Effects of Ce on the short-term biocompatibility of Ti–Fe–Mo–Mn–Nb–Zr alloy for dental materials

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Effects of Ce on the short-term biocompatibility of Ti–Fe–Mo–Mn–Nb–Zr alloy designed for implant materials were studied by acute toxicity test, hemolytic test, and MTT assay. The elements and their concentration in surface films and extraction media of Ti alloys were investigated with XPS and ICP, respectively. The primary compositions of the surface films of Ti alloys with 0.3% Ce and without Ce were TiO_2 and Nb_2O_5. There were 0.2 mg/l Fe and 0.16 mg/l Mn in the extraction medium of Ti alloy without Ce, while 0.27 mg/l Fe and 0.87 mg/l Mn in the extraction medium of Ti alloy with 0.3% Ce. The concentrations of Fe and Mn in the medium were too low to have any significant effects on human health. There was no sign of cytotoxicity in these tests. The cytotoxicity levels of Ti alloys without Ce and with 0.3% Ce were graded 0 and 1, respectively. The hemolytic degrees of Ti alloys without Ce and with 0.3% Ce were 0.558% and 0.67%, respectively. The cells being incubated in the extraction medium were normal. These phenomena indicated that Ce was innocuous within the concentration range of this study. In addition, the hemolytic ratio and toxicity level of Ti alloy with 0.3% Ce were a little higher than that of Ti alloy without Ce. This meant that Ce would slightly increase the toxicity of Ti alloy.
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1. Introduction

The necessity to substitute the hard tissues of the human body like artificial hip joints, bone, and teeth is growing because the population of those aged over 65 years of age is increasing. Because of high strength–density ratio, low elastic modulus, excellent corrosion resistance, and evident biocompatibility, the interest in applying Ti and Ti alloys to implant materials has been growing recently [1–5]. $\alpha + \beta$ Type Ti alloys such as Ti–6Al–4V ELI, Ti–6Al–7Nb, and Ti–5Al–2.5Fe have been used for orthopedic implant materials. However, the high elastic modulus of these alloys compared to teeth and the toxicity of the alloying elements such as Al and V have been pointed out [6, 7]. The researches of Ti biomaterials are focused on β-type Ti alloys recently, which are of low elastic modulus and high strength [6, 8].

The wear resistance of conventional dental Ti or Ti alloys in oral cavity condition is poor because the hardness of Ti or Ti alloys is only about half of that of the dentine, as shown in Table I. Based on cost accounting and d-electron alloy design methods, a series of new β-type Ti alloys, that is, Ti–Fe–Mn–Mo–Nb–Zr alloys were developed. The strength and hardness of these alloys were greater compared with conventional Ti alloys for dental materials, and the corrosion resistance was excellent [9].

Rare earth elements can refine the crystal structure and improve the mechanical properties of Ti alloys [10]; there have been a few reports about applying rare earth elements on artificial tooth materials. In our prior work, Ce was added into Ti–Fe–Mo–Mn–Nb–Zr alloy, the results showing that a suitable quantity of Ce can refine the crystal structure and improve the comprehensive mechanical properties of Ti alloys [9]. There were some debates about the cell toxicity of rare earth elements [14, 15]. Therefore, the effects of Ce on the short-term biocompatibility of Ti–Fe–Mo–Mn–Nb–Zr alloy were examined in this work.

2. Materials and methods

2.1. Materials

Ti–2%Fe–17%Mo–10%Mn–14%Nb–6%Zr and Ti–2%Fe–17%Mo–10%Mn–14%Nb–6%Zr–0.3%Ce alloys,

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TABLE I The properties of dens and dental alloys [5, 11–13]

Materials	Density (g/cm^3)	Yield strength (MPa)	Compress strength (MPa)	Elongation (%)	Elastic modulus (GPa)	HV
Dentine		48–105.5	232–305		12–18.6	570–600
Enamel		10–40.3	261–400		46–130	3430–4310
Co–Cr	8.7	675		6		359
316		600–700		35–65	200	170–200
316L		540–620		50–60	200	170–200
Ti–Zr	4.8	795		22	100	249 (HK)
Ti (TA2)	4.5	345		20	102	224
Ti–6A1–4V ELI	4.5	896	944	10	113–121	320
Ti–5A1–2.5Fe		942		48		
Ti–75	4.53	730		13–15	115	300

whose compositions are shown in Table II, were melted in a cold-mold non-consumable-electrode electronic arc furnace.

The specimens $(7 \times 7 \times 7\,mm^3)$ were polished with fine sand paper, cleaned with ultrasonic in physiological salt solution for 20 min, degreased with ethyl alcohol, rinsed with phosphate buffer saline thrice, and then kept at 37 °C for drying. Before testing, the specimens and physiological salt solution were disinfected with vapor at 121 °C for 1 h.

2.2. Examination methods

2.2.1. Preparation process of extraction media of Ti alloys

The extraction media of Ti alloys were obtained under standardized conditions (ISO 10993-5). For acute toxicity and hemolytic tests, the specimens were dipped in borosilicate glass tubes containing bacilli-free physiological salt solution for 120 h at 37 °C without shaking, and the medium was replaced with 1 mg/ml DMEM culture medium for MTT assay. The ratio between the specimen surface and the volume of the extraction vehicle was $3\,cm^2/ml$.

2.2.2. Acute toxicity test

Thirty healthy white male mice were used as experimental animals. The weight of each mouse was about 20 g. All of these white mice were divided into three groups at random and there were 10 white mice in each group. The mice in the first group were injected the extraction medium of alloy 1 in the tail vein, and the mice in the second group were injected the extraction medium of alloy 2. As negative controls, the mice in the third group were injected the physiological salt solution. The ratio between the volume of the extraction medium and the mass of mouse was 50 ml/kg. The general state, toxicity expression, and mortality of white mice were observed for at least three weeks. The weight of mouse was measured at 0, 24, 48, and 72 h.

2.2.3. Hemolytic test

Twenty milliliters of blood of a healthy male rabbit was gathered and then heparin sodium was added to form incoagulable rabbit blood. Eight milliliters of incoagulable rabbit blood was diluted with 10 ml physiological salt solution. Ten milliliters of the extraction medium of every specimen was mixed with 0.2 ml incoagulable rabbit blood in a glass tube. As positive and negative contrasts, the extraction medium was replaced with distilled water and physiological salt solution, respectively. Every reagent was manufactured for three copies. Every tube was centrifuged for 5 min under 750 times acceleration of gravity after being incubated at 37 °C for 60 min in 5% CO_2 atmosphere, the absorbance of the supernatant of every reagent was read at 545 nm.

2.2.4. MTT assay

After an incubation period of 48 h, L929 cell lines were digested with 0.25% trypsinase, and then were executed with 10^4 cells/ml suspension with 10% fetal bovine serum. Four pieces of 96-well plates were used. Thirty-two wells in every plate were divided into four groups, with eight wells in every group. After being added 100 µl cell suspension in every test well, the cells were seeded for 24 h to attach the cells to the surface. Then every well of the first group was added 100 µl of the extraction medium of alloy 1 and every well of the second group was added that the extraction medium of alloy 2. The third group was positive control, where every well was added 100 µl of the extraction medium of pure lead. The fourth group was negative control, where every well was added 100 µl DMEM. They were replaced into the culture box with 5% CO_2 atmosphere at 37 °C to culture continuously.

After being incubated for 24, 72, 140, and 188 h, one plate was taken out from the culture box. The shape of the cells was observed and pictures taken with an inverted microscope, and to every test well of the plate was added 50 µl 1 mg/ml MTT. The plate was replaced into the culture box for 3 h, the solutions were removed,

TABLE II Chemical compositions and properties of Ti–Fe–Mn–Mo–Nb–Zr alloys [9]

Materials	Chemical compositions (wt.%)							Compress yield strength (MPa)	HV	Elastic modulus (GPa)	Corrosion depth/year (mm/year)
	Fe	Mo	Mn	Nb	Zr	Ti	Ce				
Alloy 1	2	17	10	14	6	51	0	1258	535	13.8	0.00463
Alloy 2	2	17	10	14	6	51	0.3	1528	685	11.8	0.00230

TABLE III The original weight and weight increment [$\bar{x} \pm S$ (g)] of mice

Samples	Original weight	Weight increment		
		First day	Second day	Third day
Alloy 1	21.54 ± 0.9721	0.72 ± 0.1317	1.88 ± 0.1989	3.04 ± 0.2797
Alloy 2	21.84 ± 1.2635	1.15 ± 0.2012	2.3 ± 0.2366	4.21 ± 0.4104
Positive control	21.31 ± 0.8530	0.69 ± 0.137	1.84 ± 0.2459	2.97 ± 0.1418

and then 150 µl DMSO was added into every well. After 10 min of slow shaking, the absorbance of the solution was read at 540 nm.

2.2.5. Analyses of the surface oxide film of Ti alloys

The composition of surface oxide film of Ti alloys was analyzed with VG ESCALAB MK II X-ray photoelectron spectrograph (XPS).

2.2.6. Analyses of alloying elements in extraction medium of Ti alloys

The concentration and type of elements in the extraction medium of Ti alloys were analyzed with inductively coupled plasma-mass spectrometer (ICP-MS).

2.3. Statistical analysis

Results were expressed as mean values ± standard deviation for each group of samples. After the assessment of significant differences by one-way variance analysis, differences among groups were established by Student's *t*-test by a two-population comparison. Statistical significance was considered at a probability level of $P < 0.05$.

3. Results

3.1. Acute toxicity test

During the observation period of 21 days, no toxic symptom or bad reaction was discovered, and mortality was zero. In the observation period of 72 h, the results of the covariance analyses show that mice weights in both groups of alloys 1 and 2 increase continuously, as shown in Table III, and there was no obvious discrepancy compared with that of the positive controls. It means that the extraction media of alloys 1 and 2 do not have acute toxicity.

3.2. Hemolytic test

The hemolytic degree is expressed by the hemolytic ratio. The formula of the hemolytic ratio can be written as:

$$\text{Hemolytic ratio} = \frac{\text{Absorbency of sample} - \text{Absorbency of negative control}}{\text{Absorbency of positive control} - \text{absorbency of negative control}} \times 100\%$$

The absorbency of every group and the corresponding hemolytic ratio are shown in Table IV. According to standard [16], hemolysis will not occur if the hemolytic ratio is lower than 5%. The hemolytic ratios of alloys 1 and 2 are 0.558% and 0.67%, respectively, so hemolysis cannot be caused by both of the Ti alloys.

3.3. Morphology of cells

In the first day, partial cells of the positive control group do not paste to the wall and became a suspension and died. From the third day, many cells of the positive control group were in the poisoning state, and most of the positive control cells died on the seventh day. On the contrary, the rest groups' cells are in a normal increasing state during the whole observation period, showing no signs of direct cell toxicity.

3.4. MTT assay

According to ISO-7405, the results of the relative growth ratio (RGR) of L929 cells can be changed to toxicity level. The RGR can be estimated with the following equation:

$$\text{RGR} = \frac{\text{Absorbency of test medium}}{\text{Average absorbency of negative control}} \times 100\%$$

The absorbency of every specimen in the MMT assay is shown in Table V. RGR and toxicity levels are shown in Table VI. The result shows that the cell viability expressed by the absorbance values is higher than that of the positive control and the negative control. There are no differences among negative control, alloys 1 and 2 extraction media. It means that both of alloys 1 and 2 do not have toxic effects. The statistical analysis confirms this trend.

4. Discussion

Excellent biocompatibility is a prerequisite of materials used in clinical settings. According to ISO/TR 7406 [17], acute toxicity test, hemolytic test, and MTT assay were chosen to evaluate the effects of Ce on the biocompatibility of Ti alloys for dental materials in this paper.

The result of acute toxicity shows that the extraction

TABLE IV The absorbency and hemolytic ratio of specimens

Specimens	Absorbency			$\bar{x}$	Hemolytic ratio (%)
Alloy 1	0.005	0.008	0.007	0.0067	0.558
Alloy 2	0.001	0.01	0.003	0.0053	0.67
Positive control	0.013	0.009	0.018	0.0133	
Negative control	1.031	1.751	0.84	1.2073	

TABLE V The absorbency ($\bar{x} \pm S$) of every specimen in MMT assay

Specimens	First day	Third day	Fifth day	Seventh day
Alloy 1	0.2460 ± 0.0820	0.3863 ± 0.0437	0.5816 ± 0.1352	0.6642 ± 0.0611
Alloy 2	0.2234 ± 0.0982	0.3180 ± 0.0869	0.5466 ± 0.0848	0.5878 ± 0.0737
Positive control	0.1983 ± 0.1237	0.2428 ± 0.1356	0.3747 ± 0.1039	0.3925 ± 0.1541
Negative control	0.2156 ± 0.1513	0.3468 ± 0.1081	0.5678 ± 0.0774	0.6542 ± 0.0674

TABLE VI The RGR and toxicity level of specimens

Specimens	First day		Third day		Fifth day		Seventh day	
	RGR	Toxicity level	RGR	Toxicity level	RGR	Toxicity level	RGR	Toxicity level
Alloy 1	114	0	110	0	102	0	102	0
Alloy 2	103	0	92	0	96	1	0	1
Positive control	92	1	70	2	66	2	60	2
Negative control	100		100		100		100	

TABLE VII Elements and their molar ratio (mol %) of Ti alloys' surface oxide films

Materials	Ti	Fe	Mo	Mn	Nb	Zr	Ce	O
Alloy 1	14.52	0	2.67	1.74	1.91	1.52	0	77.64
Alloy 2	14.76	0	2.85	1.73	1.82	1.55	0	77.29

TABLE VIII Elements and their contents (mg/l) of the extraction media of Ti alloys

Materials	Ti	Fe	Mo	Mn	Nb	Zr	Ce
Alloy 1	0	0.20	0	0.16	0	0	0
Alloy 2	0	0.27	0	0.87	0	0	0

media of alloys 1 and 2 do not have acute toxicity. Ti alloy containing 0.3% Ce has no obvious discrepancy with Ti alloy without Ce in acute toxicity.

Hemolytic test can exquisitely reflect the influence of material on erythrocyte, so it is a significative test for dental materials. The hemolytic reaction level caused by the toxic material is generally larger than the toxicity reaction level produced in cell culture. If the hemolytic phenomenon is found in hemolytic experiments, it means that the material is toxic. According to ISO/TR 7406, the critical safe hemolytic ratio is 5%. The result of this study shows that hemolytic ratios of Ti alloy with 0.3% Ce and without Ce are 0.67% and 0.558%, respectively; so both of the Ti alloys cannot induce hemolysis. In addition, the hemolytic ratio of Ti alloy with 0.3% Ce was greater than that of Ti alloy without Ce. It means that Ce can increase the toxicity of Ti alloy.

The basic principle of MMT assay is that cells in culture medium proliferate at a known optimal rate, which can be reduced by chemical residua affecting mitochondria activity [18]. This result shows that extracts obtained from Ti alloy without Ce do not cause any damage on mitochondria, so the toxicity level is zero. On the contrary, on the fifth day, the toxicity level of Ti alloy with 0.3% Ce was changed from zero to one. Though this toxicity level is safe, it also shows that Ce can slightly increase the toxicity of Ti alloy.

The outstanding biological safety of Ti and its alloys has a close relationship with its passivation film TiO_2 on the surface. This oxide film can resist the corrosion of

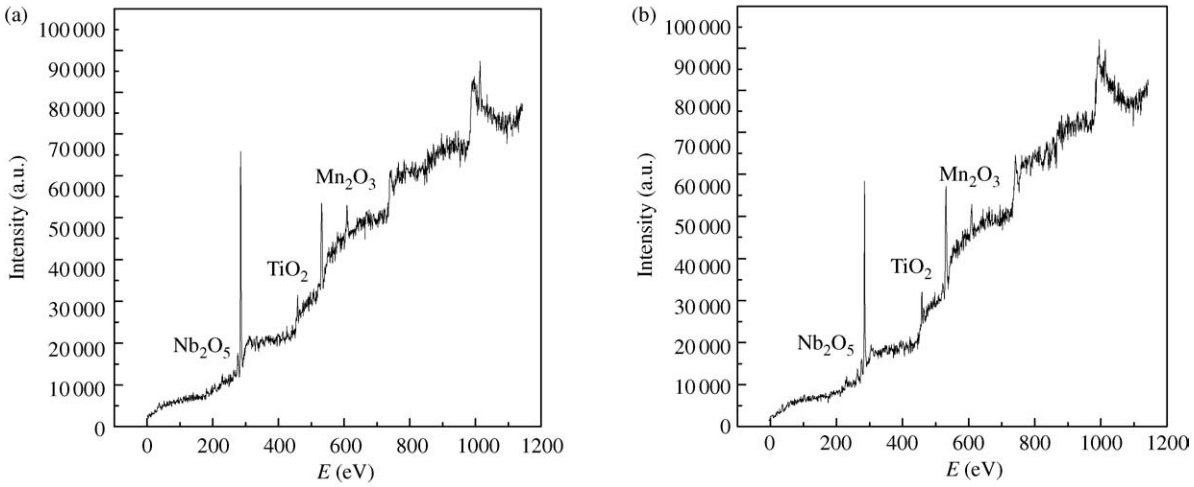

Figure 1 The results of XPS for specimen surfaces (a) alloy 1: (b) alloy 2.

various chemical materials and impede emanation of metal ions [12].

The composition of alloy surface oxide films analyzed with XPS is shown in Table VII and Fig. 1. It was clear that the surface oxide films on both of Ti alloys include TiO_2, Nb_2O_5, and Mn_2O_3, both TiO_2 and Nb_2O_5 can improve the corrosion resistance of Ti alloy. Table VIII shows the elements and concentration in extraction media of Ti alloys. There are 0.2 or 0.27 mg/l Fe and 0.16 or 0.87 mg/l Mn in the extraction media obtained from Ti alloy without Ce or Ti alloy with 0.3% Ce, respectively, and no other alloying elements in the extraction media. This is the reason why Ti alloys with or without Ce are biologically safe. In addition, the concentrations of Mn and Fe are so low that they cannot induce toxicity for the human body.

According to the discussion above, Ce can slightly reduce the biocompatibility of Ti alloys. The reason is that the contents of Fe and Mn in the extraction medium obtained from Ti alloy with 0.3% Ce are higher than those in the extraction medium obtained from Ti alloy without Ce, as shown in Table VIII.

5. Conclusions

The results of the cytotoxicity assays show that the extraction media obtained from Ti–Fe–Mo–Mn–Nb–Zr alloys with 0.3% Ce and without Ce do not have toxic effects. Ti alloys without Ce and with 0.3% Ce do not have acute toxicity. The hemolytic ratios of Ti alloys without Ce and with 0.3% Ce are 0.558% and 0.67%, respectively, and both of them are less than 5%. The cells growing in the extraction media are normal. No damage is found on mitochondria.

In addition, the hemolytic ratio and toxicity level of Ti alloy with 0.3% Ce are slightly higher than those of Ti alloy without Ce. This means that Ce can slightly increase the toxicity of Ti alloy.

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