whereas Fe + Cr + Ni solution stimulated both neutral red uptake and cell proliferation. The enzymatic assays showed that SS-corrosion products caused inhibition of both MTT reduction and acid phosphatase activity in contrast to Fe + Cr + Ni solution which stimulated their activity. Furthermore, in all biological parameters studied, a strong association was observed between the effects of Fe + Cr + Ni solution and Cr alone, suggesting that Cr was the metal ion mostly involved in the stimulatory effects of the combined solution.

1. Introduction

There is some concern over the long-term use of metallic biomaterials in orthopaedic surgery, based on the release of implant debris and metallic ions in tissues adjacent to implanted materials (reviewed in [1–4]). Several studies have demonstrated high metal concentrations in body fluids, including serum and urine, of patients with knee or hip prosthesis [3, 5–8]. Other reports have analysed the metal ions release from metallic alloys, their transport in the body and excretion in animal models [9–13].

Stainless steel (SS) corrosion products obtained by electrochemical means have been a matter of intense research at our laboratory. In fact, injection in mice of SS-corrosion products have shown to cause not only ultrastructural changes in male reproductive organs [14] and in liver parenchyma [15], but also accumulation of multinucleated giant cells and depletion of lymphocytes in spleen [16]. Further *in vitro* studies have demonstrated that such corrosion products inhibit the functional activity of human immune cells [17]. In the current study, *in vitro* cytotoxic evaluation of SS-corrosion products was performed on human fibroblasts cultured for 21 days. The cell culture parameters analysed were: metal ion accumulation (atomic absorption spectrometry), cell morphology (neutral red assay), cell proliferation (DNA assessment), and enzyme activity (both MTT reduction and acid phosphatase activity). The effects of SS-corrosion products were compared with the effects of iron, chromium and nickel ions used either separately (Fe, Cr, or Ni solutions) or combined (Fe + Cr + Ni solution).

2. Materials and methods

2.1. AISI 316L stainless steel corrosion products and metal salts

Type AISI 316L stainless steel was anodically dissolved in HBSS (Hank's Balanced Salt Solution, Sigma) through a chronoamperometric process as

previously described [16]. Atomic absorption spectrophotometry (AAS) showed that the SS-solution contained 490 µg/ml Fe, 224 µg/ml Cr and 150 µg/ml Ni. This SS-solution was diluted 1:10 in DMEM (Dulbecco's Modified Eagles Medium, Sigma). Salt solutions containing Fe ($FeCl_3 \cdot 6H_2O$) at 490 µg/ml, Cr ($CrCl_3 \cdot 6H_2O$) at 224 µg/ml or Ni ($NiNO_3$) at 150 µg/ml were also diluted 1:10 in DMEM. A mixture of the three metal solutions (Fe + Cr + Ni) was prepared and diluted 1:10 as for the previous studies. The pH of all solutions was adjusted to 7.2 with NaOH.

2.2. Culture conditions

Human skin fibroblasts were explanted and grown in control medium, i.e. DMEM containing 5% (v/v) foetal bovine serum (Sigma) and 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma). They were incubated at 37 °C in a 10% CO_2 humidified atmosphere. Cells of the fifth passage were trypsinized and seeded at a density of 2×10^4 cells/well in 24-well culture plates (Greiner 662 160) for neutral red assay, Fe, Cr and Ni analysis and DNA assessment, or at a density of 0.1×10^4 cells/well in 96-well culture plates (Greiner 655 180) for MTT assay and determination of acid phosphatase activity. The cells were cultured in control medium for a period of 3 days at 37 °C in a 10% CO_2 humidified atmosphere.

After this settling period, the control medium was replaced by testing media. These media were prepared by diluting the above solutions 1:10 in control medium to give the final concentrations presented in Table I. The osmolarities of solutions were measured by freezing point depression (Roebling Osmometer, Berlin, Germany).

Fibroblasts were re-fed with fresh testing media (Table I) three times a week, and were allowed to grow for 21 days at 37 °C in a 10% CO_2 humidified atmosphere. Evaluation of culture parameters (Fe, Cr and Ni analysis, cell morphology, cell proliferation, cell viability, and acid phosphatase activity determination) were performed on days 4, 7, 14 and 21 of culture.

2.3. Determination of Fe, Cr and Ni contents by AAS

Fibroblasts were grown in 24-well culture plates. The cells were exposed to the testing media (Table I) in triplicates. At testing days, the culture media were removed, the cells were rinsed with PBS and trypsinized with 100 µl of trypsin solution (0.25% in PBS, containing 0.1% α-D-glucose and 0.05% EDTA) per well, at 37 °C for 10 min. When cells were detached, 900 µl of HNO_3 were added to each well and the total volume was collected in Eppendorf tubes and kept at − 20 °C until being used for atomic absorption spectrometric (AAS) analysis. Each sample was diluted in triply distilled water to a final volume of 20 or 50 ml. These solutions were then used to determine the metal ions levels by AAS.

For the determination of iron, a flame chamber Model 357 was used but the iron amount in all samples was below the detection limits. The use of the graphite furnace of a Perkin Elmer Model 4100ZL, which has higher sensitivity, for iron analysis showed several technical difficulties caused by a number of interferences by contaminating iron in the analysis system. Therefore no determination of iron could be performed in the present study.

Chromium and nickel were analysed in a graphite furnace using a Perkin Elmer Model 4100ZL. The working program for the determination of both elements was in accordance with [16]. The multiple addition method was the one chosen to perform the experimental AAS measurements.

2.4. Cell morphology and viability by light microscopy

Fibroblasts were grown in 24-well culture plates. The cells were exposed to the testing media (Table I) in triplicates. At testing days, the culture media were removed, the cells were rinsed with PBS and incubated with 0.001% neutral red (Sigma) in PBS at 37 °C in a 10% CO_2 humidified atmosphere for 30 minutes. Then the vital stain was removed and the cultures were immmediately observed under the light microscope.

2.5. Determination of cell proliferation by automatic counting

Fibroblasts were grown in 24-well culture plates. The cells were cultured in control medium (Table I) in triplicates. At testing days, the medium was removed, the cells were rinsed with PBS (phosphate buffered saline, Ca and Mg free, Sigma) and trypsinized as described above. When cells were detached, 400 µl of PBS were added to each well and the total volume (500 µl) was added to 10 ml PBS. The number of cells were read by an automatic Coulter Counter.

TABLE I Metal concentrations and osmolarities of testing media

Testing medium	Metals added to medium culture	Metal concentration (µg/ml)	Osmolarity (mOsm/l)
Medium 0	None (control medium)	---	298
Medium 1	1% SS-solution	4.9(Fe) + 2.2(Cr) + 1.5(Ni)	310
Medium 2	Fe + Cr + Ni	4.9(Fe) + 2.2(Cr) + 1.5(Ni)	352
Medium 3	Fe($FeCl_3 \cdot 6H_2O$)	4.9	312
Medium 4	Cr($CrCl_3 \cdot 6H_2O$)	2.2	340
Medium 5	Ni($NiNO_3$)	1.5	364

2.6. Determination of DNA contents of fluorospectrometry

Fibroblasts were grown in 24-well culture plates. The cells were exposed to the testing media (Table I) in triplicates. At testing days, the culture media were removed, the cells were rinsed with PBS and stored at − 20 °C. The DNA contents were determined as previously described by Vrouwenvelder *et al.* [18]. Briefly, the cells of each well were detached with 100 µl of trypsin, the volume adjusted to 0.5 ml with PBS and sonified. To this solution, 1 ml of heparin solution (5000 IU of tromboliquine diluted 1 : 600 in PBS) and 0.5 ml of RNase solution (0.05 mg/ml of ribonuclease A in PBS) were added. This mixture was allowed to react for 30–60 minutes at room temperature. Then, 0.5 ml ethidiumbromide solution (0.025 mg/ml in PBS) were added. All samples and DNA-standard series were measured on a Perkin-Elmer 1000 Model LS-3B fluorescence spectrometer, using excitation at 360 nm and emission at 590 nm.

2.7. Cellular activity by assessment of MTT reduction

The yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is taken up into cells and is reduced by enzymes of metabolically active cells, producing purple formazan crystals which accumulate within the cell. The MTT assay is a simple method to estimate the viability of cells in culture and was performed according to the method described by Ciapetti *et al.* [19] with some modifications. Briefly, fibroblasts were grown in 96-well culture plates. The cells were exposed to the testing media (Table I) in eight replicates. At testing days 10 µl of freshly prepared MTT solution (5 mg/ml MTT in PBS) were added to each well and cells were incubated for a further 4 h at 37 °C in a 10% CO_2 humidified atmosphere. Then, the medium from each well was removed, 100 µl of DMSO were added and the plates were shaken for 5 min at room temperature to dissolve the formazan crystals. The plates were read at 550 nm on a scanning multiwell spectrophotometer Titertek Multiskan Model MCC 340.

2.8. Cellular activity by assessment of acid phosphatase activity

The acid phosphatase activity was assayed according to the method described by Martin and Clynes [20] with some modifications. In brief, fibroblasts were grown in 96-well culture plates. The cells were exposed to the testing media (Table I) in eight replicates. At testing days the medium was removed, the cells were rinsed with PBS and 100 µl of substrate-buffered solution (10 mM p-nitrophenyl phosphate, 0.1 M Na-acetate and 0.1% Triton X100, at pH 5.5) were added to each well. Cells were incubated for a further 2 h at 37 °C in a 10% CO_2 humidified atmosphere. Then the reaction was stopped by adding 100 µl of 1 N NaOH. The plates were read at 405 nm on a scanning multiwell spectrophotometer Titertek Multiskan Model MCC 340.

2.9. Statistical analysis

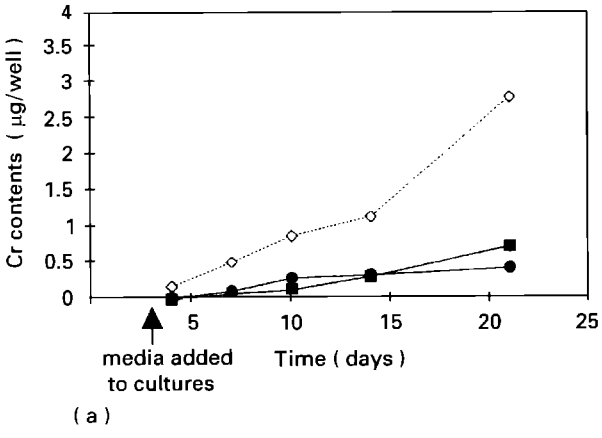
Student's *t*-test was applied to determine the statistical significance of the differences observed between means of the two groups: p values of $p < 0.05$ were considered to be significant.

3. Results

3.1. Determination of Fe, Cr and Ni in fibroblast cultures

The iron amount in all samples was below the detection limits of the flame chamber (mg/ml) and the analysis in the graphite furnace (µg/ml) was at the background level. Thus, iron accumulation in cultures could not be assessed in the present study.

In control cultures (fibroblasts grown in normal medium) chromium and nickel were not detected throughout the 21 days of the experiment. In contrast, chromium accumulated in cultures of cells exposed to SS-solution (0.5 µg/well at day 21), to Fe + Cr + Ni solution (0.8 µg/well at day 21) or to Cr solution (2.9 µg/well at day 21), as shown on Fig. 1a. Fibroblast cultures also retained nickel when exposed to SS-solution (3.7 µg/well at day 21), to Fe + Cr + Ni solution (0.3 µg/well at day 21) and to Ni solution (0.2 µg/well at day 21), as presented in Fig. 1b. These results show that fibroblast cultures preferentially retained chromium when presented as a separate ion and nickel when presented as an element of SS-corrosion products.



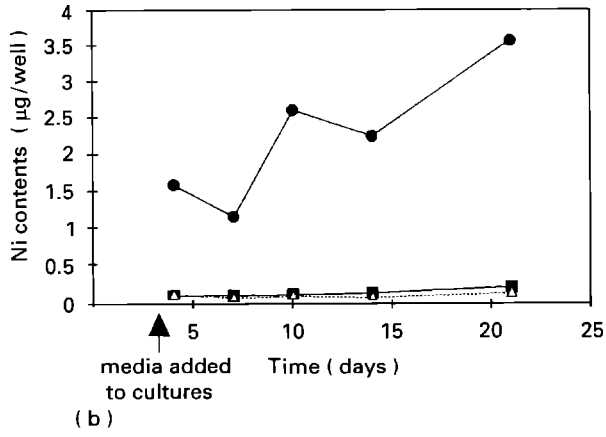


Figure 1 Concentrations of chromium (a) and nickel (b) in human fibroblast cultures grown in the presence of SS-corrosion products (—●—), Fe + Cr + Ni solution (—■—), and chromium (—◇—) or nickel solution (—△—).

3.2. Cell morphology and viability

Fibroblasts were exposed to SS-corrosion products (SS-solution) and to metal ions either combined (Fe + Cr + Ni) or separated (Fe, Cr or Ni). The ability of fibroblasts to take up the neutral red stain intracellularly was evaluated throughout the 21 days of the study. As time went on, the density of cells increased in all cell cultures. Cell viability was near 100% as all cells retained the stain intracellularly.

The control cell culture showed the normal appearance of fibroblasts as fusiform cells containing the vital stain in vesicles located in the centre of the cell, around the nucleus (Fig. 2a). Only in the case of cultures containing SS-corrosion products, deposits of metals were readily observed across the cell culture which enlarged with time; nevertheless the cells showed the normal appearance of fibroblasts (Fig. 2b). Similarly, no visible effects on cell morphology were observed on fibroblasts cultured in the presence of Ni solution (Fig. 2f). In contrast, fibroblasts exposed to Fe + Cr + Ni solution and to Fe or Cr solutions were larger and contained higher amounts of neutral red stain than control cells (Fig. 2c–e). These results show that Fe + Cr + Ni solution, but not SS-corrosion products, caused accumulation of neutral red stain intracellularly and further suggest that iron and chromium were the metal ions most involved in these morphological alterations.

3.3. Cell proliferation

Preliminary studies showed that cell proliferation could not be determined by using the automatic Coulter Counter, because the SS-corrosion products containing medium (SS-solution) caused interference in the counting system. This was probably due to the

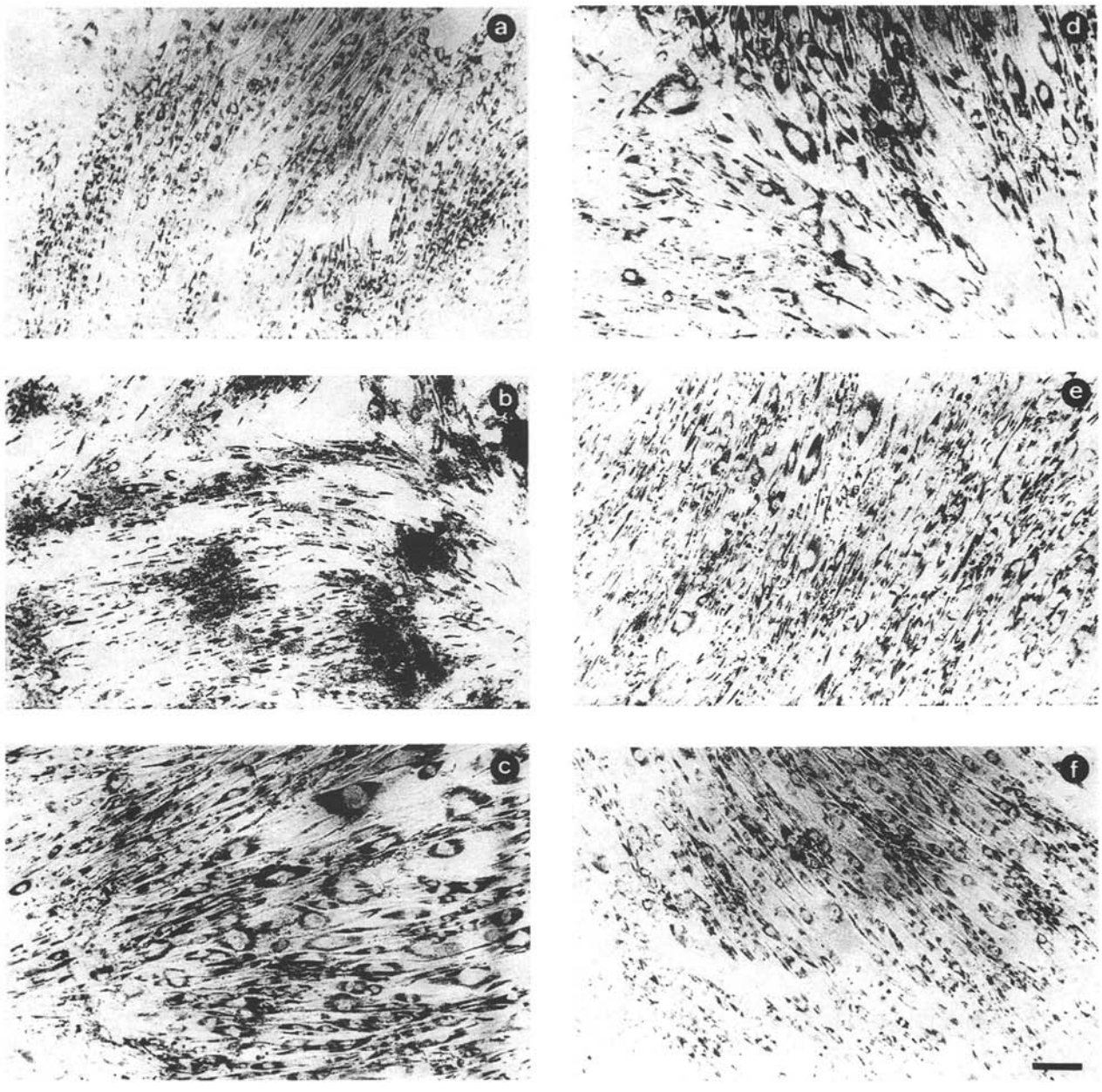


Figure 2 Human fibroblasts cultured for 21 days in control medium (a) and in the presence of SS-corrosion products (b) and metal ions either combined Fe + Cr + Ni (c) or alone Fe (d), Cr (e) or Ni (f). Bar = 20 μm.

formation of metallic particles in medium, which were counted as cells. Therefore, an alternative, biochemical method specific for DNA assessment was used.

A comparative study of the assessment of cell proliferation by either method (the number of cells by Coulter Counter, or the DNA contents by a biochemical assay) was performed in fibroblasts grown in control medium. Similar patterns of fibroblast proliferation were obtained by both methods (Fig. 3), indicating that the alternative biochemical analysis of DNA was a reliable method for evaluation of cell proliferation.

The biochemical analysis of DNA contents in cultures showed that the exposure of fibroblasts to SS-corrosion products and to metal ions either combined (Fe + Cr + Ni) or separated (Fe, Cr or Ni) caused no differences in cell proliferation up to 10 days of culture (Fig. 4). However after day 14, and when compared to the control medium, a significant increase ($p < 0.05$) of cell proliferation was observed on fibroblasts cultured in the presence of either Fe + Cr + Ni or Cr solution (Fig. 4). The Fe solution caused increase of cell proliferation later, at day 21, and none of the other metal-containing media (SS-solution and Ni solution) caused alterations ($p > 0.05$) in the cell growth throughout the experimental study (Fig. 4). These results show that Fe + Cr + Ni solution, but not SS-corrosion products, activated fibroblast cell growth and further suggest that chromium was the metal ion most involved in this stimulatory effect of cell proliferation.

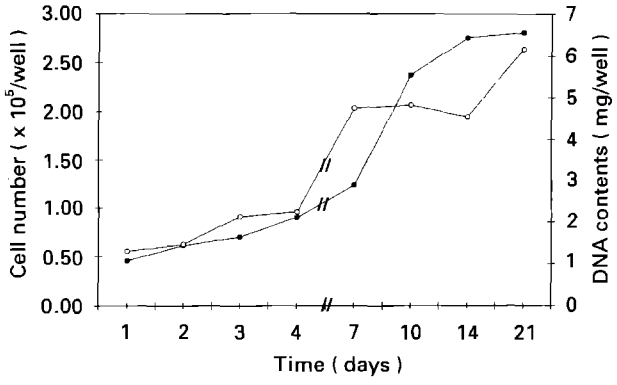


Figure 3 Cell proliferation patterns of human fibroblasts cultured in control medium, obtained by estimation of the cell numbers (by Coulter Counter) (—●—) and DNA analysis (by a biochemical assay) (-○-). The values represent means of triplicates, and SD were lower than 10% for the estimation of the cell numbers and lower than 30% for the DNA biochemical analysis.

3.4. MTT reduction

Reduction of the tetrazolium salt, MTT, by human fibroblasts was studied in cells cultured for the period of 21 days in the presence of SS-corrosion products (SS-solution) and metal ions either combined (Fe + Cr + Ni) or separated (Fe, Cr or Ni). After 7 days, and when compared to the control, significant increases ($p < 0.001$) in MTT reduction were observed in fibroblasts exposed to Fe + Cr + Ni solution or to Cr solution (Fig. 5). The Fe solution caused increase ($p < 0.001$) of MTT reduction at day 21 only. In contrast SS-corrosion products caused a significant decrease ($p < 0.05$) in MTT reduction after day 7 of culture. No significant changes ($p > 0.05$) were observed in fibroblasts exposed to Ni solution (Fig. 5). These results show that SS-corrosion products and Fe + Cr + Ni solution caused contrary effects on fibroblast viability, and further indicate that chromium is the metal ion most involved in the increase of MTT reduction caused by Fe + Cr + Ni solution.

3.5. Acid phosphatase activity

The acid phosphatase (AcP) activity of human fibroblasts grown in the presence of metal ions showed that Fe + Cr + Ni solution caused a significant increase ($p < 0.05$) whereas SS-solution ($p < 0.05$) and Fe

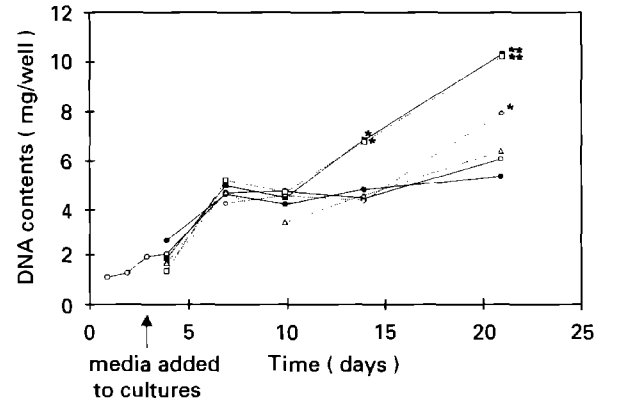


Figure 4 Cell proliferation, estimated by DNA analysis, of human fibroblasts cultured in the presence of SS-corrosion products (SS-solution) and metal ions either combined (Fe + Cr + Ni) or separated (Fe, Cr or Ni). —○— control; —●— SS-solution; —■— Fe + Cr + Ni; —◇— Fe (5μg/ml); —□— Cr (2.3 μg /ml); —△— Ni (1.5 μg/ml). *Significant differences ($0.001 < p < 0.05$) between groups of metal exposed cells and control cells. **Significant differences ($p < 0.001$) between groups of metal exposed cells and control cells.

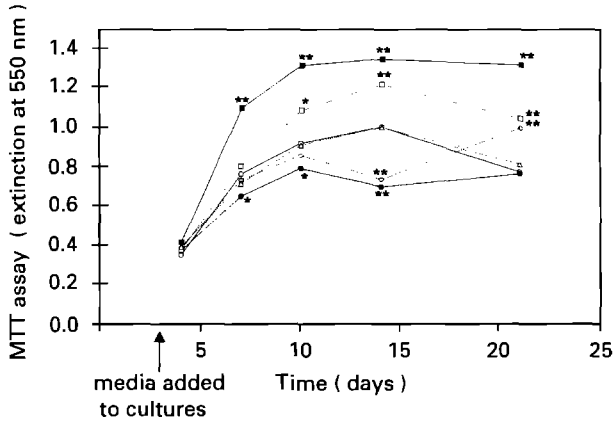


Figure 5 Cell viability, estimated by MTT reduction, of human fibroblasts cultured in the presence of SS-corrosion products (SS-solution) and metal ions either combined (Fe + Cr + Ni) or separated (Fe, Cr or Ni). —○— control; —●— SS-solution; —■— Fe + Cr + Ni; —◇— Fe; —□— Cr; —△— Ni. *Significant differences ($0.001 < p < 0.05$) between groups of metal exposed cells and control cells. **Significant differences ($p < 0.001$) between groups of metal exposed cells and control cells.

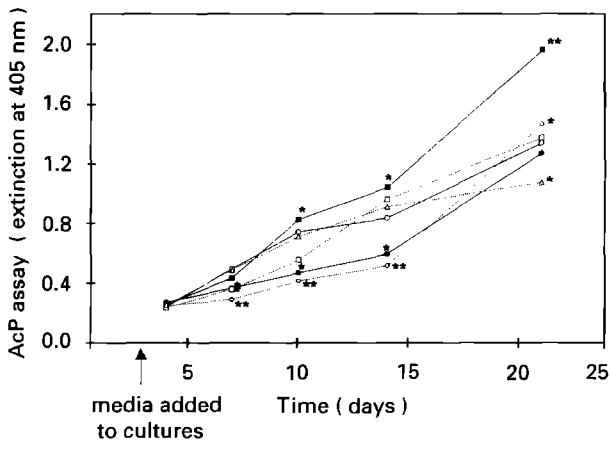


Figure 6 Acid phosphatase activity of human fibroblasts cultured in the presence of SS-corrosion products (SS-solution) and metal ions either combined (Fe + Cr + Ni) or separated (Fe, Cr or Ni). -o- control; -●- SS-solution; —■— Fe + Cr + Ni; —◇— Fe; —□— Cr; —△— Ni. *Significant differences ($0.001 < p < 0.05$) between groups of metal exposed cells and control cells. **Significant differences ($p < 0.001$) between groups of metal exposed cells and control cells.

solution ($p > 0.001$) caused a reduction of AcP activity (Fig. 6). These results show that SS-corrosion products and Fe + Cr + Ni solution caused contrary effects on AcP activity of fibroblasts, and further indicate that iron is the metal ion most involved in the decrease of AcP activity caused by SS-solution.

4. Discussion

The aim of this study was to evaluate the cytotoxicity of 316 L stainless steel corrosion products and the corresponding metal ions (iron, chromium and nickel) used either separately (Fe, Cr and Ni) or combined (Fe + Cr + Ni). The concentration of metal ions was in the range of iron, chromium and nickel found in tissues adjacent to 316 L stainless steel plates and screws [1]. Several testing methods have been developed for *in vitro* evaluation of material cytotoxicity, including dye exclusion tests [21, 22], cell counting [23, 24], incorporation [25, 26] and release [27, 28] of radioactive isotopes, protein contents [29], and enzyme activity [20, 30, 31]. These biological parameters are associated with various types of cell damage which can occur as a result of deleterious effects caused by toxic agents, and all contribute for the evaluation of cell viability.

The biological parameters selected in the present study were the following: (a) neutral red exclusion test to evaluate cell viability and to facilitate the microscopic observation of fibroblasts, (b) DNA contents to estimate cell proliferation, and (c) MTT reduction test and (d) AcP assay, both to assess the enzymatic activity of cells. The high cell viability estimated by the neutral red uptake by fibroblasts exposed to all metal ion solutions was the first evidence that all solutions used in this study were not cytotoxic. Further assessment of fibroblast proliferation and fibroblast enzymatic activities (MTT reduction and AcP activity) indicated that SS-corrosion products caused no effects on cell proliferation but inhibited enzymatic activities, whereas Fe + Cr + Ni solution stimulated neutral red uptake, cell proliferation, MTT reduction and acid phosphatase activity. These contrasting effects of SS-corrosion products and Fe + Cr + Ni solution on fibroblast cultures may be due to different valence states of oxidation of Fe (2 + or 3 +) and Cr (3 + or 6 +), or due to the effects of the ionic strength of the media, which is higher for the combined metals than for the metal ions alone. Also, several intermediates can be formed (more frequently for the Fe + Cr + Ni solution) which were not assessed experimentally but might cause kinetic and thermodynamic complexities within the cellular cultures.

In all biological parameters studied, a strong association was observed between the effects of Fe + Cr + Ni solution and Cr alone, suggesting that Cr was the metal ion mostly involved in the stimulatory effects of the combined solution. Conversely, similar effects were observed on fibroblast cultures exposed to SS-corrosion products and Ni solution. In fact the AAS analysis of cell cultures confirmed that Ni was the ion which accumulated most in cultures exposed to SS-corrosion products.

The present study indicates that stainless steel corrosion products do not interfere with fibroblast proliferation but inhibit enzymatic activities. In contrast, the corresponding metal ions from salt solutions (Fe + Cr + Ni solution) cause stimulation of cell proliferation and enzyme activities, both MTT reduction and AcP activity. Thus attention must be given not only to the metal ions alone but also to the mixture of ions which can cause diverse changes in biological activities of cells in culture.

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