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Biocompatibility in vitro tests of Zinalco™

Ma. de los Ángeles Aguilar [a,*], Saúl Espinosa [a], Leonor Rodríguez [a], Cristina Piña [b]

[a] *Departamento de Ciencias de la Salud, Universidad Autónoma Metropolitana – Iztapalapa, Apartado Postal 55-535 CP 09340, Mexico, D.F., Mexico*

[b] *Instituto de Investigaciones en Materiales, UNAM, Cd. Universitaria, D.F. CP 04510, Mexico*

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Abstract

In this study, cultures of human lymphocytes were exposed for 48 h to aqueous suspensions of Zinalco™, with different times of corrosion, to test the biocompatibility of this alloy and support its use in medical implants. The results show that the Zinalco™ concentrations assessed affect the mitotic index (MI) and some also increase the length of DNA migration measured in the comet assay in a significant manner; none of them showed clastogenic nor aneugenic effects. The authors conclude that Zinalco™ may be considered as an alternative biomaterial and suggest its use for short-term implants.

Keywords: Zinalco™; Biomaterials; Biocompatibility

1. Introduction

Information concerning unfavourable reactions among biomaterials and living systems proceeds from retrospective studies in man and current knowledge about biomaterial tissue interactions has been gained through bioassays in vivo and in vitro. In early research studies like this, attention was paid mainly to the implant location and materials were classified as ‘‘very reactive’’ or ‘‘inert’’. This simple classification has changed since some materials may induce unfavourable immune reactions, undesirable interactions with blood and other body fluids as well as damaging the genetic material at the chromosomal and DNA levels [1,2].

It is known that many alloys have been tested as potential biomaterials for orthopaedic implants or as tools for bone reconstruction; all of them must provide fracture resistance in addition to being biocompatible. Today, the most frequently used biomaterials are stainless steel, titanium and titanium alloys as well as cobalt alloys, all of which are very expensive [1,3,4].

In searching for new materials, Zinalco™, an alloy was developed at the Institute of Materials Research of the National University of Mexico (UNAM), in 1977. This alloy is made of 80% zinc, 18% aluminium and 2% copper. Due to the fact that its physical and mechanical features are very similar

* Corresponding author. Fax: +52-5-724-47-27; e-mail: maas@xanum.uam.mx

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to those of 316L stainless steel and titanium, several in vivo and in vitro tests have been performed to learn about the biocompatibility of Zinalco™ and the results showed that it was harmless [5–9].

Among the in vitro tests, Aguilar et al. (unpublished data) found that freshly prepared Zinalco™ aqueous suspensions did not affect the mitotic index (MI) nor the length of DNA migration measured in the comet assay for cultured human lymphocytes.

It has also been determined that Zinalco™ corrodes at a speed of 0.0075 mm/year with ion liberation [10,11]. Knowing that orthopaedic implants sometimes have to remain within the body for long periods (i.e., months), we decided to test if several aqueous Zinalco™ suspensions, aged 2, 4 and 6 months, could have cytotoxic and/or genotoxic effects on proliferating human lymphocytes in vitro. The selected biomarkers were MI, chromosome alterations and DNA damage.

2. Materials and methods

2.1. Zinalco™ suspensions

Stock aqueous suspensions of Zinalco™ particles ($\approx 3\ \mu$) were prepared in the following concentrations: 50, 500 and 2000 μg/ml. They were autoclaved and kept at room temperature for ageing.

2.2. Lymphocyte culture

Peripheral blood samples were obtained from six healthy volunteers (three women and three men) 25–45 years old. Cultures were prepared according to the protocol of Arakaki and Sparkes [12], placing 0.3 ml of whole blood in 60 cc flasks containing 4.5 ml of McCoy's 5a medium (Gibco) and 0.2 ml of phytohemaglutinin (Gibco). One hour before harvesting, colcemid (Microlab) was added to the cell cultures; cells were exposed to an hypotonic treatment with 0.075 M KCl and fixed with Carnoy's fixative. Slides were made using the flame dried technique and stained with Giemsa (Merck).

2.3. Treatment

After the first 24 h of incubation at 37°C, 0.5 ml aliquots from the stock suspensions of Zinalco™ were added to each culture flask to obtain the final concentrations of 5, 50 and 200 μg/ml of media; then, they were incubated again at 37°C for 48 h more. A total of 0.5 ml of distilled and autoclaved water was added to the cultures of the control group. The three experimental groups and the control were prepared in triplicate. Experiments were performed at three different periods: 2, 4 and 6 months after the Zinalco™ suspensions were prepared.

2.4. Mitotic index

The proportion of cells in metaphase was determined from a total number of 6000 cells per treatment per donor.

2.5. Chromosome alterations

Structural changes were scored in 60 good quality metaphase plates for each treatment per donor. The frequency of chromosome aberrations per cell was determined by dividing the total number of chromosome aberrations (excluding gaps) in each group by 360 cells [13,14]. The number of chromosomes in each of a total of 120 metaphases was also recorded in each group in order to detect if there was any modification in the diploid number in the experimental cultures; the modal number was determined in each case.

2.6. Comet assay for DNA damage

Before adding colcemid, an aliquot of 150 μl was taken from each culture flask and washed with PBS. The assay was performed following the Singh et al. [15] procedure. Three layers of agarose gels containing the samples were prepared on fully frosted microscope slides (Fisher) and immersed in a lysing solution for at least 1 h at 4°C. The slides were then placed in a horizontal electrophoresis box, left in the alkaline buffer for 20 min and exposed to an electric current of 300 mA for another 20-min period. After

drops of neutralisation buffer were added, the slides were stained with ethidium bromide (Sigma). A total of 150 nuclei for each donor in each group were analysed for DNA migration and measured using a fluorescent microscope (Olympus).

2.7. Statistical analyses

Absolute MI values recorded were subject to an ANOVA and to the Tukey multiple range test. The same procedure was applied to the DNA migration data. The Chi-square test was used to compare the observed and the expected frequency of chromosome aberrations in all groups.

3. Results and discussion

The MI has been widely used to evaluate cell proliferation, particularly lymphocyte proliferation, since this type of cell has proved to be a suitable experimental model to test genotoxicity in vitro and in vivo [16].

Table 1 shows that the MI average values in the experimental groups tend to diminish as a function of the concentration tested. The differences between the control and the experimental cultures are significant ($P < 0.001$). This behaviour is opposite to that observed when lymphocytes were exposed to the same concentrations of fresh Zinalco™ suspensions (Aguilar et al., unpublished data).

Table 1
MI in human lymphocyte cultures exposed to Zinalco™ suspensions aged 2, 4 and 6 months

Zinalco™ suspension age (months)	Zinalco™ concentration (μg/ml)	MI (%) (mean $\pm$ S.E.)
2	0	4.84 ± 0.26
	5	3.63 ± 0.31[a]
	50	2.76 ± 0.15[a]
	200	2.57 ± 0.14[a]
4	0	5.85 ± 0.54
	5	4.11 ± 0.32[a]
	50	4.13 ± 0.42[a]
	200	2.11 ± 0.23[a]
6	0	2.97 ± 0.21
	5	2.16 ± 0.17[a]
	50	1.89 ± 0.19[a]
	200	1.62 ± 0.14[a]

[a]Significant difference with regard to the control group (Tukey analysis) ($P < 0.05$).

Table 2
Frequency of chromosomal aberration (CA) data from cultures of human lymphocytes treated with different age Zinalco™ suspensions

Zinalco™ suspension age (months)	Zinalco concentration (μg/ml)	G′	G″	B′	B″	CA/cell[a] ($\times 10^{-2}$)
2	0	8	3	5	0	1.39
	5	19	2	3	0	0.83
	50	6	0	7	0	1.94
	200	5	1	1	0	0.30
4	0	22	2	9	0	2.50
	5	23	11	8	1	2.50
	50	23	5	8	0	2.22
	200	27	9	6	1	1.94
6	0	10	1	4	0	1.11
	5	20	2	7	1	2.22[b]
	50	24	2	3	0	0.83
	200	7	0	0	0	0.0

[a]Frequency of CA excluding gaps; CA/cell = total CA per group/360 cells $\times 10^{-2}$.
[b]Significant difference with regard to the control group (χ^2, $P < 0.01$).
G′, G″ = chromatid and isochromatid gap, respectively; B′, B″ = chromatid and isochromatid break, respectively.

In spite of the fact that the same donors participated at all sample times, it was observed that the average MI values varied depending on the month when the blood samples were cultured. The experiments to test each of the different age suspensions were performed during three different months, December 1996 and January and February 1997. As in other studies [17–19], inter-individual variations were also found, most probably due to the different levels of sensitivity of each donor.

Since corrosion might occur but at a low speed, we consider improbable that Zinalco™ particles from a prosthesis could reach concentrations in the body fluids as high as those tested in this study. Moreover, in preliminary in vivo experiments performed in rats, Pérez-Gallardo et al. [20] found that the organisms isolated the implanted Zinalco™ plates by covering them with connective tissue.

It was found that the frequency of chromosomal aberrations per cell (excluding gaps) was similar in all groups (Table 2). Gaps were the most frequent

Table 3
Percentage of cells with $2n = 46$ in human lymphocyte cultures treated with aged Zinalco™ concentrations

Zinalco™ suspension age (months)	Zinalco™ concentration (μg/ml)	Cells with $2n = 46$ (%)
2	0	92
	5	90
	50	94
	200	92
4	0	86
	5	89
	50	90
	200	88
6	0	91
	5	91
	50	92
	200	94

alterations recorded, the chromatid type was more frequent than the isochromatid one; this suggests that Zinalco™ particles may affect the chromosome structure during the G_1 phase of the cell cycle [21]. The χ^2 test showed that there are not significant differences ($P > 0.05$) between the control and the exposed groups to the suspensions aged 2, 4 and 6 months, except for the significant difference at the concentration of 5 μg/ml of the 6-month old suspension.

It is known that each component of Zinalco™ may alter chromosome structure [22–24] but our observations show that in the alloy form they are not clastogenic. This fact could be interpreted as evidence of the chemical stability of the material.

According to our data, Zinalco™ did not have an aneugenic effect ($\chi^2 = 0.29$; $P > 0.05$) since in all groups, nearly 90% of the scored cells showed a diploid number of $2n = 46$ (Table 3). In the remaining 10%, the number varied from 40 to 90.

The highly sensitive comet assay also revealed that Zinalco™ does not have a drastic effect on DNA (Table 4). The highest percentage of cells showing the comet appearance were found in the 2-month old suspension group with 52–58% of damaged cells followed by the 6-month old group with 38–51%.

The lowest proportion of cells, 24–28%, with DNA migration was found in those cultures treated with the 4-month old suspension. The difference between the control group and the one exposed to the concentration of 200 μl/ml was significant ($\chi^2 = 4.26$; $P < 0.05$). Thus, excepting for this case, the alloy does not affect the proportion of cells showing damage with the comet assay.

The distribution of the DNA migration length observed in control and exposed cultures was similar; most of the damaged cells fell in the length range from 1 to 20 μm, followed by a smaller percentage with a length from 21 to 40 μm or longer (Table 4).

The average tail length data of damaged cells in control and experimental cultures are shown in Table

Table 4
Percentage of cells exposed to different age Zinalco™ suspensions that showed DNA migration and distribution of DNA migration

Zinalco™ suspension age (months)	Zinalco™ concentration (μg/ml)	Cells with DNA damage	Distribution of DNA migration (μm)					
			1–20	21–40	41–60	61–80	81–100	101–
2	0	54	78.67	15.67	3.56	1.67	0.44	0
	5	57	70.67	20.56	6.22	2.00	0.44	0.11
	50	52	71.88	22.00	4.94	0.94	0.24	0
	200	58	68.89	21.67	7.33	1.56	0.56	0
4	0	19	94.00	5.78	0.22	0	0	0
	5	24	89.44	9.89	0.67	0	0	0
	50	25	94.33	5.33	0.33	0	0	0
	200	28[a]	94.22	5.44	0.33	0	0	0
6	0	45	92.47	6.12	1.29	0.12	0	0
	5	51	88.33	10.56	0.67	0.33	0.11	0
	50	38	78.33	10.56	5.56	4.00	1.22	0.33
	200	49	77.78	12.22	5.78	2.44	1.44	0.33

[a] Significant difference with regard to the control group (χ^2, $P < 0.05$).

Table 5
DNA damage (mean DNA migration) in cultures of human lymphocytes treated with aged Zinalco™ suspensions

Zinalco™ suspension age (months)	Zinalco™ concentration (μg/ml)	Average tail length (+S.E.) (μm)
2	0	23.21 ± 0.64
	5	25.76 ± 0.70[a]
	50	24.48 ± 0.57
	200	26.95 ± 0.65[a]
4	0	19.12 ± 0.61
	5	20.71 ± 0.51
	50	17.47 ± 0.47
	200	17.21 ± 0.44[a]
6	0	16.07 ± 0.47
	5	17.61 ± 0.44
	50	28.80 ± 1.73[a]
	200	27.30 ± 0.99[a]

[a]Significant difference with regard to the control group (Tukey analysis) ($P < 0.05$).

5. The highest concentration used (200 μg/ml) differed significantly from the control cultures in the 2- and 6-month old groups which can be interpreted as an evidence of its toxicity. However, in the 4-month old group, the behaviour was distinct since the exposed cells to this concentration showed the lowest DNA damage of the group although their MI was also the lowest. This might be due to the triggering of a different kind of response such as a selective killing process or to the proliferation of a heterogeneous cell population as suggested by Nacci and Cayula [25]. The exposure to the Zinalco™ suspensions for aged 2 and 6 months at the concentrations of 5 and 50 μg/ml, respectively, induced significant differences in tail length with regard to their controls. This observation may be explained in terms of variations in the individual sensitivity.

Because of the stability of the material, there are few possibilities of finding particles of this alloy suspended in the body fluids at the concentrations tested in our experiments.

On the basis of this study, we conclude that Zinalco™ shows cytotoxic and genotoxic effects but it is not a clastogenic agent. This alloy may be considered as an alternative biomaterial for designing medical implants and we would recommend its use for prosthesis that would remain in the body for periods of no longer than 2 months.

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