IN VITRO TESTS OF THE Ti-35Nb-5Ta-7Zr ALLOY.

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Abstract. *This work studied the cytotoxicity test (In Vitro Methods) of alloy Ti-35Nb-5Ta-7Zr. Cytotoxicity test methods are useful for screening materials that may be used in medical devices because they serve to separate reactive from nonreactive materials, providing predictive evidence of material biocompatibility. Cytotoxicity test is based in the international norms ISO 10993-5 Biological Evaluation of Medical Devices. The cytotoxicity test was accomplished through diffusion in agar, where an amount of the material under test, in the case the alloy was put on a agar layer that recovers a cell monolayers. The material will be able to liberate or not visible signs of toxicity substances, these they are diffused through the layer dies of agar, killing or breaking cells and evidencing a presence or absence of a zone of cellular effects beneath and surrounding the sample. For this assay, cells were used McCoy CRL-1696 ATCC-USA) in the concentration of 3x105 cells/ml. In the elution test method, extracts are obtained by placing the test in separate cell culture media under standard conditions. The extract was with the following concentrations: 0%, 10%, 20%, 30%, 50%, 65%, 80%, 100%. The assay was analyzed in Elisa Bio-Rad in 550 nm, where the percentage of dead cell was verified. The result for cytotoxicity test: negative for diffusion in agar and elution test method.*

Keywords*: biomaterial; cytotoxicity; Ti-35Nb-5Ta-7Zr alloy.*

1. INTRODUCTION

Some metallic alloys have been used with success as biomaterial since they are materials capable to reconstitute or to substitute tissue and corporal functions without that negative react to the host. Application's areas including orthopaedics, cardiology (as part of devices in valves) and odontology (Willians, 1987). Biomaterials is a growing industry and provoking the search of new possibilities of alloys that can improve in the quality of life on the peoples that need to be attended with these implant device (Ramos, 2001). To verify the material interactions versus biological enviroment, tests in-vitro and in vivo have been performed. In the course of biocompatibility process the material must reach a required clinical result (Kawachi, 2000).

Among the alloys of titanium more studied, one is sought that has a closer Young's modulus to the bone (E = 10-30 GPa), with prominence for: Ti-12Mo-6Zr-2Fe, Ti-15Mo-5Zr-3Al, Ti-15Mo-3Nb-3O, Ti-15Zr-4Nb-2Ta-0. Of this generation the alloys appeared Ti-13Nb-13Zr (E = 79 GPa) in the which all of the constituent elements are biocompatible. (Niinomi, et al.1999; Wang, 1996). In sequence the Ti-35Nb-5Ta-7Zr alloy with a module of elasticity of 55 GPa, very close of the bone, therefore, the use for biomedical applications (Oliveira, et al. 2010) Tests for biocompatibility are accomplished, firstly in vitro test, like for example, cytotoxicity test.

The main objective of the cytotoxicity test is to identify the material or device has the capacity in producing lethal effects in the environment biological. The cytotoxicity test can be accomplished through diffusion in ágar and/or for elution. In the diffusion test an amount of the material in test, it is put on ágar layer that recovers a confluent monolayer of cells, in case the material liberates toxics substances, will be diffused through the ágar layer, killing or breaking cells of the monolayer and evidencing a sign of cellular degradation. In the elution test, the extract of the material is added in general in varied concentrations in cellular cultures and the evaluation, it is accomplished through the observation of the inhibition or not of the growth of the same ones through incorporations of coloring vital (Daguano, et al., 2007). The index of cellular survival is the parameter more used capable to identify toxicity levels, through coloring vital as the red of neutral, that it is soluble in water and it has passage for the cellular membrane concentrating on the lysosomes. Some substances are harmful to the cellular membrane, what results in a smaller absorption of the neutral red for the cell, that facilitates the visualization of the alive cells compared to the deads or harmed, through the color of the cellular culture that it can be measured (Rogero, et al., 2003; Ciapetti, et al., 1999). The cytotoxicity test is a fast test, standardized, sensitive and of low cost, capable to inform a material it presents significant amounts of elements toxics and it is standardized in the norms ISO 10993-5 (ISO, 1992). The sensibility of the test is in the fact that the cellular cultures stay viable in controlled atmosphere and without the mechanisms of existent cellular defense in the biological system.

2. MATERIALS AND METHODS

2.1. Sample preparation

As test samples were used, alloy Ti-35Nb-5Ta-7Zr and Ti-6Al-4V (obtained of commercial orthopedical devices), with diameter and respective weights of 3 and 1,5 cm and 3 and 2 g. the surface of those materials was sanded with sandpapers: 220 - 320 - 600 - 1200 - 2000 and polished with oxide of chrome and alumina - 0,3µm. After that procedure the material was clean and soon afterwards sterilized. For the cellular culture, cells of the cellular lineage ATCC-CRL-1696 (mouse McCoy-fibroblasto) they were sowed in plates of Petri, sterile and disposable (Corning) in middle of cellular culture Iscove's and incubated to 37 ±1 °C, with controlled atmosphere (5% of CO_2 and 95% of humidity) for 48 hours.

In the prepare of the extracts (cytotoxicity test), the extraction vehicle used was saliva artificial formula of Fusayama (Fusayama, et al. 1963) as it demonstrates the Tab. 1.

Table 1. Formula of the artificial saliva of

KCl	NaCl	$CaCl_2.2H_2O$	$NaH_2PO_4.2H_2O$	$Na_2S.9H_2O$	Urea
0,4	0,4	0,906	0,690	0,005	1

The norm ISO 10993-11 (ISO, 1992) for the confection of the extracts, where for each 20ml of extraction vehicle it was used 2-4g of material. As that experiment had the intention of demonstrating the behavior of extracts the base of artificial saliva, simulating the buccal atmosphere, it was added to the artificial saliva, fractions of salts of NaF 0,1% (concentration found in rinse oral and toothpaste). The artificial saliva was filtered inside of the flow chapel to laminate in instruments and sterilized containers. After filtering the artificial saliva, it was added at to samples of the alloys metallic (Pereira, et al. 2008). Then, the flasks containing the vehicles with and without the samples of alloys they were conditioned 37 °C (± 2 °C) for 72 hours (± 2 h). After this period, the extracts were flowed in sterile flasks, to separate the alloys of the extraction vehicles and these were stored until the moment of use. That procedure was also accomplished inside of the flow chapel to laminate. The separated samples in 4 sterile flasks, identified in the Tab. 2.

Table 2. Extracts and your components

Extracts	**Component**
A	Artificial saliva + NaF 0,1%
B	Artificial saliva l + NaF 0,1% + Ti-35Nb-5Ta-7Zr
C	Artificial saliva + NaF 0,1% + Ti-6Al-4V
D	Control negative - artificial saliva + no-toxic filter fragments
E	Control positive – artificial saliva + latex fragments

2.2. Cytotoxicity test

The tests of cellular cytotoxicity for diffusion in ágar, where an amount of the material in test (Ti-35Nb-5Ta-7Zr) they were put on a ágar layer that recovers a confluent monocamada of cells. The cellular lineage was used ATCC-CRL-1696 (mouse McCoy-fibroblasto). The cellular concentration is of 3x10-5 células/ml, that were sowed in petri plates (15 x 60 mm), in a volume of 5ml, being incubated later by a period of 48 hours, with humid atmosphere to 5% of CO_2 and temperature of 37˚C . Finished the process described above, in flow chapel to laminate, after growth of the cellular layer, the middle of culture was despised (Iscove's) and added 5ml of the half "overlay" for each petri plate. The "overlay" is composed by parts similar of minimum of Eagle (MEM) and ágar to 1,8 % with 0,01% of red neutral. The temperature of mixture of MEM and ágar was to 44˚C. When the ágar was practically solidified was added surface the alloys. Consecutively the plates were incubated in 5% of CO_2 and temperature of 37˚C for a period of 24 hours (Rogero, et al. 2003) In case there is liberation of toxins, a sign of cellular degradation is evidence. The samples were triplicated and, as positive control, they were added on the same ágar type, latex fragments and, for the negative control, no- toxic filter fragments, were used with medium size of superficial area of 0,25 cm^2. The evaluation of the results of the diffusion test in ágar, it is done by the observation of the index of the sign of cellular degradation, this is represented by the area no red-faced for the vital color. The indexes, presented in the Tab. 3, it is numbered and made a correlation, at the answer index.

Table 3. Degradation Index

Degradation Index	Description
0	absence of cellular degradation
1	limited area under the sample
2	less that 0,5 cm around of the sample
3	among 0,5 – 1,0 cm around of the sample
4	larger than 0,5 – 1,0 cm around of the sample
5	Total degradation

In the elution test, the extract of the material is added in varied cellular concentrations, in general, it is accomplished through the observation of the inhibition or not of the growth of the same ones through incorporations of coloring vital. In this test the cellular lineage was used ATCC-CRL-1696 (mouse McCoy-fibroblasto), following the norm ISO 10993-5, it was used for that test the originated extracts (Tab.2) of the alloys in contact with the artificial saliva of Fusayama. The used cellular amount was of 1x105 cells/ml in Iscove's and licensed in plate with 96 wells, after, incubated it a temperature of 37˚C C with 5% of CO_2. After a period of 24 hours, removed Iscove's by aspiration and the monolayer of cells was washed with PBS. The extracts in the following concentrations: 100, 80, 65, 50, 30, 20, 10%, after substitution of the middle for the fractional extracts, the cells were incubated in the 96 wells for a period of 24 hours (Fig. 1).

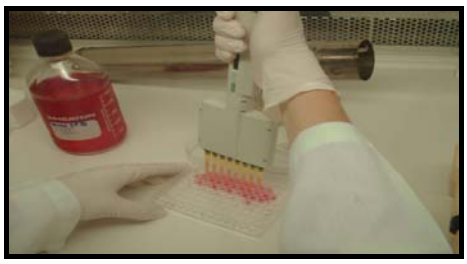

Figure 1. Incubated cells

Finished the time of incubation, the cells were treated with 3-(4,5 dimetil) tiazol-2-il-2,5-difenil tetrazolio bromide (MTT), with the intention of development of a blue color, because that method of cellular counting, bases on the reduction of MTT (yellow composition) to a product farmazan, that has violet coloration and that it can be measured by absorbance up to 570nm. The existent reduction is due the mitochondrion desidrogenases, only present in the cells that continued alive after the process. [58-60] After a period of 3 hours of incubation, the middle with MTT is removed and 50µl of PBS are added with isopropanol in the proportion of 1:1. The calibration of ELISA BIO-RAD'S reader was in 550nm, for read the test, demonstrated in the Fig. 2.

Figure 2. ELISA BIO-RAD'S reader

2.3. Results and Conclusions

The result of the diffusion test in ágar was obtained through macroscopic analysis of the plates, where it can observe the formation or not of sign cellular degradation. The sign formation with clear color around of the tested sample, it means that there was toxicity process, elapsed by the material inserted to the cellular way. The sign can only be observed when there is cellular death, because only like this, it happens liberation of the neutral red color, that it was incorporate for the cells, resulting in a transparent aspect in the place.

The cytotoxicity was evaluated through the index of the formed sign of cellular degradation (Tab.3). The results are demonstrated in the Tab. 4 and Fig. 3.

Table 4. Index results – Diffusion test

Sample	Index	Diameter (cm)
Positive Control	4	3,0
Negative Control	0	0,0
Ti-35Nb-5Ta-7Zr	0	0,0
Ti-6Al-4V	0	0,0

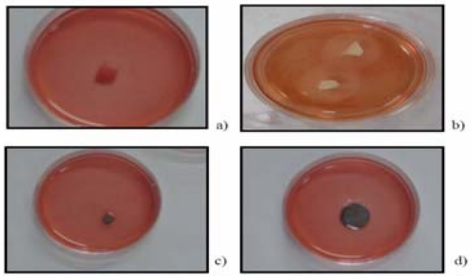

Figure 3. Diffusion test – Sign cellular degradation
a) Negative control; b) Positive control; c) Ti-6Al-4V; d) Ti-35Nb-5Ta-7Zr

The result of the diffusion test for the Ti-35Nb-5Ta-7Zr (Fig. 3. d), it doesn't indicate any sign of cellular lesion, whit degradation index of diameter 0,0 cm. Equally were the rehearsals controls Fig. 3 a) and c). That result is confirmed by the comparison to the positive control, a sign cellular degradation of 3cm can be observed, what puts the sample in index same to 4 (Tab. 4 and Fig. 3 b), due the material (latex) your toxicity was identified with the death local cellular. That result demonstrates that the Ti-35Nb-5Ta-7Zr in direct contact with the cells, didn't cause cellular toxicity.

For the elution test (Fig. 4), it confirmed that the extract originated B of the contact of the artificial saliva (with additions of NaF 0,1%) to the alloy Ti-35Nb-5Ta-7Zr , independent of the concentration that is, it doesn't cause cellular toxicity, demonstrating doesn't liberate toxic components, observed by the registered absorbance in the alive cells. Was used, the negative control (extract D), positive control (extract E), the extract A (artificial saliva with NaF 0,1%) and also the extract C (artificial saliva + NaF 0,1% + Ti-6Al-4V) as parameter. As expected, the positive control (extract E) presented toxicity character in the cells, and your concentration went increasing, more evident it was toxicity tenor, reaffirming that the other used extracts (especially the extract B) it doesn't cause cellular toxicity.

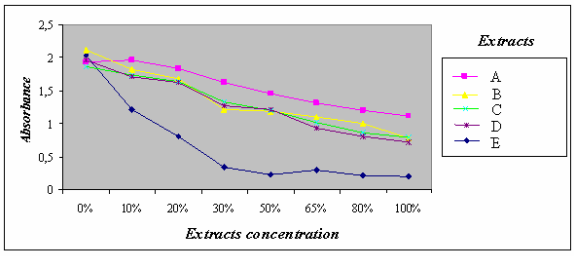

Figure 4. Alive cells absorbance versus extracts concentration.

The elution test, complements the results of the diffusion test, demonstrating that the Ti-35Nb-5ta-7Zr (be as material or derived of the same) is not toxic for cells. The preliminary demonstrated that the alloy it is a material with biocompatible character in presence the saliva with Na 1%, could be suggested like biomaterial candidate, for used in odontological applications.

3. ACKNOWLEDGEMENTS

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