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APPLICATION OF INAA IN THE STUDY OF METALLIC IONS RELATED TO TOXICITY

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ABSTRACT

The 316L stainless steel which is commonly used as biomaterial in metallic implants has shown toxic effect in cytotoxicity *in vitro* assay by neutral red uptake methodology. Instrumental neutron activation analysis was applied to evaluate metal composition in the steel and in the extract composed by cell culture medium (MEM) where the sample remained immersed during 10 days at 37 ^{o}C. The aim of this study was to determine the level and identify the elements related to cytotoxicity, in solutions containing metallic ions with different associations and concentrations. The results showed Co, Cr and Ni elements in the extract which are metallic elements previously associated to toxicity. The association of Cr and Ni resulted in toxicity although these elements when individually present in the medium did not show any toxicity effect. On the other hand, the association of Co to Cr + Ni reduced the cytotoxic effect.

1. INTRODUCTION

Stainless steels (SS) are commonly used as biomaterials for orthopedic implants. The 316L SS is widely used for surgical implants due to its good mechanical properties, low cost and conformability. However, it is susceptible to pitting corrosion. Corrosion of SS might lead to the release of nickel ions into body fluids and cause adverse effects, such as allergenic reactions. Nickel and chromium are the main alloying elements of stainless steels and this last element has also been associated to toxic effects.

In this study, instrumental neutron activation analysis was applied to evaluate the stainless steel composition and the composition of ions in the extract obtained from the cytotoxicity assay. The aim was to determine the elements and their concentrations associated to toxicity.

2. MATERIALS AND METHODS

2.1. Material

The material used in this study was a 316L austenitic stainless steel whose composition was determined by Instrumental Neutron Activation Analysis (INAA).

2.2. Preparation of Extract

Samples sheets of stainless steel sterilized by autoclaving were immersed in physiological like solution, MEM (minimum Eagle´s medium) supplemented with 10% fetal calf serum, the same medium used for cellular growth in the cytotoxicity assay. The extraction time was 10 days in an incubator at 37^{o}C, according to International Standardization Organization (ISO) [1]. After incubation time the obtained extract was divided to chemical analysis and cytotoxicity test.

2.3. Chemical Analysis

The elemental composition of the 316L stainless steel and of the extract was determined by instrumental neutron activation analysis according to procedure described previously [2].

2.3.1. 316L Stainless steel

A piece of sample was conditioned in a clean polyethylene capsule for irradiation in the IEA-R1 nuclear reactor. The sample and elemental synthetic standards were irradiated together under a thermal neutron flux of 5 x 10^{12}n.cm^{-2}.**s^{-1}** for 16h. The sample and standards were measured in a **Model GMX20195** hyper-pure Ge detector coupled to an EG&G Ortec ADCAM 918A Multichannel Buffer, and this to a microcomputer, after adequate decay times varying from 5 to 10 days. Counting times of 5400 s was used for standards and of 10 000 to 40000 s for samples. The distances source-detector utilized were of 2 and 30 mm. The gamma ray spectra were processed using appropriate software and the concentrations of the elements were calculated by a comparative method.

2.3.2. Extract

500μL of extract and the blank of culture medium MEM were pipetted and dried in a clean polyethylene capsule for irradiation in the IEA-R1 nuclear reactor. The procedures used for irradiation and gamma ray activity measurement were the same described in 2.3.1.

2.4. Cytotoxicity Assay

The cytotoxicity assay was performed by neutral red uptake method according to ISO [1] and the described methodology in a previous paper [3-4]. It was used mouse fibroblastic cell line NCTC L929 from ATCC bank.

Based in the result of cytotoxicity assay Co, Cr and Ni standard solutions from Spex Certiprep, USA diluted in MEM to simulate the extracts were used to verify which metal element is responsible for toxicity.

3. RESULTS AND DISCUSSION

The elemental composition of the 316L stainless steel obtained by INAA is shown in Table 1.

The elemental concentrations of the metals released in the MEM culture medium (extract) were determined by INAA and the results obtained are shown in Table 2. Ni was not detected

and detection limit value was evaluated. Results of a blank sample are also presented in this Table.

Table 1. Elemental composition of 316L stainless steel by INAA

As ($\mu g.g^{-1}$)	Co ($\mu g.g^{-1}$)	Cr (%)	Fe (%)	Mn (%)	Mo (%)	Ni (%)	V ($\mu g.g^{-1}$)
46.8 ± 0.4	1229 ± 16	17.80 ± 0.03	57.8 ± 0.2	1.44 ± 0.02	1.97 ± 0.12	12.9 ± 0.1	120 ± 23

Table 2. Elemental concentration in extract and in blank solution obtained by instrumental neutron activation analysis

Element	Extract [a]	Blank
Co ($ng.mL^{-1}$)	6.6 ± 0.2	0.0014 ± 0.0001
Cr ($ng.mL^{-1}$)	174 ± 6	618 ± 3
Ni ($ng.mL^{-1}$)	< 0.61	-

a- The contribution of the blank value was discounted

The results of cytotoxicity assay with 316L SS and positive and negative control are shown in Figure 1. The negative control (Ti) did not present cytotoxicity, but the positive control (0.02% Phenol solution) and the 316L SS sample showed toxicity effects. The cytotoxic index $IC_{50\%}$ obtained in the graphic for the 316L SS sample tested and the positive control was 42 and 91, respectively.

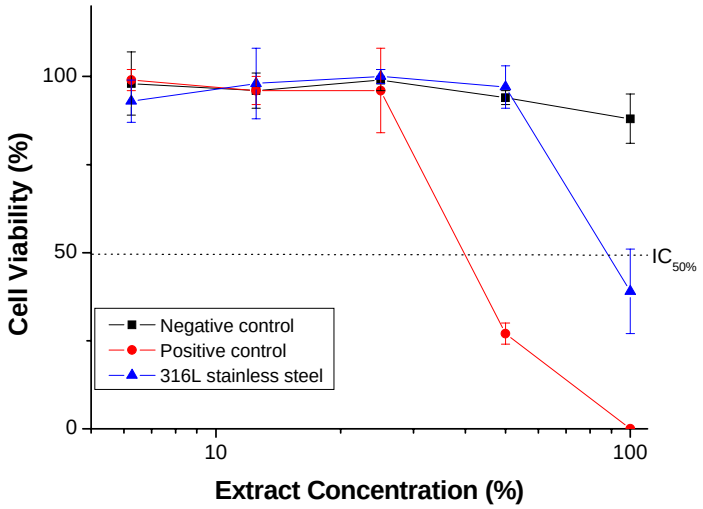

Figure 1. Cytotoxicity assay viability curves obtained by neutral red uptake methodology.

As chromium and nickel are known as toxic metal elements [5] cytotoxicity studies were carried out with Co, Cr and Ni standard solutions. In previous studies [6-7] solutions with the same concentration of the extract and 100 times more concentrated were prepared, either with the Co, Cr and Ni elements being added individually or in association, and the results showed no cytotoxic effects related to the tested solutions. It was proposed that the reason for these results was the short time of contact between the metal ions and the culture medium (MEM) components, such as amino-acids, proteins and other complexes. The metallic ions have to bind to some biochemical components (similar to metal transporters) to form complexes that might provoke cellular alterations leading to cell injuries [8].

Solutions prepared with Co, Cr and Ni addition, either individually or in association to MEM were kept 10 days in an incubator at 37°C. After incubation time, these solutions were used as extract in the cytotoxicity assay carried out according to the neutral red uptake method.

The solutions with individual addition of metal elements did not show any toxicity. For the solutions prepared with association of two elements, specifically Co+Cr; Co+Ni; and Cr+Ni, only the solution with Cr+Ni showed high cytotoxicity. In this solution, death of all cell population occurred, even for 1% extract dilution, as shown in Fig. 2. The cell viability curve shows values very close to zero, well below the $IC_{50\%}$ line. The association of the three elements Co, Cr and Ni in the solution also presented toxic effect, however the viability was much greater than that of the solution with only Cr and Ni, suggesting that the addition of Co to Cr+Ni decreased the cytotoxicity. The cell viability curve crossed the $IC_{50\%}$ line at an extract concentration of approximately 90%, showing an $IC_{50\%}$ of 90.

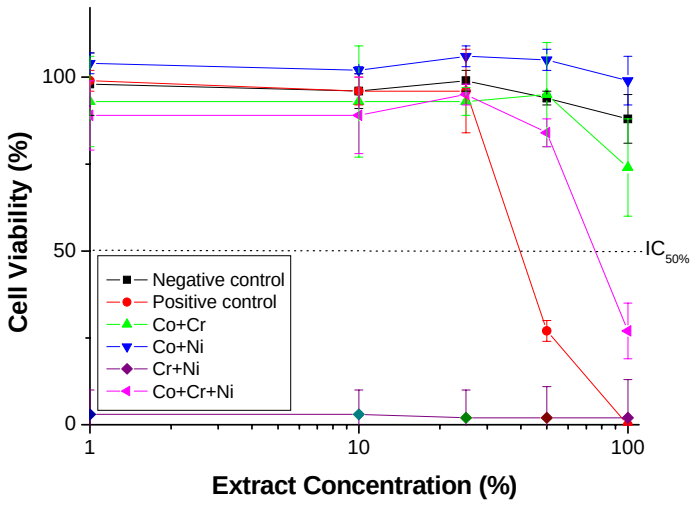

Figure 2. Cytotoxicity assay viability curves obtained by neutral red uptake methodology of Co, Cr and Ni solutions

4. CONCLUSION

Instrumental neutron activation technique is an useful method to determine the composition of stainless steels and of their corrosion products released in physiological environments.
Metal elements released from this type of steels, such as cobalt, chromium and nickel, when individually in solution, even at concentration 100 times higher than that usually detected in their extracts (corrosion products in culture medium), did not show cytotoxic effect by the neutral red uptake methodology. On the other hand, the association of chromium and nickel in the culture medium caused high cytotoxicity. The addition of cobalt to the solution containing Cr+Ni reduced its cytotoxic effect, although the combination of the three elements still led to cytotoxicity. The results lead to the conclusion that the toxic effects of the 316L stainless steel in the cytotoxicity assay is due to the lixiviation of metal elements when in combination, mainly Cr and Ni.

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