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Plasma and chemical treatments of zirconia surface for cell response improvement: A comparative study

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ABSTRACT

Zirconia is an inert implantable biomaterial with poor or null cell response. To enhance this condition of inertness, surface modification can be used to induce bioactivity improving the implant-tissue interaction. For bioactivation, a zirconia surface was modified by means of two treatments, one with argon plasma and the other with calcium and phosphate ions in addition to plasma. *In vitro* tests were performed to determine cell response on both surfaces and on a control. The results showed similar evidence of bioactivation in the treated samples. The novelty of this paper lies in the cellular response studies carried out in zirconia treated surfaces with two different bioactivation processes and its comparative evaluation, including cell viability, morphology and spreading. To our knowledge, no prior studies have reported these experiments, which may have many applications in the field of biomaterials.

1. Introduction

Biomaterials are man-made materials intended for medical applications such as implantable devices, and body part replacements. Currently, an attempt is being made to evolve once inert biomaterials to become bioactive [1,2].

For a long time, zirconia has been used as material for implants in dentistry, maxillary and orthopedic contexts [3,4]. However zirconia is an inert material, meaning it does not interact with soft or hard tissue [2,4], leading to the formation of fibrous encapsulation [5]. Thus, modification of surfaces offers a good alternative method for inducing bioactivity in zirconia [3,6,7].

In a previous study, we described cell bioactivity on biofunctionalized zirconia surfaces. We implemented several steps, designed to modify the zirconia surface: a) mechanical polishing, b) argon plasma activation, c) chemical functionalization with phosphate and calcium ions and d) biofunctionalization with a protein coating of laminin 5, which is an adhesion protein of epithelial cells [6]. This study revealed that cell viability, adhesion and proliferation were more feasible on the biofunctionalized surfaces than on those solely activated with plasma or chemically functionalized. However, the two intermediate steps prior to biofunctionalization, were not adequately analyzed and compared to each other. In fact, the viability results indicated cell activity on their surfaces, indicating a positive result that may be worth investigating. Therefore, in the present study we analyzed and compared these two plasma-treated and chemically-functionalized surfaces, testing viability and cell adhesion response for each one, looking to shed some light on the behavior of epithelial cells on these treated surfaces. In this way, looking to contribute to the development of better bioactive biomaterials for implants.

2. Materials and methods

2.1. Materials

The materials used included a dental pre-sintered zirconia (Natura Zir®), $CaCl_2$ and $NAHPO_4$ (Sigma Aldrich). The materials used for the biological tests were Dulbecco's modified eagle's medium (DMEM), Tripsin EDTA 0.05 % and fetal bovine serum (Sigma Aldrich). For cell staining, we used Phalloidin (Biotium CF®488A) for F-Actin and Hoechst (Sigma Aldrich) for nucleus staining.

2.2. Zirconia substrate preparation

Zirconia substrates were disc shaped of 3 mm thickness and 6 mm diameter for the viability tests and 8 mm diameter for cell adhesion tests.

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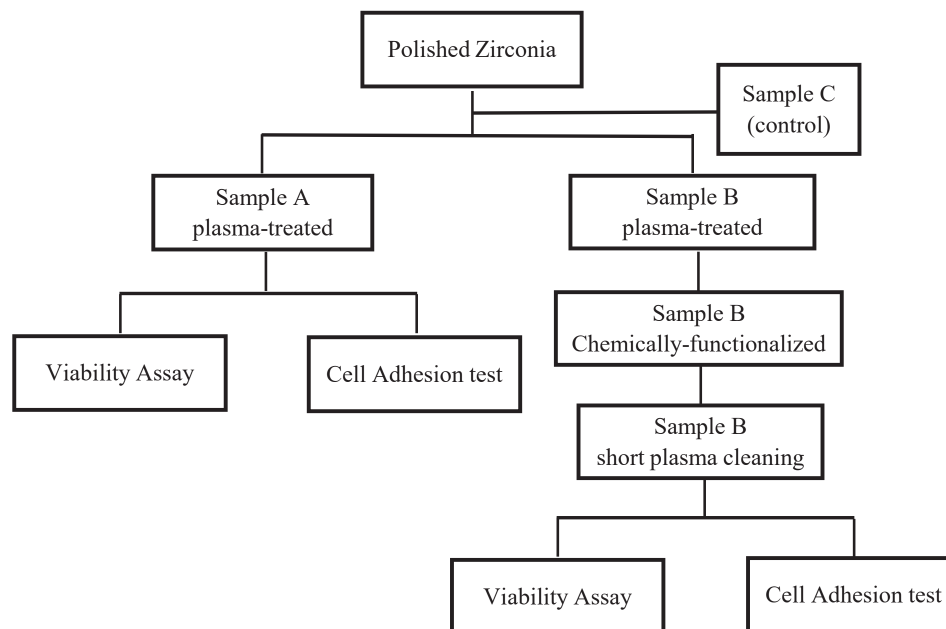

Fig. 1. Scheme for the experimental procedure.

The discs were designed in Solid Works and milled in a Roland dental milling machine DWX5 (Germany). Later, they were ground using a diamond pad (9 μm) for 5 min and polished with a diamond suspension (1 μm), using a nylon pad for 5 min in a rotator GPX 200 Leco polisher at 250 rpm (India). At this point, some discs were separated and taken as controls (sample C). Once the substrates were ready, we followed the experimental procedure as described in Fig. 1.

2.3. Surface treatments

After polishing, zirconia substrates were treated with argon plasma for surface activation using a Diener Plasma Technology model Femto Standard Version (Germany) [6]. Briefly, surfaces were treated with argon plasma for 20 min, at 0.4 mbar pressure, power 70 W and gas flow 10 sccm. After this step, half of the substrates were removed and labeled as sample A. The remaining substrates were chemically functionalized by placing phosphate and calcium ions over their surfaces, following a protocol established by Sunarso, et al. [7]. In summary, the substrates were submerged for 24 h in a 0.1 M NaH2PO4·H2O aqueous solution and for 1 h in a 0.01 M CaCl2 solution at 80°C [6]. Finally, we applied a short plasma cleaning (3 min.). These samples were named sample B.

To evaluate and compare the effect of these surface treatments on zirconia, wettability tests were carried out using a FTA200 wettability equipment (Virginia, USA). For this, a distilled water drop of 2 μL was automatically deposited on the sample surface using a surgical syringe with a precision flow control valve, then static water contact angles were measured for each sample at room temperature.

2.4. In vitro studies

2.4.1. Viability assays

The cell viability tests for the two treated zirconia surfaces and the control, labeled as A, B and C, were carried out by means of MTT assays. MTT assay is a colorimetric test that measures cellular metabolic activity through a reduction of MTT, a yellow tetrazolium salt, to a purple formazan crystalized salt catalyzed by mitochondrial succinate dehydrogenase. Since only viable cells with active metabolism convert MTT into a purple colored formazan product, color change can be a quantifiable marker of live cells [8]. The experiment was completed as follows: (1) Three different substrates from each sample A, B, and C were prepared (9 in total). (2) The substrates were placed in untreated 96-well plates, which don't allow cell attachment, and 1.5×10^4 cells were seeded on each substrate in a culture medium of DMEM supplemented with 10% FBS and incubated for 20 h at 37°C and 5% CO_2. (3) Culture medium was withdrawn and each substrate was very gently washed with PBS. (4) DMEM and MTT (0.5 mg/ml in PBS) was added to each substrate. The substrates were protected from light and incubated at 37°C and 5% CO_2 for 4 h. (5) The medium was withdrawn and the formazan crystals were solubilized in acidified isopropanol. (6) Afterward, 100 μL of solubilized formazan were collected from each substrate and deposited in to a 96-well plate for spectroscopic analysis. This was done by means of UV–vis absorbance measurements at 570 nm using a microplate reader Varioskan Lux VLBLATD2 Thermo Fisher Scientific (USA).

The experiment was repeated three times and were labeled as assay 1, 2 and 3 respectively; therefore, nine cell viability measurements were obtained for each sample.

To calculate cell viability, which is the percentage of live cells, a positive control is necessary, so the same number of cells used in the essay (in our case 1.5×10^4 cells) are seeded in a treated plate that assures a 100% of viability and the absorbance is measured in a UV-vis spectrometer. The absorbance measured is taken as the positive control and is used as reference to calculate the viability in the following essays. Thus, cell viability is calculated dividing the measured sample absorbance by the positive control absorbance as follows:

$$\%Cellviability = \frac{sampleabsorbance}{positivecontrolabsorbance} * 100$$

2.4.2. Cell adhesion and morphology

Samples A, B and C were deposited in a 24-well plate. Then, 1.5×10^4 cells were seeded on each surface. DMEM supplemented with 10% of fetal bovine serum was added to each well and the plate was incubated

Table 1
Contact angle measurements.

	Sample A	Sample B	Sample C
Contact angle immediately after surface treatment	3.81° (±0.17°)	31.41° (±3.03°)	62.44° (±2.51°)
Contact angle 48 h after surface treatment	48.04° (±7.12°)	51.18° (±5.1°)	Not measured

Table 2
Viability values from MTT assays.

Sample	assay 1			assay 2			assay 3			Average	
	% Viability			% Viability			% Viability			% Viability	std dev
	substrate 1	substrate 2	substrate 3	substrate 1	substrate 2	substrate 3	substrate 1	substrate 2	substrate 3		
A	71	51	65	49	46	67	70	48	64	59	10
B	58	70	65	55	72	59	58	66	71	64	6
C	43	27	35	22	20	29	45	33	37	32	9

for 24 h at 37°C, 5% CO_2 and 80% humidity. Subsequently, the DMEM was removed, all samples were gently washed with PBS and the cells were staining following a protocol previously reported [6]. Finally, samples were analyzed using a confocal fluorescence microscope (Zeiss LSM-700, Germany). The number of adhered cells per image field (0.1024 μm^2) was counted and averaged, analyzing six images per sample and three samples per group (n = 18), using the image J software (USA). Amplification 20x.

3. Results

3.1. Wettability tests

Wettability tests done on samples A, B and C are summarized in Table 1. The average contact angle was obtained from three measurements. Taking the control sample C (untreated) as reference, it can be observed that the surface treatments on samples A and B significantly impacted on its surface polarity, reducing dramatically the contact angle for the water drop. Additionally, in an effort to get some information about the surface activation lasting time, measurements 48 h after treatment were made on samples A and B, the results indicated that surface activation actually decays in time.

3.2. Viability assays

Results obtained are shown in Table 2 and summarized in Fig. 2. The percentage of viability was obtained averaging the nine measurements for each sample and its standard deviation was calculated as well. The average numbers showed very similar cellular viability for samples A and B 59% ± 10 and 64% ± 6 respectively, with no significant differences. Contrarily, the control sample C showed a much lower viability of 32% ± 9.

3.3. Cell adhesion and morphology

The images show cells cultivated on zirconia surfaces, Fig. 3; the nuclei are stained in blue and the actin filaments in green. Samples A and B yielded similar results, showing cell spreading and an analogous cellular density Fig. 4. Contrarily, the control sample C shows a rounded-shape with non-spreading morphology cells. This non-flat morphology impedes focusing, causing blurry images. Furthermore, cellular density was also lower than in the treated samples.

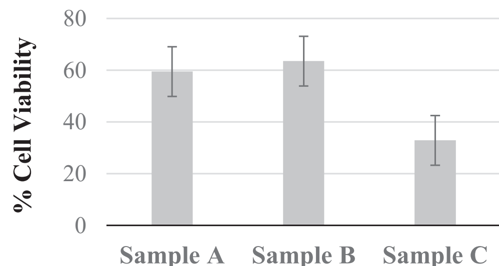

Fig. 2. Graphical representation of MTT assays. Mean values are the average of nine measurements for each sample and bars represent standard deviation.

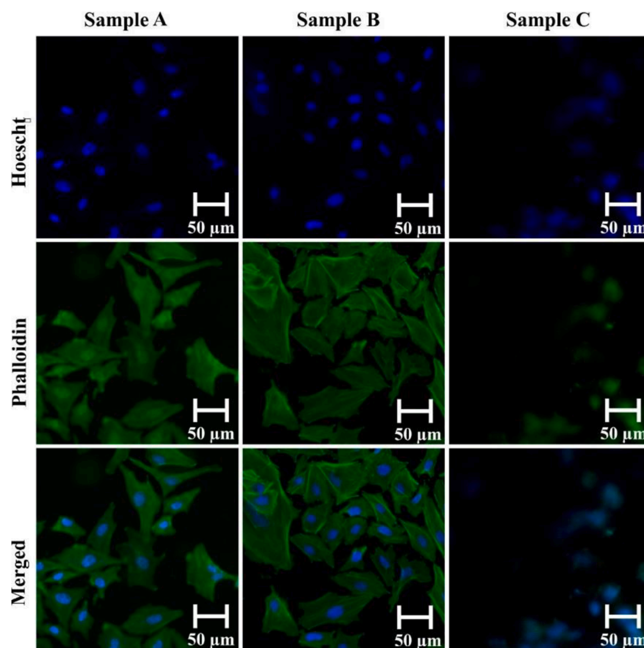

Fig. 3. Cell adhesion on modified zirconia surfaces after 24 h, (a) Sample A, (b) Sample B and (c) Sample C. Hoechst for nucleus and phalloidin for actin staining.

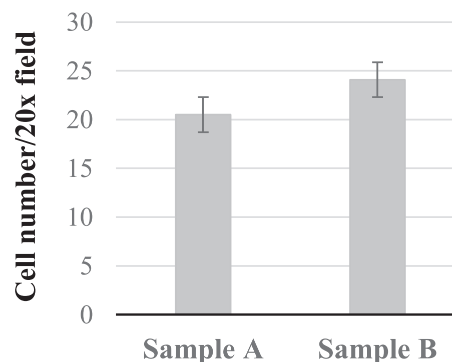

Fig. 4. Cell adhesion on modified zirconia surfaces after 24 h. The number of adhered cells per image field (n = 18) was counted and averaged.

4. Discussion

The results obtained from these experiments concur with those reported previously [6]. Both surface treatments; activation with argon plasma or treatment with ions considerably increased zirconiás bioactivity, compared to polished surfaces (untreated). This can be explained by changes in polarity, which is neutral due to the balance between Zr and O [9], and the consequent increase in surface energy. This neutrality

was modified through plasma that rises the amount of oxygen at the surface by bond breaking and incorporation into reactive sites [10] as well as through chemical treatment adding phosphate and calcium ions [6]. This phenomenon can be macroscopically observed through changes in wettability or measuring Z potential, reported in our previous work [6]. The experimental results demonstrate that cell response is significantly enhanced by these changes at the zirconia surface. Also, the cell adhesion test provides information about how cells interact with a surface; good interaction leads to a spreading morphology [2]. Unexpectedly both treatments; plasma and chemical functionalization, exhibited a similar degree of cellular response, in terms of viability, adhesion and spreading. This may indicate that the cellular response was instigated by the improvement in surface polarity rather than by chemical modification. About polished surfaces, the fact that cells maintain their viability can be explained because zirconia is an inert biocompatible material, thus it is non-toxic for cells. Likewise, polishing may alter surface polarity to some extent. However, this surface does not offer apt conditions for cellular anchorage.

5. Conclusions

The results obtained from *in vitro* studies with cell cultures demonstrate that zirconia surfaces can be bioactivated by surface treatments that are able to modify their polarity or surface energy. In this particular case, the similar behavior of cells on both surfaces; plasma-treated and chemically- functionalized, may indicate that cellular response is caused by changes in polarity rather than chemistry. Although, our previous report indicated that surfaces biofunctionalized with laminin 5 protein manifest significantly better cell response and viability than those activated with plasma or chemically functionalized, these intermediate treatments still present enough cell activity to be considered as a feasible option for bioactivation; the main advantage being that the preparation process is simpler, shorter, and cheaper than those that use adhesion of biomolecules, such as proteins or peptides.

CRediT authorship contribution statement

Lillian V. Tapia-Lopez: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Antonia Luna-Velasco:** Resources, Writing - review & editing, Supervision, Visualization, Funding acquisition. **Hilda E. Esparza-Ponce:** Writing - review & editing, Supervision. **Javier S. Castro:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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