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Effects of Co–Cr corrosion products and corresponding separate metal ions on human osteoblast-like cell cultures

H. TOMÁS*, G. S. CARVALHO‡, M. H. FERNANDES#§, A. P. FREIRE*, L. M. ABRANTES*
*Faculdade de Ciências da Universidade de Lisboa, Campo Grande, C1, Piso 5, 1700 Lisboa Portugal
‡CEFOPE, Universidade do Minho, 4719 Braga codex Portugal
#Faculdade de Medicina Dentária da Universidade do Porto, R. Dr Roberto Frias, 4200 Porto Portugal
§Instituto de Engenharia Biomédica, Praça Coronel Pacheco, 1, 4000 Porto Portugal

The cytocompatibility of the degradation products of a Co–Cr orthopaedic alloy was investigated with particular focus on the dose-effect of an electrochemically dissolved alloy extract and of the corresponding separate metal ions on human osteogenic bone marrow derived cells. The extract solution contained 15 ppm of Co and 8 ppm of Cr as analysed by atomic absorption spectroscopy. Stock salt solutions of $CoCl_2 \cdot 6H_2O$, $CrCl_3 \cdot 6H_2O$ and Na_2CrO_4 at corresponding concentrations were also prepared. Several dilutions of the above metallic solutions were tested for a period of 21 days on cells (third subculture) cultured in α-minimal essential medium containing foetal bovine serum and supplemented with antibiotics, dexamethasone ascorbic acid and β-glycerophosphate. The osteoblast response to the presence of metal ions was evaluated by several biochemical parameters: cell viability (MTT reduction by intracellular enzymes), alkaline phosphatase activity (an osteoblast marker) and protein production (both intracellular and extracellular). Co–Cr corrosion products showed opposite effects to their respective metal salts only on day 1. With time the different metal solutions presented a similar pattern of inhibition. These results suggest that impaired bone formation *in vitro* can occur in the presence of Co–Cr corrosion products.

1. Introduction

Despite the good corrosion resistance of cobalt–chromium alloys which are currently used in orthopaedics, the accumulation of metal ions at the implant site and their distribution throughout the body, via systemic circulation, has been cited as a cause of health concern [1–9]. Corrosion has been associated to the formation of the fibrous capsule that frequently involves Co–Cr implants keeping osteogenesis at a considerably long distance [10]. Osteolysis, which is a phenomenon of bone loss, has also been reported to occur around Co–Cr components used in total hip arthroplasty [9]. Among other factors, metallic debris resulting from corrosion and wear have been considered as a cause of bone loss [11, 12].

The cellular events taking place at the bone/material interface can be determinant for the success of an implant. These aspects can be better studied through *in vitro* models using well-characterized osteoblasts [13, 14], as they are ideal systems for dose–effect quantification of biomaterial degradation products [15].

The objective of the present work was to study the dose- and time-effect of Co–Cr corrosion products and the corresponding metal ions, Co^{2+} and Cr^{3+} or Cr^{6+}, on human osteoblast-like cells from bone marrow origin, by analysing several biochemical parameters: MTT reduction by intracellular enzymes, alkaline phosphatase (ALP) activity and protein production.

2. Materials and methods

2.1. Metallic solutions

A suitable electrochemical technique [16] was used to dissolve (in NaCl 0.15 M) a sample of a Co–Cr–Mo orthopaedic alloy with composition (percentage by weight): Co(balance), Cr(28), Mo(5.5), Ni(1), Si(0.95), Fe(0.7), Mn(0.65) and C(0.25). An extract solution containing 15 ppm Co and 8 ppm Cr was obtained as determined by atomic absorption spectrophotometry (in a Pye Unicam SP9 spectrophotometer with an air–acetylene flame).

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TABLE I Culture time and metallic concentrations (ppm) tested

Solution number	Culture days	Extract [Co]	Extract [Cr]	Co^{2+} [Co]	Cr^{3+} [Cr]	Cr^{6+} [Cr]
1	1,14	7×10^{-4}	4×10^{-4}	7×10^{-4}	4×10^{-4}	4×10^{-4}
2	1,7,14,21	3×10^{-3}	2×10^{-3}	3×10^{-3}	2×10^{-3}	2×10^{-3}
3	1,14	1×10^{-2}	6×10^{-3}	1×10^{-2}	6×10^{-3}	6×10^{-3}
4	1,7,14,21	5×10^{-2}	3×10^{-2}	5×10^{-2}	3×10^{-2}	3×10^{-2}
5	1,14	2×10^{-1}	1×10^{-1}	2×10^{-1}	1×10^{-1}	1×10^{-1}
6	1,7,14,21	8×10^{-1}	4×10^{-1}	8×10^{-1}	4×10^{-1}	4×10^{-1}
7	1,14	3	1.6	3	1.6	1.6

$CoCl_2 \cdot 6H_2O$, $CrCl_3 6H_2O$ and Na_2CrO_4 stock solutions were also prepared in NaCl 0.15 M at extract equivalent concentrations.

After pH adjustment (to 7.4) and sterilization in an autoclave, further solutions were obtained by successive dilutions (1:4).

2.2. Cell culture

Human bone marrow cells (third subculture) were seeded at an initial density of 10^4 cells/cm^2. The control (NaCl 0.15 M) and all the above metallic solutions were diluted (1:5) in α-MEM (α-minimal essential medium, Sigma) with 10% foetal bovine serum (Sigma), 2.5 μg/ml fungizone (Gibco), 50 μg/ml gentamicin (Gibco), 10^{-8} M dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma) and 50 μg/ml ascorbic acid (Sigma). These media were initially added to cells and then renewed twice a week. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 and were carefully watched throughout the experiments using an Olympus CK2 phase contrast inverted optical microscope.

2.3. Biochemical assays

Biochemical assays were carried out at days 1 and 14 for all tested solutions and also at days 7 and 21 for selected metallic concentrations. Table I shows the final Co and Cr concentrations in the culture (numbered from 1 to 7) and the testing days for each case.

2.3.1. MTT assay

The cellular reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to produce a purple formazan product was used to estimate cell viability and proliferation. At the end of the culture time, osteoblasts (cultured in 96-well plates) were incubated with MTT (0.5 mg/ml in culture medium) for 4 h at the culture conditions. The culture medium was then decanted, the formazan salts were dissolved with DMSO (dimethylsulphoxide) and the optical density (OD) was measured at 600 nm. Results are the mean of eight replicates.

2.3.2. Alkaline phosphatase activity and protein contents

At the end of culture time, osteoblasts cultured in 24-well plates were rinsed twice with PBS (phosphate buffered solution) and stored at − 20 °C. Later, PBS with Triton X-100 was added to each well, the cells were scraped from the plastic surface and the suspension was sonified. The sample was then stored again at − 20 °C.

The total protein contents were evaluated according to the Lowry method [17]. The quantities of all involved reagents were adapted to 40 μl protein-containing samples and direct absorbance measurement (600 nm) in 96-well plates. Bovine serum albumin was used for standards preparation.

ALP activity was determined by incubating, at 37 °C, 100 μl of the sample with 100 μl of 20 mM p-nitrophenylphosphate prepared in a pH = 10.3 buffer solution (1 M diethanolamine and 1 mM $MgCl_2$). The reaction was stopped after 15 min by addition of 0.5 ml of NaOH 0.2 M and the p-nitrophenol (yellow product) content was measured by comparing the absorbance at 405 nm with a series of standards.

For both ALP activity and protein content, the results are the mean of three replicates.

2.4. Histochemical characterization

Cultures were fixed with 1.5% glutaraldehyde (prepared in 0.14 M sodium cacodylate buffer, pH = 7.4) and rinsed with distilled water.

ALP staining: a solution prepared in Tris buffer (pH = 10) and containing a mixture of Na-α-naphtyl phosphate (2 mg/ml) and fast blue RR salt (2 mg/ml) was added to fixed cells. After 1 h in the dark, cells were rinsed in tap water and observed in an Olympus BH-2 optical microscope. ALP positive cells were stained brown.

Phosphates: fixed cultures were covered with a 1% silver nitrate solution and kept for 1 h under UV light. After rinsing, a 5% sodium thiosulphate solution was added for 2 min and cultures were washed again. Phosphate deposits gave a black reaction.

Calcium: fixed cultures were covered with a 1% S alizarin sodium sulfonate solution (0.028% v/v in NH_4OH), pH = 6.4, for 2 min and then rinsed with water and ethanol (0.01% v/v in HCl). Calcium deposits were red stained.

2.5. Statistical analysis

The percentage alteration of MTT reduction relative to the control values was calculated according to the formula:

$$\% \text{ alteration} = (OD_{\text{test, 600 nm}}/OD_{\text{control, 600 nm}}) \times 100 - 100$$

Student's t-test was applied to determine the statistical significance of the differences between groups of results: p values lower than 0.05 were considered significant.

3. Results and discussion

The osteogenic nature of the primary culture and of the first and second subcultures of bone marrow derived cells was confirmed histochemically by showing a strong intracellular ALP reaction and extracellular matrix mineralization.

In the present work, the third subculture was used to study the cytocompatibility of Co–Cr alloy corrosion products and the corresponding metal ions, Co^{2+} and Cr^{3+} or Cr^{6+}. Both chromium valences were tested due to the doubts concerning the oxidation state of chromium release from such Co–Cr implants. This third subculture also showed strong histochemical reaction for intracellular ALP in the control and metal exposed cultures, confirming the osteoblast phenotype of the cells (data not shown). On day 14, a few nodules of mineralization were observed only in the control culture, indicating the onset of such a process [18]. However, the aim of this paper was to evaluate metal ions cytocompatibility by studying several biochemical parameters. Thus, cell viability/cytotoxicity was estimated using the MTT assay, and specific cytocompatibility, associated to osteoblast phenotypic expression, was evaluated by protein production and ALP activity.

3.1. Cell viability/cytotoxicity

Despite the controversy and the lack of knowledge related to the enzyme systems which reduce MTT, and to the localization of this reaction inside cells [19], the MTT assay has been widely used to measure cells viability/proliferation and to estimate the toxicity of several agents [20, 21]. Reduction of the MTT by human osteoblasts was then studied in cells either exposed to several concentrations of metal ions and cultured for 1 and 14 days (Fig. 1), or exposed to selected concentrations (Nos 2, 4 and 6 – see Table I) and cultured for 1, 7, 14 and 21 days (Fig. 2).

The effects of metal ions on human osteoblast-like cells were shown to be dose- and time-dependent. In fact, at the beginning of cell culture, day 1, only the highest extract concentration (No. 7) caused significant inhibition of the MTT assay (Fig. 1a). In contrast, Co^{2+} (Fig. 1a), Cr^{3+} and Cr^{6+} (Fig. 1b) caused stimulation of the MTT assay, particularly significant at lower concentrations. These opposite effects of Co–Cr corrosion products, as compared to the corresponding metal ions on osteoblast cell cultures, are in agreement with previous studies in which MTT and other enzymatic activities of human fibroblasts are inhibited by stainless steel corrosion products but stimulated by metal salts [22].

On the day 7 of culture the MTT assay was lower than on day 1, and similar in all cultures (Fig. 2) suggesting that during this first week the cells were

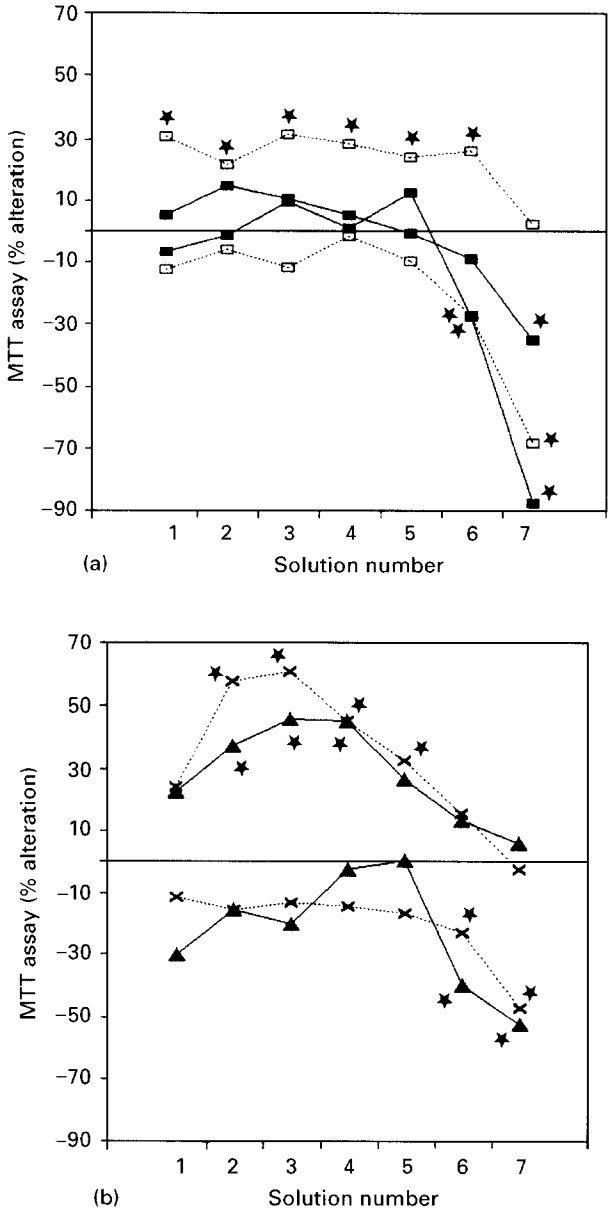


Figure 1 Percentage alteration in the MTT assay relative to the control. Effects on days 1 and 14 of several concentrations of the Co–Cr extract and Co^{2+} (a). Cr^{3+} and Cr^{6+} (b) salts are shown (MTT results standard deviations were in general lower than 20%). *Significant differences ($p < 0.05$) between groups of metal exposed cells and control cells. Day 1: ext —■—; Co -⊟-; Cr^{3+} —▲—; --×-- Cr^{6+}. Day 14: ext —■—; Co -⊟-; Cr^{3+} —▲—; Cr^{6+} --×--.

adapting (lag phase) to the culture conditions and that some cells probably died. After day 7 the viable cells entered an exponential growth phase (Fig. 2). The highest concentrations (Nos 6 and 7) of all metallic solutions caused significant decrease in the MTT assay at day 14 (Figs 1 and 2), which was even more evident at day 21 (Fig. 2). Significant differences were found in the inhibitory effects caused by the highest concentrations (No. 6) of the metallic solutions (Fig. 2).

3.2. Specific cytocompatibility

The total protein content detected in cultures is both intracellular and extracellular (the latter mainly collagen, according to [23]). Total protein was estimated in the control and in cultures exposed to the

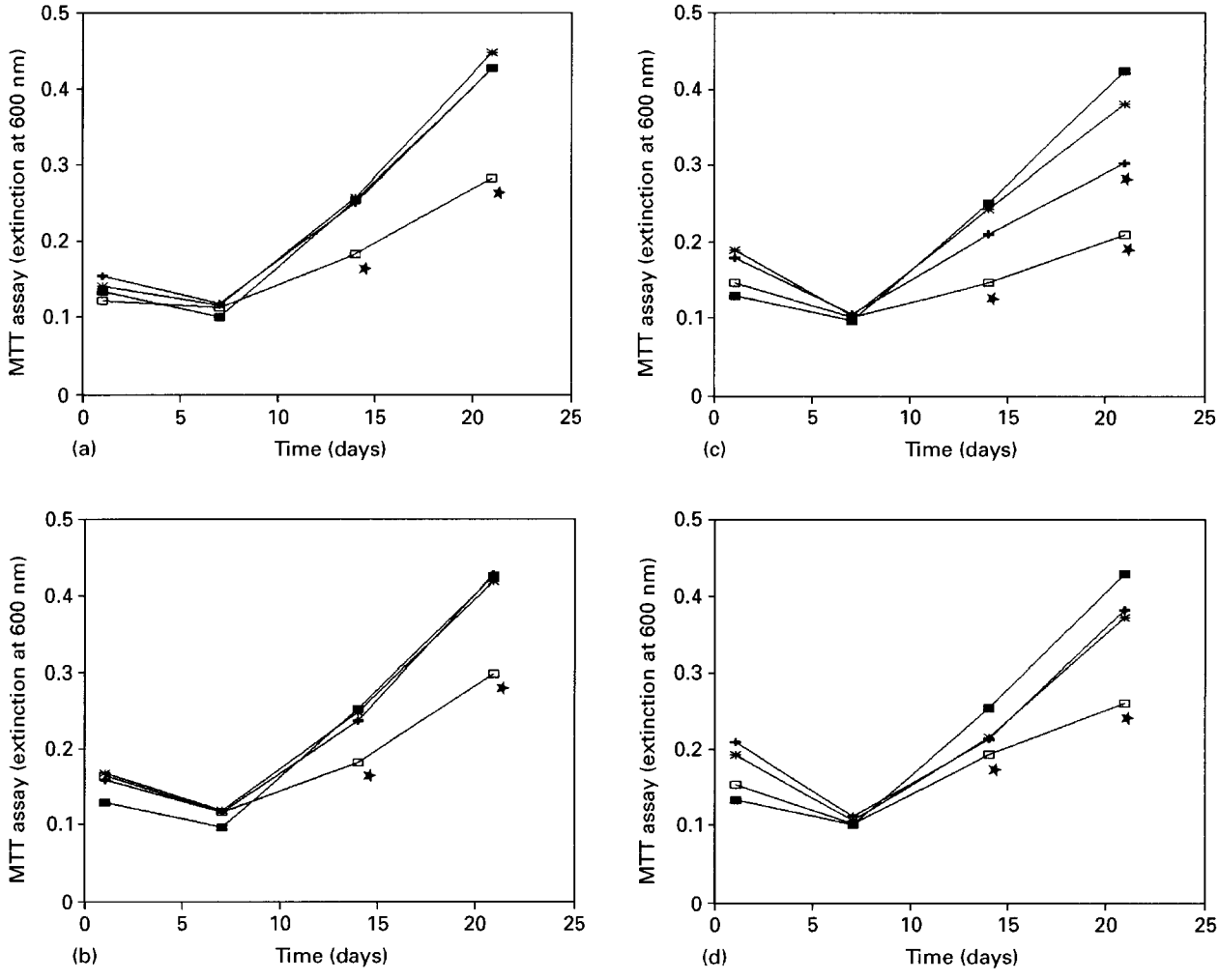


Figure 2 Cell viability estimated by the MTT assay. Effects over 21 days of selected concentrations (Nos 2, 4 and 6) of the Co–Cr extract (a), Co^{2+} (b), Cr^{3+} (c) and Cr^{6+} (d) salts are shown (MTT results standard deviations were in general lower than 20%). *Significant differences ($p < 0.05$) between groups of metal-exposed cells and control cells. Control —■—; extract 2/Co 2/Cr^{3+} 2/Cr^{6+} 2 —+—; extract 4/Co 4/Cr^{3+} 4/Cr^{6+} 4 —✳—; extract 6/Co 6/Cr^{3+} 6/Cr^{6+} 6 —□—.

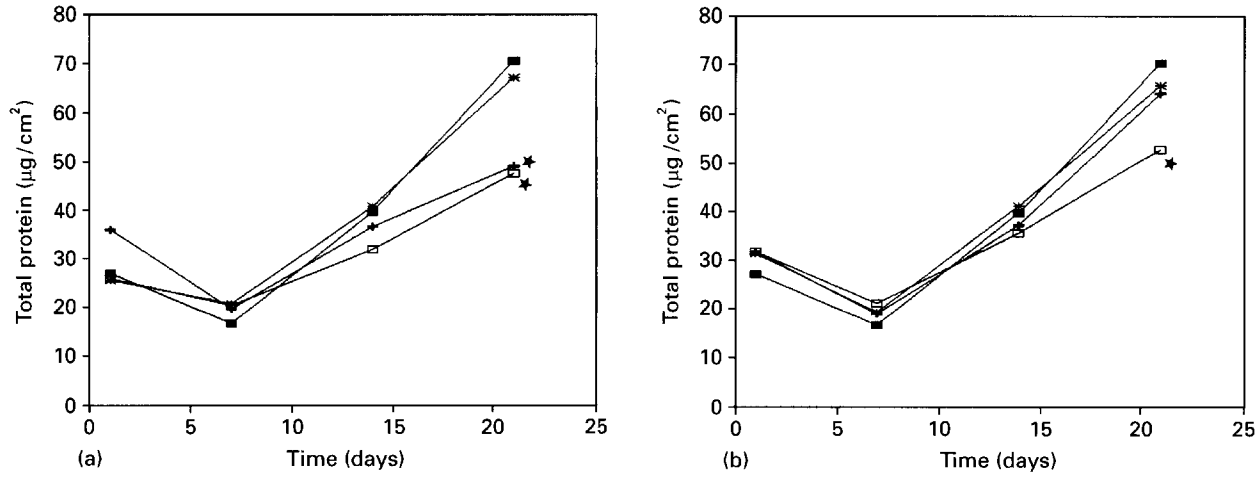
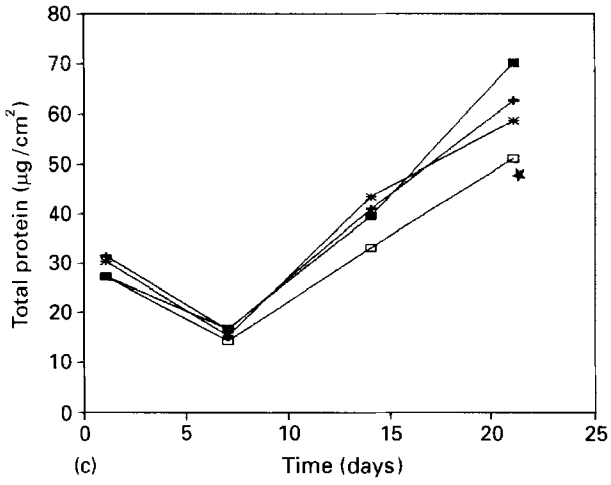


Figure 3 Total protein contents. Effects over 21 days of selected concentrations (Nos 2, 4 and 6) of the Co–Cr extract (a) and Co^{2+} (b), Cr^{3+} (c), and Cr^{6+} (d) salts are shown (standard deviations were in general lower than 35%).*Significant differences ($p < 0.05$) between groups of metal-exposed cells and control cells. Control —■—; extract 2/Co 2/Cr^{3+} 2/Cr^{6+} 2 —+—; extract 4/Co 4/Cr^{3+} ^{4}/Cr^{6+} 4 —✳—; extract 6/Co 6/Cr^{3+} 6/Cr^{6+} 6 —□—.

selected concentrations of metal solutions (Nos 2, 4, 6) as shown in Fig. 3. Statistically significant differences in protein production were observed only at day 21 for the Co–Cr extract with Co^{2+} and Cr^{3+} at the highest concentrations (Fig. 3). Interesting is the fact that a lower protein content was observed on day 7 compared to day 1 (Fig. 3), in accordance with the above data on MTT (Fig. 2). Similarly, the ALP activity on day 7 was lower than that observed on day 1 (Fig. 4). At day 21 the Co–Cr extract, Cr^{3+} and Cr^{6+}, caused inhibition of the ALP activity of the cultured osteoblasts (Fig. 4).



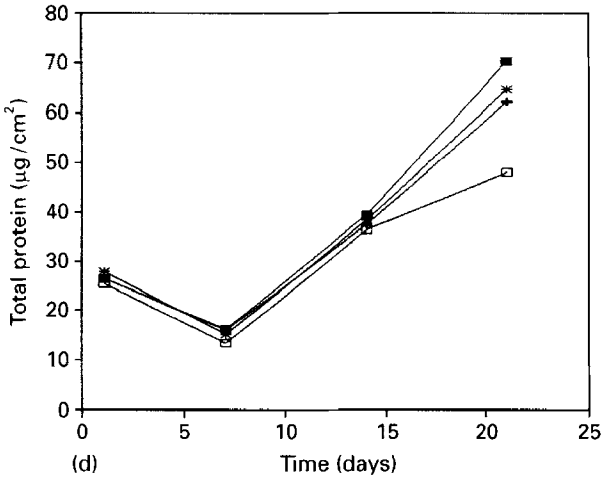
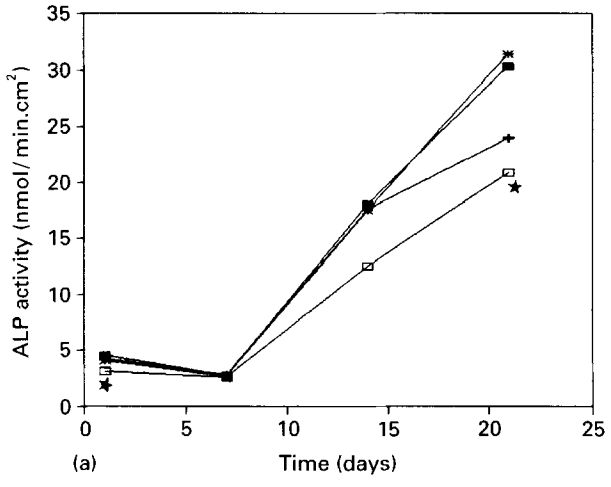
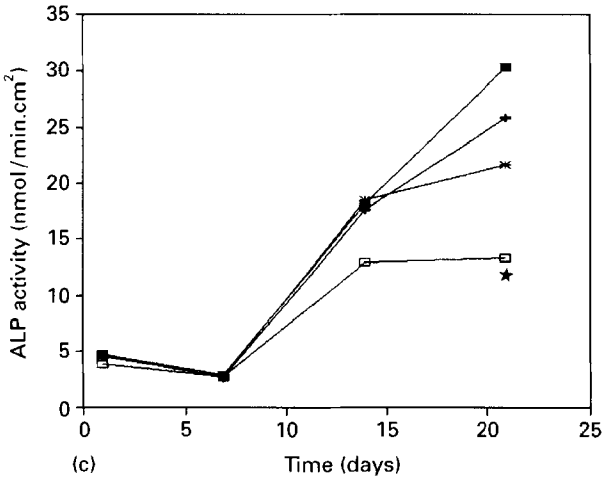
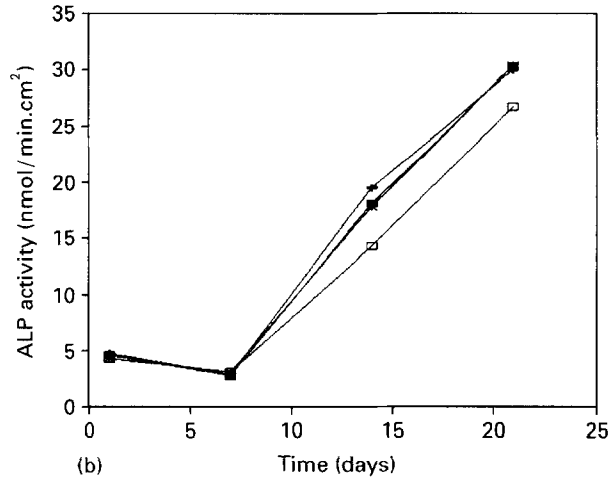


Figure 3 (Continued).







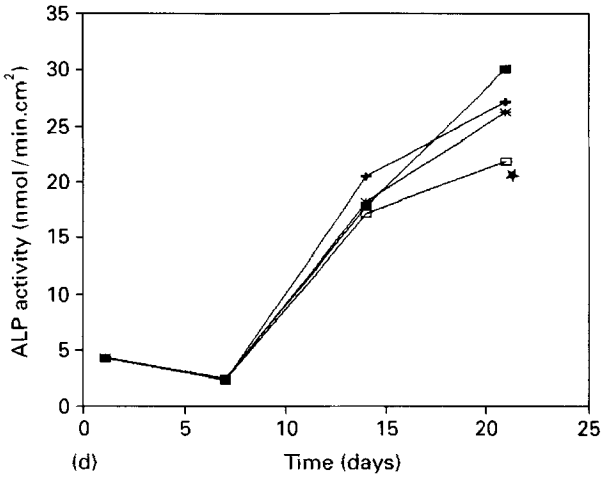


Figure 4 ALP activity. Effects over 21 days of selected concentrations (Nos 2, 4 and 6) of the Co–Cr extract (a) and Co^{2+} (b), Cr^{3+} (c), and Cr^{6+} (d) salts are shown (standard deviations were in general lower than 30%).*Significant differences ($p < 0.05$) between groups of metal-exposed cells and control cells. Control —■—; extract 2/Co 2/Cr^{3+} 2/Cr^{6+} 2 —+—; extract 4/Co 4/Cr^{3+} 4/Cr^{6+} 4 —✳—; extract 6/Co 6/Cr^{3+} 6/Cr^{6+} 6 —□—.

4. Conclusions

At the experimental conditions described in this paper, Co–Cr corrosion products showed opposite effects to their respective metal salts only on day 1. With time, all the different metal solutions presented a similar pattern of inhibition as estimated by cell viability (MTT reduction by intracellular enzymes), ALP activity (a specific marker of osteoblasts) and protein production (both intracellular and extracellular). Since ALP plays a crucial role in the initiation of mineralization [24], the findings presented in this work indicate that defective bone formation occurs above a certain

level of Co–Cr corrosion products concentration. Thus, these *in vitro* results are in agreement with the finding of bone loss around Co–Cr components used in total hip arthroplasty [9].

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References

1. O. E. M. POHLER, in "Biomaterials in reconstructive surgery" (C. V. Mosby, St. Louis, 1983) p. 158.
2. J. BLACK, P. OPPENHEIMER, D. M. MORRIS, A. M. PEDUTO and C. C. CLARK, *J. Biomed. Mater. Res.* **21** (1987) 1213.
3. F. W. SUNDERMAN, Jr., S. M. HOPFER, T. SWIFT, W. N. REZUKE, L. ZIEBKA, P. HIGHMAN, B. EDWARDS, M. FOLCIK and H. R. GOSSLING, *J. Orthop. Res.* **7** (1989) 307.
4. M. TRAISNEL, D. MAGUER, H. F. HILDEBRAND, *Clin. Mater.* **5** (1990) 309.
5. R. M. MOORE, Jr., S. HAMBURGER, L. J. JENG and P. M. HAMILTON, *J. Appl. Biomater.* **2** (1991) 127.
6. E. F. HENNING, H. J. RAITHEL, K. H. SCHALLER and J. R. DÖHLER, *J. Trace Elem. Electrolytes Health Dis.* **6** (1992) 239.
7. S. A. BROWN, K. ZHANG, K. MERRITT and J. H. PAYER, *J. Biomed. Mater. Res.* **27** (1993) 1007.
8. J. ALBORE-SAAVEDRA, F. VUITCH, R. DELGADO, E. WILEY and H. HAGLER, *Amer. Surg. Pathol.* **18** (1994) 83.
9. F. F. BUECHEL, D. DRUCKER, M. JASTY, W. JIRANEK and W. H. HARRIS, *Clin. Orthop. Related Res.* **298** (1994) 202.
10. J. D. DE BRUIJN, PhD thesis, Leiden (1993) p. 9.
11. S. NASSER, P. A. CAMPBELL, D. KILGUS, N. KOSSOVSKY and H. C. AMSTUTZ, *Clin. Orthop.* **261** (1990) 171.
12. P. A. REVELL, B. WEIGHTMAN, M. A. R. FREEMAN and B. V. ROBERTS, *Arch. Orthop. Trauma Surg.* **91** (1978) 167.
13. J. VILAMITJANA-AMEDEE, R. BAREILLE, F. ROUAIS, A. I. CAPLAN and M. F. HARMAND, *In Vitro Cell. Dev. Biol.* **29A** (1993) 699.
14. R. BIZIOS, *Biotechnol. Bioeng.* **43** (1994) 582.
15. A. PIZZOFERRATO, G. CIAPETTI, S. STEA, E. CENNI, C. R. ARCIOLA, D. GRANCHI and L. SAVARINO, *Clin. Mater.* **15** (1994) 173.
16. H. TOMÁS, A. P. FREIRE and L. M. ABRANTES, *J. Mater. Sci. Mater. Med.* **5** (1994) 446.
17. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. Biol. Chem.* **193** (1951) 256.
18. C. MANIATOPOULOS, J. SODEK and A. H. MELCHER, *Cell Tissue Res.* **254** (1988) 317.
19. M. V. BERRIDGE and A. S. TAN, *Arch. Biochem. Biophys.* **303** (1993) 474.
20. G. CIAPETTI, E. CENNI, L. PRATELLI and A. PIZZOFERRATO, *Biomaterials* **14** (1993) 359.
21. A. MARTIN and M. CLYNES, *Cytotechnology* **11** (1993) 49.
22. G. S. CARVALHO, M. CASTANHEIRA, I. DIOGO, A. M. ABREU, J. P. SOUSA, J. A. LOON and C. A. VAN BLITTERSWIJK, *J. Mater. Sci. Mater. Med.* (in press).
23. Y. GOTOH, K. HIRAIWA and M. NAGAYAMA, *Bone and Mineral* **8** (1990) 239.
24. C. G. BELLOWS, J. E. AUBIN and J. N. M. HEERSCHE, *ibid.* **14** (1991) 27.

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