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Effects of AISI 316L corrosion products in in vitro bone formation

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Abstract

Rat bone marrow cells were cultured in experimental conditions that favour the proliferation and differentiation of osteoblastic cells (i.e., 2.52×10^{-4} moll^{-1} ascorbic acid, 10^{-2} moll^{-1} β-glycerophosphate and 10^{-8} moll^{-1} dexamethasone) in the absence and in the presence of stainless-steel corrosion products, for a period of 18 days. An AISI 316L stainless-steel slurry (SS) was obtained by electrochemical means and the concentrations of the major metal ions, determined by atomic absorption spectrometry, were 8.78×10^{-3} moll^{-1} of Fe, 4.31×10^{-3} moll^{-1} of Cr and 2.56×10^{-3} moll^{-1} of Ni. Bone marrow cells were exposed to 0.01, 0.1 and 1% of the SS and at the end of the incubation period, control and treated cultures were evaluated by histochemical assays for the identification of the presence of alkaline phosphatase and also calcium and phosphate deposition. Cultures were further observed by scanning electron microscopy. Levels of total and ionised calcium and phosphorus in the culture media collected from control and metal exposed cell cultures were also quantified. Histochemical staining showed that control cultures presented a strong reaction for the presence of alkaline phosphatase and exhibited formation of calcium and phosphates deposits. The presence of 0.01% SS caused no detectable biological effects in these cultures, 0.1% SS impaired osteoblastic behaviour and, 1% SS resulted in cell death. In the absence of bone cells, levels of total and ionised calcium and phosphorus in the control and metal added culture medium were similar throughout the incubation period. A significant decrease in the levels of ionised calcium and phosphorus were observed in the culture medium of control cultures and also in cultures exposed to 0.01% SS after two weeks of incubation, an event related with the formation of mineral calcium phosphate deposits in these cultures. In cultures grown in the presence of 0.1 and 1% SS corrosion products, levels of calcium and phosphorus were similar to those observed in the absence of cells.

Results showed that stainless-steel corrosion products above certain concentrations may disturb the normal behaviour of osteoblast-like rat bone marrow cell cultures.

Keywords: Osteoblast-like cells; Mineralisation; Stainless-steel corrosion products

1. Introduction

The cellular events taking place at the bone/material interface can be determinant for the success of the implant long-term performance [1–4]. There is some concern over the use of metallic biomaterials in orthopaedic surgery, based on the release of implant debris and metallic ions in tissues adjacent to implanted materials and their distribution throughout the body via systemic circulation [5, 6]. Several studies have demonstrated high metal concentrations in body fluids (including serum and urine) of patients with knee and hip prosthesis and other authors have further suggested a potential role for ions and particles released from metal implants in various pathological bone and tissue conditions [7–12].

Well characterised osteoblast-like cell cultures have been used as a suitable in vitro model to study the interactions of biomaterials and their degradation products with bone cells at the tissue-implant interface [13–16]. Previous work performed in osteogenic cell cultures suggest acute and long-term effects of metal ions, found in commonly used orthopaedic implants, on the

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proliferation and function of osteoblast lineage cells [17–21].

AISI 316L stainless-steel corrosion products obtained by electrochemical means have been a matter of intense research at this laboratory. Both in vitro and in vivo studies have shown deleterious effects of stainless-steel corrosion products in several organs and tissues [21–26]. In this work, rat bone marrow cultures obtained in experimental conditions that favour the proliferation and differentiation of osteoblastic cells were cultured in the presence of AISI 316L stainless-steel corrosion products at different concentrations, for a period of 18 days. Control and treated cultures were evaluated by histochemical assays for the identification of the presence of alkaline phosphatase and calcium and phosphates deposition and examined by scanning electron microscopy (SEM); levels of total and ionised calcium and phosphorus in the culture media collected from control and metal added cultures during the incubation period, were also quantified. The concentrations of the major metal ions present in the SS were in the range of iron, chromium and nickel levels found in tissues adjacent to stainless-steel implants [27].

2. Materials and methods

2.1. Metallic slurries

A metallic AISI 316L stainless-steel slurry was anodically dissolved in Hank's balanced salt solution (HBSS) by imposing a constant potential of ca. 4 V during 5 h, and the resulting concentrations of the major metal ions were determined by atomic absorption spectrometry (AAS): 8.78×10^{-3} moll^{-1} of Fe, 4.31×10^{-3} moll^{-1} of Cr and 2.56×10^{-3} moll^{-1} of Ni. After pH adjustment (to 7.4 by addition of aqueous solution of sodium hydroxide) and sterilisation in an autoclave, further solutions were obtained by successive dilutions.

2.2. Cell culture

A primary culture of osteoblast-like cells was established using a young adult male Wistar rat bone marrow suspension obtained accordingly with the method described by Maniatopoulos et al. [28]. Briefly, the femora were excised aseptically, cleaned of soft tissues, and passed through 2 washes, 10 min each, of α-MEM (α-minimum essential medium, Sigma M 0894) containing antibiotic antimycotic solution (Sigma A 9909 1700 units of penicillin per mL, 1.7 mg ml^{-1} of streptomycin and 4.2 μg ml^{-1} of amphotericin B). Then, the epiphyses were removed, and the marrow flushed out using a 10 ml syringe with a 20G needle and containing culture medium. The cell suspension obtained was distributed, after homogenisation, through three 50 mL culture flasks and incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Medium was changed 24 h later to remove non-adherent cells. The culture medium consisted of α-MEM supplemented with 10% foetal bovine serum, antibiotic antimycotic solution (170 units of penicillin per ml, 0.17 mg ml^{-1} of streptomycin and 0.42 μg ml^{-1} of amphotericin B), 10^{-8} moll^{-1} dexamethasone, 2.52×10^{-4} moll^{-1} ascorbic acid and 10^{-2} moll^{-1} β-glycerophosphate and was changed twice a week.

Cells of the first subculture (obtained by tripsinisation of cells 7 days after the beginning of the incubation) were seeded at a concentration of 10^4 cells cm^{-2} in 35 mm diameter tissue culture dishes and cultured in the same experimental conditions that those used in the primary culture (control cultures) and also in the presence of 0.01, 0.1 and 1% of the stainless-steel slurry (metal-treated cultures) for a period of 18 days. Control culture medium and also metal-treated culture medium (containing 0.01% SS, 0.1% SS and 1% SS) were incubated in the absence of bone cells in the same experimental conditions as those used for cell cultures. The culture media were changed every 2 days, collected and analysed for quantification of total and ionised calcium and phosphorus. At the end of the incubation period (18 days) control and metal-treated cultures were evaluated by histochemical assays for the identification of the presence of alkaline phosphatase and calcium and phosphates deposition and examined by SEM.

2.3. Histological methods

2.3.1. Light microscopy

The presence of ALP positive cells and phosphate or calcium deposits were visualised by histochemical staining. The cultures were fixed with 1.5% glutaraldehyde in 0.14 moll^{-1} sodium cacodylate buffer and rinsed with distilled water.

ALP staining: Fixed cultures were incubated during 1 h in the dark with a mixture, prepared in Tris buffer pH = 10, containing 2 mg ml^{-1} of Na-α-naphthyl phosphate (reacting substrate) and 2 mg ml^{-1} of fast blue RR salt. The incubation was stopped by rinsing the samples with water and then, the cells were observed in a Olympus BH-2 optical microscope. ALP positive cells stained brown.

Phosphates staining: Phosphates deposits were assessed by the von Kossa technique [29], i.e., the 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 stained black.

Calcium staining: The fixed cultures were covered with a 1% S alizarin sodium sulphonate solution (0.028% in NH_4OH), pH = 6.4, for 2 min and then rinsed with water and acid ethanol (ethanol, 0.01% HCl) [29]. Calcium deposits stained red.

2.3.2. Scanning electron microscopy and X-ray microanalysis

Cultures were fixed as for light microscopy and dehydrated in a graded ethanol series. Critical point drying of the samples was followed by gold sputtering. The preparations were examined in a Philips SEM 525M at 15 kV.

2.4. Ionised and total calcium and phosphorus quantifications

2.4.1. Ionised and total calcium

The free and total calcium contents in cell culture media were evaluated by potentiometric means accordingly with the procedure described elsewhere [30]. The potential was monitored using a calcium selective electrode and an Ag/AgCl reference electrode, and the values of potential were recorded when the readings stabilised within $\pm$ 0.10 mV for 5 min. Calibration curves were prepared with standards with a composition similar to the culture medium and using the appropriate set of concentrations (in the same range as the measured calcium levels in the culture media). The ionic strength of the standard calcium solutions as well as of the samples was adjusted and maintained at ca. 0.17 $mol\,l^{-1}$ which corresponds to the value of the ionic strength of culture medium in order to keep the equilibrium of the system unchangeable [30].

2.4.2. Ionised and total phosphorus

The inorganic (Pi) and total phosphorus (P) were determined colorimetrically using the Fiske and Subbarow procedure [31]. For Pi quantification, the culture medium was treated with tricloroacetic acid to precipitate protein and lipid-bound phosphates. The supernatant fluid was reacted with ammonium molybdate in an acid solution to form phosphomolybdate. A mixture of sodium bisulphite, sodium sulphite and 1-amino-2-naphtol-4-sulfonic acid was then added to reduce the phosphomolybdate to form a phosphomolybdenum blue complex. The intensity of the colour was measured at 660 nm in a Shimadzu UV-VIS spectrometer.

For P quantification, samples of cell culture medium were, first, digested in a microwave oven (CEM Model MDS-2000) with nitric acid (suprapure 65%) in order to destroy organic matter. Then, the solutions obtained were treated as for Pi quantification (obviously the trichloroacetic acid step was not performed). All values were calculated from standard curves read at the same wavelength.

All data presented correspond to the mean of, at least, three measurements of the same sample. Standard deviations were not represented in Fig. 3 to avoid superposition of data and symbols, however the minimum and maximum values for the coefficient of variation of the levels of total and ionised calcium and phosphorus measured in the culture media, calculate as the standard deviation divided by the mean of the set of data and multiplied by 100 [32], are represented in Table 2.

3. Results and discussion

Previous studies have shown that rat bone marrow cultures obtained in the experimental conditions described above present osteoblast features, namely, high alkaline phosphatase activity and formation of a mineralised extracellular matrix [28, 33–34]. The presence of 2.52×10^{-4} $mol\,l^{-1}$ ascorbic acid, 10^{-2} $mol\,l^{-1}$ β-glycerophosphate and 10^{-8} $mol\,l^{-1}$ dexamethasone favours the formation of cultures of osteoblast phenotype in several bone cell culture systems [28, 35–39].

In this work, rat bone marrow cells (first subculture) were cultured during 18 days in control culture medium and in the presence of 0.01, 0.1 and 1% of the SS prepared as described in Section 2. At the end of the incubation period, control and treated cultures were evaluated by histochemical assays and examined by SEM. Quantification of total and ionised calcium and phosphorus in the control and metal added culture media (incubated in the absence and in the presence of bone cells) during the 18 days culture period was also performed.

3.1. Histochemical assays

Bone marrow cultures grown in control conditions showed the formation of three-dimensional nodular structures that were opaque in appearance and increased in size with incubation time. Histochemical assays showed a strong positive reaction for the presence of ALP and also for calcium phosphates deposition. Histochemical staining was mainly associated with the formation of three-dimensional nodules. Bone marrow cells cultured in the presence of the lowest concentration of the stainless steel slurry tested (0.01%, i.e., 8.78×10^{-7} $mol\,l^{-1}$ of Fe, 4.31×10^{-7} $mol\,l^{-1}$ of Cr and 2.56×10^{-7} $mol\,l^{-1}$ of Ni) showed a similar behaviour; no marked differences were observed in the intensity of the histochemical reactions in this experimental situation, as compared with control cultures. In the presence of 0.1% of SS corrosion products, cultures exhibited a positive histochemical reaction for the presence of alkaline phosphatase (although weaker than that observed in control cultures) but calcium and phosphates deposition was not observed. The presence of 1% SS slurry in the incubation medium (corresponding to 8.78×10^{-5} $mol\,l^{-1}$ of Fe, 4.31×10^{-5} $mol\,l^{-1}$ of Cr and 2.56×10^{-5} $mol\,l^{-1}$ of Ni) was toxic and resulted in cell death; only few cells with an altered morphology were observed in this situation. Fig. 1 shows the results concerning the histochemical staining for the presence of ALP and calcium deposition in cell cultures grown

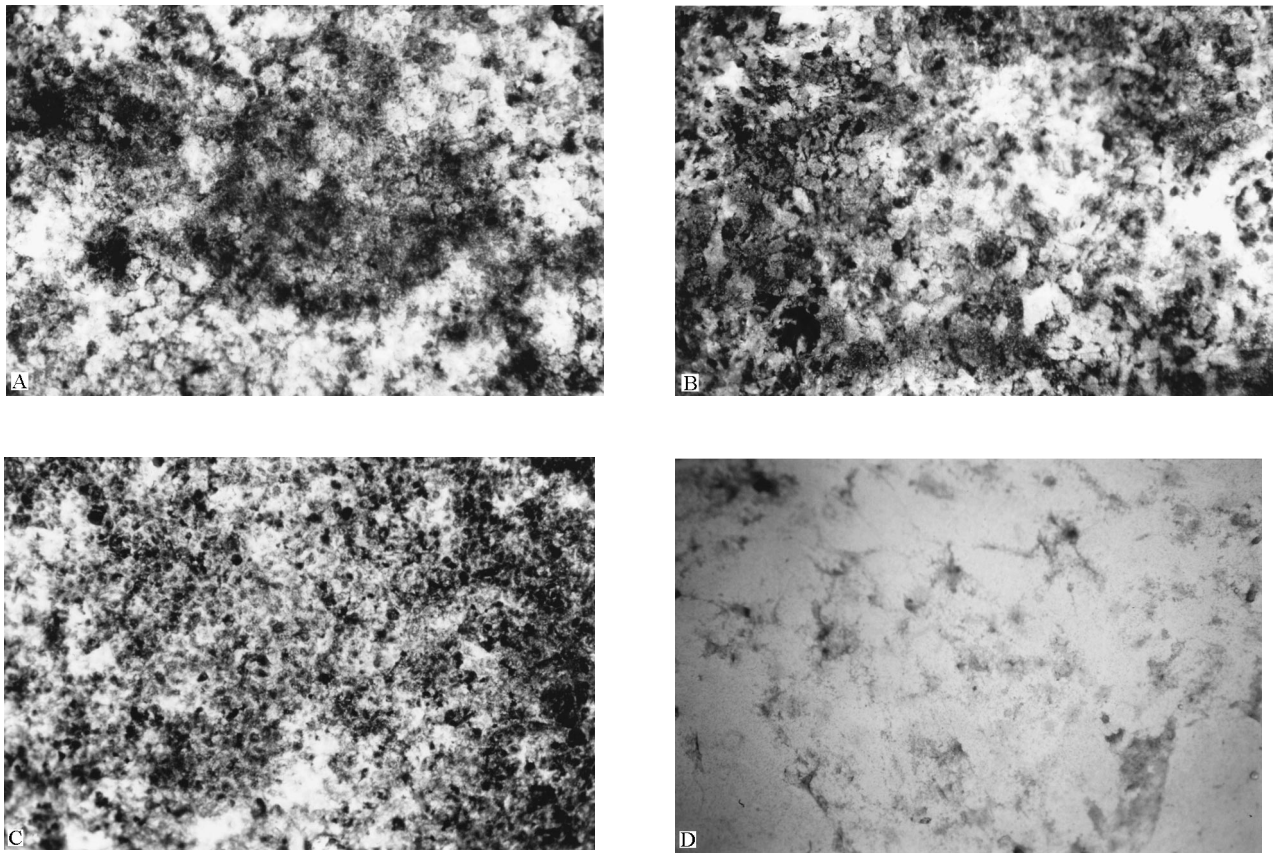

Fig. 1. Histochemical assays for the identification of the presence of ALP and calcium deposition on 18 days rat bone marrow cultures (×100). Cells grown in control conditions: A (ALP); B (calcium deposition). Cells grown in the presence of 0.1% SS corrosion products: C (ALP); D (calcium deposition).

in control conditions (Fig. 1A and B) and also in the presence of 0.1% SS slurry (Fig. 1C and D).

3.2. Scanning electron microscopy

SEM observation of control and metal-treated cultures confirmed the results observed on the histochemical assays. Control cultures showed the presence of cells and numerous globular deposits that seemed to fuse in regions of high density. This aspect is in accordance to other previously reported studies in similar culture systems [28, 33, 34]. In the presence of 0.01% SS cultures presented globular deposits similar to those observed in control cultures. In both cases, X-ray microanalysis of the globular deposits showed the presence of Ca and P peaks. These formations were absent in bone marrow cultures exposed to 0.1% SS, where only cells and a network of collagen fibers could be observed. Cultures treated with 1% SS showed evident signs of deterioration as compared to control cultures. Fig. 2 shows the SEM appearance of cell cultures grown in control conditions and the X-ray spectrum of the mineral spherules (Fig. 2A and B) and also in the presence of 0.1% SS slurry (Fig. 2C and D).

3.3. Total and ionised calcium and phosphorus in the culture media

Culture media from control and metal added cultures were collected every two days (and cultures refed with fresh medium) during the 18 days incubation period and analysed for total and ionised calcium and phosphorus concentration. Quantification of these species in control and metal-treated culture media incubated in the absence of bone cells in the same experimental conditions as cell cultures, was also performed. Levels measured of total and ionised calcium and phosphorus were not cumulative, as culture medium was totally replaced every 2 days; values observed reflect changes occurring in intervals of 2 days throughout the culture period.

In the absence of bone cells, levels of total and ionised calcium and phosphorus in control and metal-treated culture media were similar throughout the 18 days of incubation (Table 1A and B). Ionised calcium and phosphorus originate from calcium and phosphates compounds present in α-MEM (2.4 and 0.90 $mmol\,l^{-1}$, respectively) and in foetal bovine serum (ca. 3.8 and ca. 4.0 $mmol\,l^{-1}$, respectively). Addition of β-glycerophosphate (10 $mmol\,l^{-1}$) was responsible for the increased

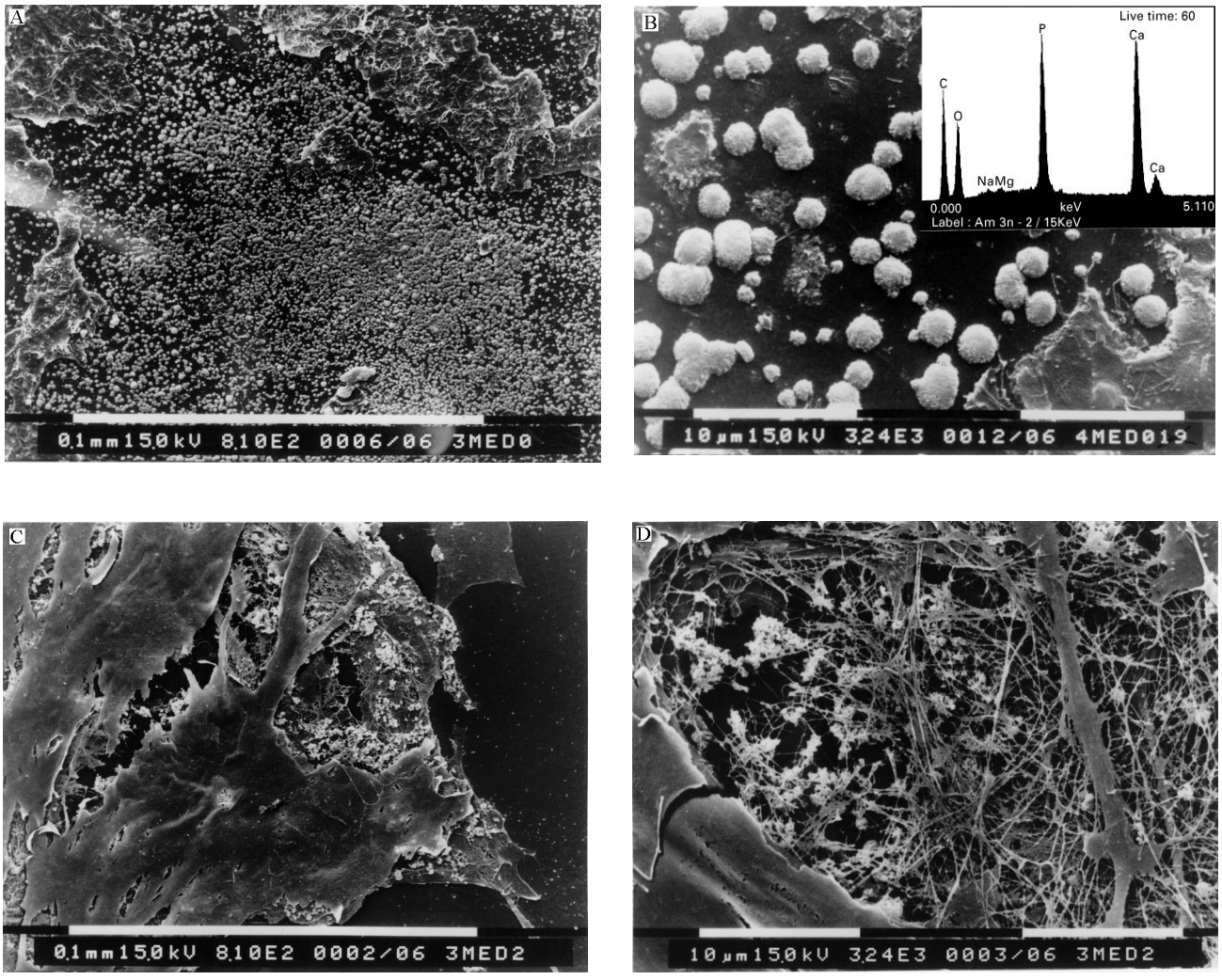

Fig. 2. SEM appearance of rat bone marrow cultures grown in control conditions for 18 days (A: Bar = 0.1 mm; B: Bar = 10 μm) and in the presence of 0.1% SS corrosion products (C: Bar = 0.1 mm; D: Bar = 10 μm). Inset: Energy-dispersive X-ray spectrum of mineral spherules present in cultures grown in control conditions showing the presence of calcium (ca. 62.5 at %) and phosphorus (P, 37.5 at %) peaks.

value observed for total phosphorus concentration, as compared to that found for ionised phosphorus. One can notice that calcium and phosphorus content in culture medium varied slightly during the time course of the experiment. This fact may be explained bearing in mind that the culture medium has to be prepared several times during the assay and that is practically very difficult to pipette the culture medium uniformly due to the presence of proteins (which bind calcium and phosphorus). So, discrepancies may come basically from two steps: during medium introduction in the culture dishes, and during collection of aliquots for quantification. The possibility of the incorrect application of the selected methods may be discarded since good precision and accuracy were obtained in preliminary comparative studies [30] and also, other authors [40, 41] reported similar variations in calcium and phosphorus content using other techniques.

Results concerning levels of total and ionised calcium and phosphorus in the culture media collected from control cultures and also metal treated cultures are shown in Fig. 3; standard deviations were not represented to avoid superposition of data and symbols; however, the minimum and maximum values for the coefficient of variation of the measured levels are represented in Table 2. In the first two weeks of incubation, culture media from control cultures and also cultures exposed to 0.01% SS showed increasing concentrations of ionised phosphorus (maximal levels were about 8.0–9.5 $mmol l^{-1}$, compared with 1.3–1.8 $mmol l^{-1}$ present in the absence of bone cells), although concentration of total phosphorus remained constant. These results suggest that most of the β-glycerophosphate added is converted to inorganic phosphate, most probably by alkaline phosphatase (known to have high efficacy in hydrolysing this ester phosphate), present in high levels,

Table 1
Levels (mean ± standard deviation) of total and ionised calcium (A) and phosphorus (B) measured in control culture medium and SS slurry treated culture medium incubated in the absence of bone cells and collected during the 18 days incubation period
(A)

(days)	Control		0.01% SS		0.1% SS		1% SS	
	Total Ca ($mmol l^{-1}$)	Ionised Ca ($mmol l^{-1}$)	Total Ca ($mmol l^{-1}$)	Ionised Ca ($mmol l^{-1}$)	Total Ca ($mmol l^{-1}$)	Ionised Ca ($mmol l^{-1}$)	Total Ca ($mmol l^{-1}$)	Ionised Ca ($mmol l^{-1}$)
2	1.87 ± 0.022	1.24 ± 0.014	2.01 ± 0.044	1.61 ± 0.070	2.13 ± 0.093	1.33 ± 0.040	2.25 ± 0.12	1.67 ± 0.012
4	2.22 ± 0.15	1.55 ± 0.011	2.25 ± 0.099	1.46 ± 0.012	2.09 ± 0.078	1.48 ± 0.011	2.22 ± 0.018	1.44 ± 0.061
6	2.01 ± 0.031	1.67 ± 0.10	2.11 ± 0.10	1.61 ± 0.011	2.19 ± 0.13	1.66 ± 0.050	2.04 ± 0.095	1.51 ± 0.054
8	2.12 ± 0.089	1.48 ± 0.065	2.12 ± 0.064	1.67 ± 0.10	2.03 ± 0.11	1.52 ± 0.034	2.10 ± 0.090	1.41 ± 0.038
10	2.45 ± 0.078	1.69 ± 0.070	2.43 ± 0.17	1.65 ± 0.023	2.54 ± 0.062	1.71 ± 0.040	2.30 ± 0.127	1.44 ± 0.048
12	2.48 ± 0.064	1.54 ± 0.080	2.47 ± 0.031	1.54 ± 0.034	2.23 ± 0.044	1.68 ± 0.040	2.29 ± 0.082	1.62 ± 0.083
14	2.44 ± 0.082	1.43 ± 0.044	2.25 ± 0.036	1.71 ± 0.041	2.00 ± 0.066	1.71 ± 0.025	1.81 ± 0.099	1.39 ± 0.049
16	2.21 ± 0.078	1.64 ± 0.053	1.90 ± 0.099	1.49 ± 0.050	1.96 ± 0.099	1.36 ± 0.021	2.09 ± 0.060	1.40 ± 0.080
18	2.26 ± 0.13	1.70 ± 0.023	2.07 ± 0.062	1.60 ± 0.060	2.19 ± 0.064	1.60 ± 0.057	2.15 ± 0.064	1.62 ± 0.034

(B)

(days)	Control		0.01% SS		0.1% SS		1% SS	
	Total P ($mmol l^{-1}$)	Ionised P ($mmol l^{-1}$)	Total P ($mmol l^{-1}$)	Ionised P ($mmol l^{-1}$)	Total P ($mmol l^{-1}$)	Ionised P ($mmol l^{-1}$)	Total P ($mmol l^{-1}$)	Ionised P ($mmol l^{-1}$)
2	10.0 ± 0.13	1.45 ± 0.045	10.8 ± 0.083	1.59 ± 0.005	11.3 ± 0.083	1.63 ± 0.005	10.9 ± 0.065	1.40 ± 0.015
4	10.1 ± 0.055	1.61 ± 0.015	9.70 ± 0.21	1.79 ± 0.017	10.6 ± 0.084	1.54 ± 0.006	10.7 ± 0.063	1.65 ± 0.063
6	11.8 ± 0.25	1.53 ± 0.006	10.3 ± 0.16	1.44 ± 0.008	11.3 ± 0.045	1.68 ± 0.015	10.5 ± 0.036	1.50 ± 0.012
8	11.3 ± 0.15	1.80 ± 0.014	9.04 ± 0.13	1.35 ± 0.008	11.0 ± 0.020	1.70 ± 0.045	9.02 ± 0.045	1.80 ± 0.018
10	12.0 ± 0.10	1.67 ± 0.063	10.4 ± 0.045	1.25 ± 0.018	10.4 ± 0.038	1.71 ± 0.046	9.02 ± 0.040	1.49 ± 0.009
12	10.0 ± 0.30	1.40 ± 0.010	9.89 ± 0.10	1.66 ± 0.015	10.5 ± 0.075	1.52 ± 0.007	9.88 ± 0.16	1.47 ± 0.002
14	9.87 ± 0.045	1.53 ± 0.020	9.90 ± 0.15	1.54 ± 0.063	10.0 ± 0.046	1.33 ± 0.008	9.16 ± 0.10	1.57 ± 0.045
16	10.6 ± 0.032	1.39 ± 0.048	9.76 ± 0.10	1.47 ± 0.048	9.74 ± 0.046	1.41 ± 0.002	11.1 ± 0.034	1.39 ± 0.010
18	9.65 ± 0.047	1.65 ± 0.015	10.5 ± 0.032	1.63 ± 0.010	9.84 ± 0.037	1.70 ± 0.010	10.1 ± 0.027	1.70 ± 0.007

according to the results observed in the histochemical assays (Fig. 1A). Ability of the cultures to hydrolyse this compound appeared to be maximal between 8 and 12 days of culture. As ALP activity has been regarded as an in vitro marker of osteoblastic differentiation [36, 37], this observation suggests that, at this stage of the culture, more cells differentiate and therefore have high ALP activity which more rapidly cleaves the β-glycerophosphate. Levels of total and ionised calcium in the culture media did not change significantly during this phase of the culture. However, after approximately two weeks of culture, levels of calcium and phosphorus (almost all ionised at this stage of the culture) in the culture media decreased significantly. This consumption of ionised calcium and phosphorus (inorganic phosphate) is most probably related to the formation of mineral deposits (calcium phosphates deposition), as previous work has shown that mineralisation of the extracellular matrix in this culture system (control conditions) is observed from two weeks onward [28, 33, 34].

In cultures grown in the presence of 0.1% SS, part of the β-glycerophosphate added was hydrolysed and measured levels of inorganic phosphate in the incubation medium increased throughout the incubation period. Hydrolysis of this compound was probably performed by alkaline phosphatase, as this enzyme is present in these cultures (although, at lower levels than those observed in control conditions, Fig. 1). However, levels of total calcium and phosphorus in the incubation media were similar throughout the 18 days incubation period and close to those found in the absence of cells, contrasting with the significant decrease observed in control and 0.01% SS cultures, after two weeks of incubation.

Cultures grown in the presence of 1% SS did not show significant changes in the concentration of total and ionised calcium and phosphorus during the incubation period and levels were similar to those measured in the absence of cells. These results were expected as histochemical assays and SEM observation of the cultures have shown that the presence of SS at this concentration was too toxic and resulted in cell death.

Results presented in Fig. 3 show that values found for total calcium and phosphorus concentrations are, in majority, lower than the expected theoretical values

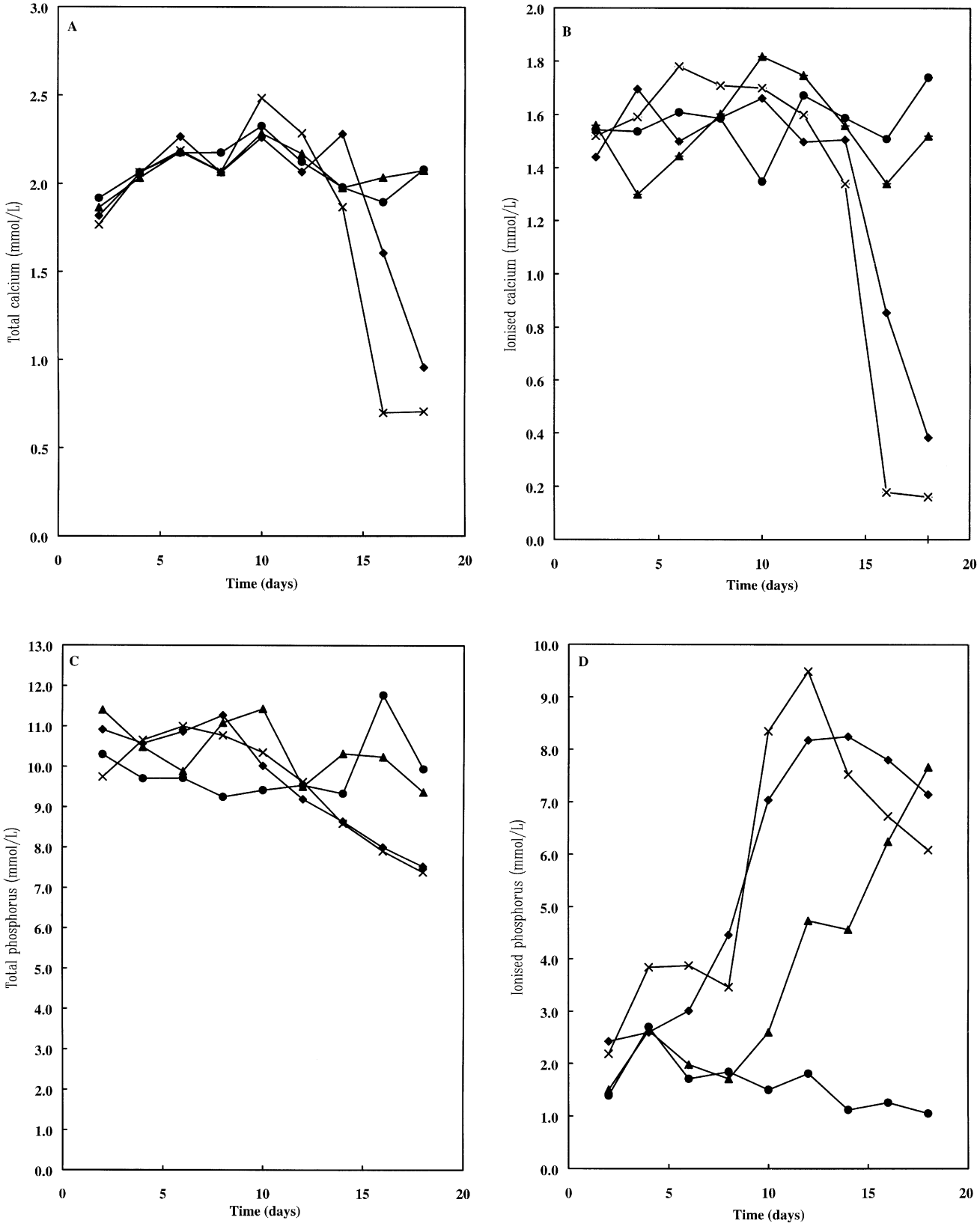

Fig. 3. Levels of total and ionised calcium (A, B) and phosphorus (C, D) in the culture media from control and metal added cell cultures, collected every 2 days throughout the 18 days incubation period. (◆) culture medium from control cultures; (×) culture medium from 0.01% SS cultures; (▲) culture medium from 0.1% SS cultures; (●) culture medium from 1% SS cultures.

Table 2
Minimum and maximum values for the coefficient of variation (%) of the levels of total and ionised calcium and phosphorus, measured in the culture media collected from control and SS-treated cell cultures during the 18 days incubation period

	Coefficient of variation (%)			
	Total Ca	Ionised Ca	Total P	Ionised P
Control cultures	3.9–6.3	1.5–6.5	0.11–1.6	0.040–0.58
0.01% SS cultures	4.2–6.8	1.1–4.4	0.14–2.8	0.11–2.1
0.1% SS cultures	3.0–6.7	0.70–4.4	0.33–2.2	0.17–3.0
1.00% SS cultures	2.6–6.3	0.70–6.6	0.00–2.7	0.12–4.1

based upon the proportion of each compound in the culture medium (α-MEM + 10% foetal bovine serum + 10 $mmol l^{-1}$ β-glycerophosphate) i.e., ca. 2.8 $mmol l^{-1}$ for calcium and ca. 11.3 $mmol l^{-1}$ for phosphorus suggesting that some constant precipitation occurs in all culture plates during the 18 days of incubation. However, this non-biological mineral deposition seems to be insignificant when compared with the high consumption of calcium and phosphorus observed during the biomineralisation that occurred in control and 0.01% SS cultures from approximately two weeks onward (Fig. 3).

The behaviour observed in control cultures concerning levels of calcium and phosphorus in the culture medium during the culture period appears to be in agreement with that reported in the literature in studies performed in similar culture systems (cultures forming mineralised bone nodules in these experimental conditions) [28, 40, 42, 43]. These studies have shown that alkaline phosphatase and organic phosphate play a crucial role in the initiation of the mineralisation process. Studies performed in the presence and in the absence of the alkaline phosphatase inhibitor levamisole showed that β-glycerophosphate is utilised as a substrate for this enzyme and that it is rapidly converted to inorganic phosphate, required for the formation of calcium phosphate [40, 42]. In addition, it was also shown that incorporation of calcium in the cell culture occurs during the mineralisation process [40–43]. The results reported in this work suggest that evaluation of the concentration of total and ionised calcium and phosphorus in the incubation medium throughout the culture period may be regarded as a measure of the mineralisation process occurring in these cell cultures, as calcium phosphates deposition requires consumption of calcium and phosphate ions from the culture medium.

The results reported and presented in Figs. 1–3 showed that the presence of 0.01% SS (corresponding to 8.78×10^{-7} $mol l^{-1}$ of Fe, 4.31×10^{-7} $mol l^{-1}$ of Cr and 2.56×10^{-7} $mol l^{-1}$ of Ni) caused no detectable effects in the normal behaviour of rat bone marrow cultures obtained in the experimental conditions described, 0.1% SS impaired osteoblastic behaviour of these cultures and 1% SS resulted in cell death. Previous work has shown that in rabbit bone marrow cell cultures obtained in similar experimental conditions, stainless-steel corrosion products (and also solutions of the corresponding major metal ions prepared from inorganic salts and used at the same concentration) affect proliferation and function of the osteoblast-like cells [21]. Also, metal ions found in others commonly used orthopaedic implants such as Co–Cr–Mo and Ti–6Al–4V alloys were found to cause acute and long-term toxic effects in rat and human bone marrow cultures [17–20]. Results obtained in this kind of studies suggest that metal ions and particles released from metallic implants may play a role in the bone loss reported to occur surrounding orthopaedic implants.

4. Conclusions

The present study showed that stainless-steel corrosion products above certain concentrations disturb the normal behaviour of rat bone marrow cultures obtained in experimental conditions that favour the proliferation and differentiation of osteoblastic cells.

Metal ions may interfere with the proliferation and differentiation of osteoblastic cells or/and with the function of the differentiated cell. Further studies are required in order to clarify the effect of stainless-steel corrosion products in the behaviour of rat bone marrow cell cultures.

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