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TITLE:

Accessing the Cytotoxicity and Cell Response to Biomaterials

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biocompatibility, biomaterials, cell culture, cytotoxicity, conditioned medium, extracts.

SUMMARY:

This methodology aims to evaluate biomaterial cytotoxicity through the preparation of soluble extracts, using viability assays and phenotypic analysis, including flow cytometry, RT-PCR, immunocytochemistry, and other cellular and molecular biology techniques.

ABSTRACT:

Biomaterials contact directly or indirectly with the human tissues, making it important to evaluate its cytotoxicity. This evaluation can be performed by several methods, but a high discrepancy exists between the approaches used, compromising the reproducibility and the comparison among the obtained results. In this paper, we propose a protocol to evaluate biomaterial cytotoxicity using soluble extracts, which we use for dental biomaterials. The extract preparation is detailed, from pellet production to its extraction in a culture medium. The biomaterial cytotoxicity evaluation is based the following: metabolic activity using the MTT assay, cell viability using the Sulphorhodamine B (SBR) assay, cell death profile by flow cytometry, and cell morphology using May-Grünwald Giemsa. Additional to cytotoxicity evaluation, a protocol to evaluate cell function is described based on the expression of specific markers assessed by immunocytochemistry and PCR. This protocol provides a comprehensive guide for biomaterials cytotoxicity and cellular effects evaluation, using the extracts methodology, in a reproducible and robust manner.

INTRODUCTION:

Biocompatibility can be defined as the capacity of a material to integrate tissue and induce a favorable therapeutic response, free of local and systemic damages[1–3]. Biocompatibility evaluation is crucial for the development of any material intended for medical use. Therefore, this protocol provides a systematic and comprehensive approach for every researcher aiming to develop new biomaterials or studying new applications for existing biomaterials.

In vitro cytotoxicity tests are widely used as the first phase for biocompatibility evaluation, using primary cell cultures or cell lines. The results constitute a first indicator of potential clinical application. Besides being vital for the biomaterial development, this testing is mandatory to comply with current regulations for market introduction, from EUA and EU regulators (FDA and CE certification)[4,5–8]. Moreover, standardized testing in biomedical research provides a significant advantage in terms of reproducibility and comparison of results from different studies on similar biomaterials or devices[9].

International Organization for Standardization (ISO) guidelines are widely used by multiple independent commercial, regulatory, and academic laboratories for testing materials in an accurate and reproducible manner. The ISO 10993-5 refers to the in vitro cytotoxicity assessment and the ISO 10993-12 reports to sampling preparation[10, 11]. For biomaterial testing, three categories are provided, to be selected according to the material type, contacting tissues, and the treatment goal: extracts, direct contact, and indirect contact[8, 11–13]. Extracts are obtained by enriching a cell culture medium with the biomaterial. For the direct contact tests, the biomaterial is placed directly on the cell cultures, and, in indirect contact, incubation with the cells is performed separated by a barrier, such as an agarose gel[11]. Appropriate controls are mandatory, and a minimum of three independent experiments should be performed[5, 8, 10, 11, 14].

It is critical to simulate or exaggerate clinical conditions to determine the cytotoxic potential. For extract testing, test the following: the material's surface area; the medium volume; the medium and the material pH; the material solubility, osmolarity and diffusion ratio; and the extraction conditions such as agitation, temperature, and time

influence media enrichment[11].

The methodology allows the quantitative and qualitative evaluation of cytotoxicity of several pharmaceutical formulations, both solid and liquid. Several assays can be performed, such as neutral red uptake test, colony formation test, MTT assay, and XTT assay[5, 10, 14].

Most cytotoxicity assessment studies published use simpler assays, namely MTT and XTT, which provide limited information. Evaluating biocompatibility should not only involve the assessment of cytotoxicity but also bioactivity of a given test material[2], as this protocol endorses. Additional evaluation criteria should be used when justified and documented. Thus, this protocol aims to provide a comprehensive guide, detailing a set of methods for the biomaterial cytotoxicity evaluation. Besides, the evaluation of different cellular processes, namely the type of cell death, cell morphology, cell function in the synthesis of specific proteins, and specific tissue production, are described.

PROTOCOL:

1. Pellet preparation

1.1. Prepare the molds of polyvinyl chloride (PVC) by using PVC plates with circular-shaped holes of known dimensions.

NOTE: PVC moldings can be made of different sizes. Calculate the contact surface of PVC molds, using the formula $A= 2\pi r(r+h)$ (r: radius of the cylinder; h: height of the cylinder).

1.2. Prepare the biomaterial to be tested according to the manufacturer's instructions and as close as possible to the beginning of the experiment.

NOTE: For the preparation of biomaterials paste/paste formulation, an adequate amount of base paste and catalyst are mixed manually with a mixing spatula. For other materials with liquid and powder formulations, manual spatulation or mechanical mixing with vibration should be performed, following the manufacturer's instructions or the adequate for new materials. For liquid materials, this step is not necessary. Start the protocol in step 2.

1.3. Place the biomaterial on the molds with a spatula and let them set for the appropriate time.

NOTE: The setting time and setting conditions of the biomaterials must follow the manufacturer's instructions or the adequate for new materials.

1.4. After setting, remove the biomaterial' pellets from the PVC molds and place them in a container (a 6 well plate or a Petri dish can be used).

1.5. Sterilize the pellets by placing them under an ultraviolet light (UV) lamp for 20 minutes for each side.

2. Obtaining soluble extracts of biomaterials

NOTE: All procedures should be performed under strict sterile conditions.

2.1. Determine the necessary number of pellets by calculating the pellet surface area based on the formula described in 1.1.

NOTE: As a reference value, the contact surface area of 250 mm^2/mL[11, 15] is achieved by adding 9 pellets (r 3 mm x h 1.5 mm) *per* mL of the medium.

2.2. Prepare the soluble extracts (extract enriched with the biomaterial).

2.2.1. Place the pellets in a 50 mL tube and add the corresponding of the cell culture medium, appropriate for the cell cultures to be used. Place the tubes for 24 hours in the incubator at 37°, in constant rotation.

NOTE: Use the cell culture medium appropriate for the cell cultures.

2.2.2. After 24 hours, remove the tubes from the incubator. At this point, the extracts correspond to a concentration of 1/1 or 100%.

2.2.3. Make dilutions of the extract by sequential addition of the cell culture medium in a 1:2 ratio.

NOTE: No pH adjustment should be made to the media.

2.2.3.1. Add 1 mL of culture media to 1 mL of 100% extract to obtain a 50% extract. Add 1 mL of culture media to 1 mL of 50% extract to obtain a 25% extract, and so on (**Figure 1**).

NOTE: Use the concentrations found relevant for each compound.

[Please place Figure 1 here.]

3. Cell culture preparation

3.1. Prepare a cellular suspension and plate it in an adequate cell container, such as a multiwell plate, according to the number of cells needed for the experiments.

3.1.1. Start with a flask of the desired cells with 80% to 90% confluence.

3.1.2. Discard the cell culture media, wash with phosphate-buffered saline solution (PBS) and detach the cells with trypsin-EDTA (1 to 2 mL for a 75 cm^2 cell culture flask).

3.1.3. Add the cell culture media (2 to 4 mL for a 75 cm^2 cell culture flask) and centrifuge at 200 x *g* for 5 min.

3.1.4. Suspend the pellet in a known volume of cell culture media.

NOTE: This protocol is designed for the use of adherent cell cultures; however, simple adaptations can be made to work with suspension cell cultures.

3.1.5. Count the cells in the hemocytometer and calculate the cell concentration of the cell suspension.

3.1.6. Suspend the determined amount of cell suspension in culture medium and transfer to multiwell dishes. As a reference value for seeding density, consider 5 – 20 x 10^5 cells/cm^2.

NOTE: The appropriated number of cells must be calculated according to the cell type and cell characteristics, namely cell doubling time.

3.2. Incubate the cells for 24 hours to allow cell adhesion.

3.3. After this period, administer the soluble extracts into the culture plates.

3.3.1. Aspirate the cell culture medium.

3.3.2. Add the biomaterials' extracts to each well, according to the sequence of concentrations, as described previously. Add fresh cell culture medium to the control wells.

3.3.3. Incubate the plates for 24 h or longer.

NOTE: Negative controls must be performed in each assay, corresponding to untreated cells, maintained in the culture medium. The incubation times can be selected accordingly to the study goals.

4. Evaluation of the metabolic activity

4.1. After the step 3.3.3, aspirate the medium from the plates and wash each well PBS.

4.2. Place, in each well, the adequate volume of 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazole bromide (MTT) prepared in PBS, pH 7.4.

4.3. Incubate the plates for 4 h or overnight in the dark at 37 ° C.

4.4. To solubilize the obtained formazan crystals, add the adequate volume of 0.04 M solution of hydrochloric acid in isopropanol to each well and stir plates for 30 minutes.

NOTE: Adjust the amount of MTT and isopropanol according to the size of the wells.

4.5. Stir and homogenize the contents of each well, if necessary, by pipetting up and down until no crystals are seen.

4.6. Quantify the absorbance at a wavelength of 570 nm with a 620 nm reference filter, in the spectrophotometer.

4.7. To calculate the metabolic activity, divide the absorbance of the treated cells by the absorbance of the control cultures. To obtain percentage values multiply by 100.

5. Cell death evaluation

NOTE: To perform this evaluation a minimum of 10^6 cells per condition should be used.

5.1. Use centrifuge tubes properly identified.

5.2. After soluble extracts incubation, described in step 3.3.3, collect the culture media to the respective tube.

5.3. Detach the cells and add the suspension to the respective tubes.

5.4. Concentrate the suspensions by centrifugation at 120 x *g* for 5 minutes.

5.5. Wash the pellets with PBS. Remove the PBS by centrifugation at 1,000 x *g* for 5 minutes.

5.6. Add 1 mL of PBS and transfer the cell pellets to identified cytometry tubes.

5.7. Remove the PBS by centrifugation at 1,000 x *g* for 5 minutes.

5.8. Incubate with 100 µL of binding buffer (0.01 M HEPES, 0.14 mM NaCl and 0.25 mM $CaCl_2$)[16], and allow the cells to rest for about 15 minutes for cell membrane recovery.

5.9. Add 2.5 µL of Annexin-V Fluorescein isothiocyanate (FITC) and 1 µL of IP for 15 minutes at room temperature in the dark.

5.10. After incubation, add 400 µL of PBS and analyze it on the cytometer. For the analysis and quantification of the information use appropriate software.

5.11. Present results as a percentage of living cells, apoptosis, late apoptosis/necrosis, and necrosis.

6. Morphology evaluation

6.1. Select the appropriate size of sterilized glass coverslips that fit inside the multiwell plate.

6.2. Place each slide in a well using sterile tweezers.

6.3. Distribute a cellular suspension at an adequate concentration into the wells and let overnight in an incubator at 37 °C in a humidified atmosphere with 95% air and 5% CO_2.

6.4. Expose the cell cultures to the extracts, as described in step 3.3.

6.5. Aspirate the extracts.

6.6. Let the coverslips dry at room temperature and then add a sufficient volume of May-Grünwald solution to cover the coverslips; incubate for 3 minutes.

6.7. Remove the dye and wash with distilled water for 1 minute.

6.8. Remove the water and add a sufficient volume of Giemsa solution to cover the coverslips for 15 minutes.

6.9. Wash the coverslips in running water.

6.10. Transfer the coverslips to a slide with forceps.

6.11. Look under a microscope. Take the photographs with the chosen magnification.

7. Cell function assessment through reverse transcription polymerase chain reaction (RT-PCR)

NOTE: Accordingly, to the study goal, select the specific markers to be evaluated. As an example, alkaline phosphatase is presented as a gene of interest for odontoblasts activity evaluation. Other genes of interest can be seen in **Table 1**.

7.1. Distribute 20,000 cells/cm^2 of control medium, 30,000 cells/cm^2 of biomaterials groups that slightly affect cell viability, and 50,000 to 100,000 cells/cm^2 to biomaterials that drastically affect cell viability into cell culture flasks.

NOTE: The adjustment of cell number depends on the cell type.

7.2. Incubate with soluble extracts, as described in 3.3.

7.3. Detach the cells to obtain a suspension as described in 3.1.2, 3.1.3, and 3.2.4.

7.4. Wash approximately 2 million cells with PBS twice; for this centrifuge at 200 x g for 5 minutes at room temperature.

7.5. Lyse the cells by suspending the pellet in 1 mL of RNA purification solution (e.g., NZYol), intense stirring, and successive pipetting.

7.6. Incubate the samples for 5 minutes at room temperature.

7.7. Add 200 µL of chloroform and shake the tubes by hand for 15 seconds.

7.8. Incubate for 3 minutes at room temperature.

7.9. Centrifuge lysates at 4 ° C for 15 min at 12,000 x *g*. During this centrifugation, two phases originate in the sample, leaving the RNA in the aqueous (upper) phase.

7.10. Remove the aqueous phase to a new tube and add 500 µL of cold isopropanol to precipitate RNA.

7.11. Incubate samples at room temperature for 10 minutes and centrifuge at 12,000 x *g* for 10 minutes at 4 ° C.

7.12. Remove the supernatant and wash the pellet with 1 mL of 75% ethanol by centrifugation at 7,500 x *g* for 5 minutes at 4 ° C.

7.13. Dry the pellet at room temperature until ethanol evaporation.

7.14. Suspend in RNase-free water.

7.15. Quantify and determine the degree of purity of the samples using absorption spectrophotometry. Determine the purity of the RNA by the ratio of the results obtain with the wavelength 260 nm and 280 nm and use only samples with a purity ratio (A260/280) around 2.0.

7.16. Store samples at -80 ° C.

7.17. Proceed to perform RT-PCR following manufacturer's protocol[17].

8. Cell function assessment through protein identification

NOTE: According to the study goal, select the specific proteins to be evaluated. As an example, dentin sialoprotein (DSP) is presented as a protein of interest for odontoblasts activity evaluation. Other proteins of interest can be seen in **Table 1**.

8.1. Culture cells in coverslips and expose to the extracts; for this, repeat step 6.1 to 6.5.

8.2. Wash the cell cultures with PBS.

8.3. Fix with 3.7% paraformaldehyde for 30 minutes at room temperature.

8.4. Wash twice with PBS.

8.5. Permeabilize with 0.5% Triton in PBS for 15 minutes.

8.6. Block the peroxidase with 0.3% hydrogen peroxide in PBS for 5 minutes.

8.7. Wash twice with PBS.

8.8. Wash twice with 0.5% bovine serum albumin (BSA).

8.9. Block cell cultures with 2% BSA for 45 minutes.

8.10. Wash with 0.5% BSA in PBS.

8.11. Incubate cultures with the primary antibody according to the select protein for 60 minutes at room temperature.

NOTE: This protocol uses the primary antibody DSP(M20) Antibody (1:100) and the secondary antibody Polyclonal Rabbit Anti-goat immunoglobulins/HRP (1:100).

8.12. Wash five times with 0.5% BSA in PBS.

8.13. Incubate with secondary antibody for 90 minutes at room temperature.

NOTE: Make the antibody dilutions using 0.5% PBS in BSA.

8.14. Wash five times with 0.5% BSA in PBS for 1 minute in each wash.

8.15. Incubate cultures with a substrate and chromogen mixture at a concentration of 20 µL chromogen/mL substrate for 25 minutes.

8.16. Wash twice with 0.5% BSA in PBS.

8.17. Counterstain with Hematoxylin for 15 minutes.

8.18. Wash with a sequence of 0.037 mol/L ammonia and distilled water for 5 minutes to remove excess dye.

8.19. Mount the coverslips on the slides. Use glycerol as the mounting medium.

8.20. Allow drying overnight.

8.21. Look under a microscope. Take the photographs with the chosen magnification.

9. Mineralized formation assessment through alizarin red s assay

9.1. Prepare an Alizarin Red S solution at a concentration of 40 mM[18] Stir the solution for homogenization for 12 hours in the dark.

NOTE: To prepare 100 mL of Alizarin Red S solution, solubilize 14.4 g of alizarin powder (Molecular weight: 360 g/mol) in ultrapure water, protected from light. For this solution, the pH value is critical and should be between 4.1 and 4.3.

9.2. Incubate cell culture with soluble extracts, as described in 3.3.

9.3. Wash cell cultures with PBS three times.

9.4. Fix with 4% paraformaldehyde for 15 minutes at room temperature.

9.5. Wash three times with PBS.

9.6. Stain with Alizarin Red Staining solution for 20 minutes at 37 °C in the dark.

9.7. After staining, wash the plates with PBS until complete dye removal.

9.8. Look under a microscope. Take the photographs with the chosen magnification.

9.9. Add an extraction solution, composed by 10% (w/v) acetic acid and 20% (w/v) methanol, to each well, and let stirring for 40 minutes at room temperature.

9.10. Measure the absorbance at 490 nm wavelength on a spectrophotometer[19].

REPRESENTATIVE RESULTS:

The representative results here refer to the study of dental biomaterials. The extract methodology allows to obtain a cytotoxicity profile and cell function after exposition to the dental materials, regarding effects on metabolic activity (**Figure 2**), cell viability, cell death profile and cell morphology (**Figure 3**), and specific proteins expression (**Figure 4**).

The MTT assay is used to obtain a quick overview of the cytotoxicity of the materials in a quick and straightforward way. A comparison between two or more materials can be made (**Figure 2**), where a severe reduction of the metabolic activity, even when low (6.25%) and medium concentrations (50%), indicates higher toxicity (**Figure 2a**). At the same time, less cytotoxic materials present only lighter or no reduction (**Figure 2b**). Comparisons between different time points allow determining more immediate cytotoxic effects or at later stages.

Effects on cell viability provide important information about viable cell reduction, which can compromise the tissues' capacity to recover after a damaging effect. The determination of the percentage of viable cells allows comparing material cytotoxicity; more cytotoxic materials induce higher cell death for the same concentration (**Figure 3a and 3b**). Reductions superior to 30% are critical and define materials at risk of low biocompatibility (**Figure 3a**). This information is completed with the cell death profile (**Figures 3a and 3b**). In the representative results, more cytotoxic materials are characterized by an accentuated decrease in cell viability and for a late apoptosis and necrosis cell death profile (**Figure 3a**), while less cytotoxic ones present less cell death and a more apoptotic and late apoptotic profile (**Figure 3b**).

The information obtained from the cellular morphology evaluation (**Figure 3c**) complements the cell viability evaluation. Changes from the cell´s typical morphology can indicate an apoptotic or necrotic profile[16]. Also, additional information can be obtained from this protocol, like the observation of material particles (red arrows, **Figure 3C**).

Specific markers, fundamental to cell function, affected by the extract exposure can be evaluated by several techniques, as immunohistochemistry, PCR, flow cytometry, blotting, or colorimetric assays (**Table 1**). Representative results of the DSP expression after exposure to extracts are shown in **Figure 4a**, and it can be seen that some materials (tricalcium silicates cements) stimulate the cells to increase protein expression. In contrast, others (calcium hydroxide cements) promote a significant decrease in protein expression, independently of viability loss. In both cases, the concentration of the extracts directly influences the protein expression.

In the MDPC-23 cell line of the odontoblast phenotype, the formation of mineralization deposits is characteristic. The protocol for the mineralized deposits identification and quantification allows evaluating the specific function of this type of specialized cells. In the presented case, it was observed that besides being less cytotoxic, tricalcium silicates cement stimulates the cell function, once an increase in mineralized deposits was observed (**Figure 4b**). On the opposite, the more cytotoxic calcium hydroxide cement led to reduced mineral deposition due to cell impairment and death (**Figure 4b**). Additional to a qualitative evaluation, a quantitative determination can be performed (**Figure 4c**).

FIGURE AND TABLE LEGENDS:

Figure 1: Scheme of the preparation and dilutions of soluble extracts.

Figure 2: Metabolic activity. Metabolic activity of MDPC-23 cells treated with calcium hydroxide cement [**a**)] and tricalcium silicate cement [**b**)] soluble extracts for 24, 72, and 120 hours. The results are normalized to the control cell cultures, with a value of 100%. Significant differences are represented by *, where * means $p<0.05$, ** means $p<0.01$, and *** means $p<0.001$. Part of this Figure has been modified from a previous publication with permission from the publisher[20].

Figure 3: Cell viability, death profile, and cell morphology. Cell viability, cell death profile, and cell morphology in MDPC-23 cells subjected to treatment with calcium hydroxide and tricalcium silicate biomaterials at 6.25% and 50% concentration, after 120 hours of exposure. **a**) and **b**) Results are plotted as the percentage of living cells in apoptosis, late apoptosis or necrosis, and necrosis. Significant differences with respect to control or between conditions are represented with *, where * means $p <0.05$, ** means $p <0.01$, and *** means $p <0.001$. **c**) Cells stained with May-Grünwald Giemsa after treatment with a 50% concentration of biomaterials soluble extracts. The control group represents cells in culture in DMEM with 10% FBS. Images in the left column were obtained with a magnification of 100x, and the images in the column on the right were

obtained with a magnification 500x. Figure bars represent 100 µm. Part of this Figure has been modified from a previous publication with permission from the publisher[20].

Figure 4: DSP expression and mineralized nodule formation. a) MDPC-23 cells labeled by immunocytochemistry for the detection of DSP expression when subjected to treatment with calcium hydroxide and tricalcium silicate at concentrations of 50% and 6.25% after 96 hours of incubation. **b**) Images from cultured MDPC-23 cells stained with Alizarin Red S stain when treated with calcium hydroxide and tricalcium silicate biomaterials at concentrations of 50% and 6.25% after 120 hours of incubation. All the photographs were obtained with a magnification of 100x. Both Figure bars represent 150µm. **c**) Formation of calcium deposits from MDPC-23 cells treated with calcium hydroxide and tricalcium silicate after 120 hours of exposure. The results are the relationship between the absorbances obtained between the study condition and the control. Significant differences are represented by *, where * means $p<0.05$, ** means $p<0.01$, and *** means $p<0.001$. Part of this Figure has been modified from a previous publication with permission from the publisher[20].

Table 1: List of odontoblastic differentiation/function markers [47-79]**.** This table provides a list of odontoblastic markers and detection methods; some of these markers are also expressed by other tissues.

DISCUSSION:

This protocol was designed taking into consideration the ISO 10993-5, which refers to the evaluation of in vitro cytotoxicity of biomaterials that contact with the tissues, to evaluate the biocompatibility and to contribute to studies reproducibility[21]. This is a growing concern in science, and many authors are already following these recommendations in the experimental design of their in *vitro* studies[15, 22–28].

The methodology proposed was selected to screen the most relevant aspects of cell biology. Thus, this protocol goes beyond the recommendations, once it provides a complete approach to evaluate cytotoxicity using common assays and a complementary evaluation, including several cell parameters from phenotype to function. This complementary evaluation is important to truly assess the biomaterials effect, once viability may not translate alterations at the level of gene and protein expression, cell cycle, or secretome.

The extracts are advantageous, particularly in adherent cell lines, because there is no interference with cell attachment to the substrate and optimal culture conditions, in opposition to some direct contact approaches where materials are placed on the surface of the culture plate[22, 28].

Moreover, extracts allow cell exposure to different concentrations[29], mimicking diffusion of substances in tissues, which simulates the clearance they undergo in vivo, particularly when they are applied in contact with extremely irrigated tissues. Direct contact tests may not accurately assess different concentrations, and indirect contact tests demonstrated potential difficulties with non-diffusion, incomplete diffusion through membranes, or reaction with agar.

Tests providing a quantitative assessment are preferred, with cell viability reduction by more than 30% being considered cytotoxic[11, 30]. In the development of new biomaterials, if such reduction occurs, it determines the need for reformulation or abandonment. If encouraging results are achieved, further studies should be performed envisioning in vivo evaluation[29, 31].

In vitro tests should simulate or exaggerate the clinical conditions. Thus, the determination of appropriate surface volume ratios for extract preparation is critical. Surface to volume ratios of 1.25–6 cm^2/mL were suggested. In the case of materials with surface irregularities like foams 0.1–0.2 g/mL or 6 cm^2/mL are a starting point [15, 20 2]. The *ratio* of 250 mm^2 *per* mL of medium was used in representative results used in this protocol and other studies[15, 20].

Even if not used in this way in the clinics, the samples must be sterilized by methods that do not alter their properties. UV irradiation is frequently a good choice. This is of paramount importance to prevent microbial contamination of cell cultures[11, 24, 32].

Extraction media include cell culture medium with or without serum, physiological saline solution, dimethylsulfoxide, or purified water, selected according to the biomaterials chemical characteristics [11, 33]. Aiming for cell culture studies, the use of the cell culture medium is preferred since it avoids further processing steps. The conditions for extraction should be adjusted to the experimental model. In the representative results shown in this protocol, the DMEM culture medium supplemented with FBS was used for 24 ± 2 hours at 37 ± 1 °C.

Some biomaterials may leave residues in the extraction media, which may negatively affect the cell cultures. While filtration and centrifugations should be avoided, a possibility is to allow the particles to sediment before using. Another issue is the pH that may suffer alteration after extraction. Since it is not recommended to perform further adjustments [11], the pH of the extracts must be measured, registered, and additional controls to isolate the pH effect must be included in the experimental design if necessary.

While this protocol was described for adherent cell cultures, simple modifications can be performed to use suspension cultures. Similarly, besides using solid biomaterials, it is possible to adapt the procedure, essentially the extraction steps, to study liquids, gels, or foams [34–37].

The preparation of cell cultures with appropriate density is critical, especially on cell cultures with high duplication rate[31] . According to the recommended seeding density range of the cells used, if long-time incubations are planned, the reduction of the initial seeding density must be performed to avoid the problems associated with excessive confluence. In addition, highly cytotoxic materials may require higher initial seeding densities.

Besides the advantages of the extract methodology, it is not the best choice for materials

where the evaluation of cell adherence is relevant. In this case, the direct contact studies must be performed [38–41]. Although this is a comprehensive approach, it is important to keep in mind it is an in vitro assessment, which does not totally reflect the in vivo conditions[42].

A biomaterial should only not cause damage to the tissue but stimulate some of the anti-inflammatory and immunomodulant processes[43–46]. Thus, this protocol goes further, with the evaluation of cellular mechanisms, including cell viability and cell death profile, as well as other mechanisms of protein synthesis. The evaluation performed should allow concluding on the biomaterial bioactivity in living tissues, besides cytotoxicity.

With the explosion of new materials for medical applications, not only for dentistry but also for orthopedics, surgery, ophthalmology, cardiology, etc., the initial screenings should be made systematically. This protocol might be an important tool for researchers aiming to develop and characterize novel biomaterials.

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The authors have no competing financial interests or other conflicts of interest.

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Figure 1

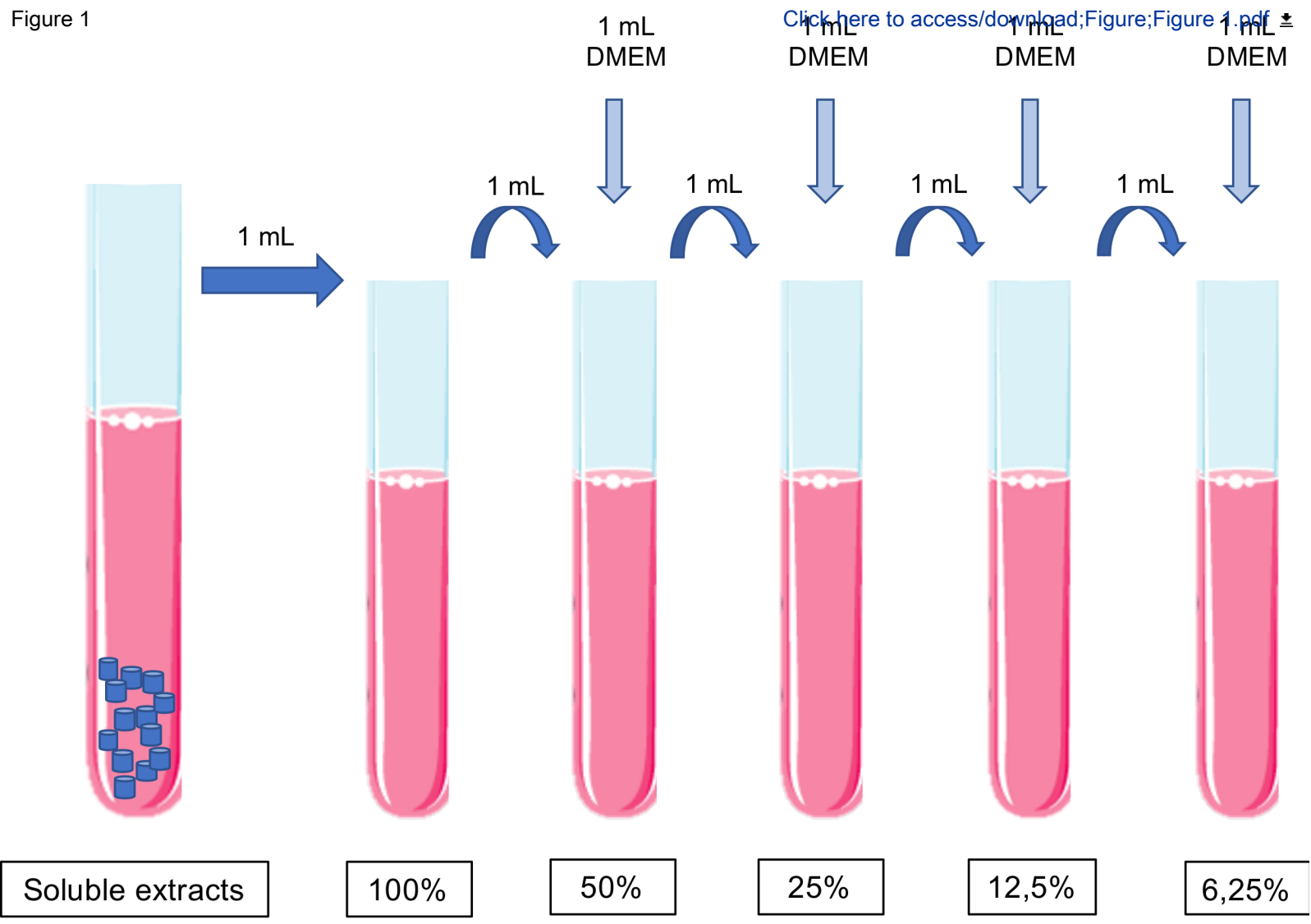


Figure 2

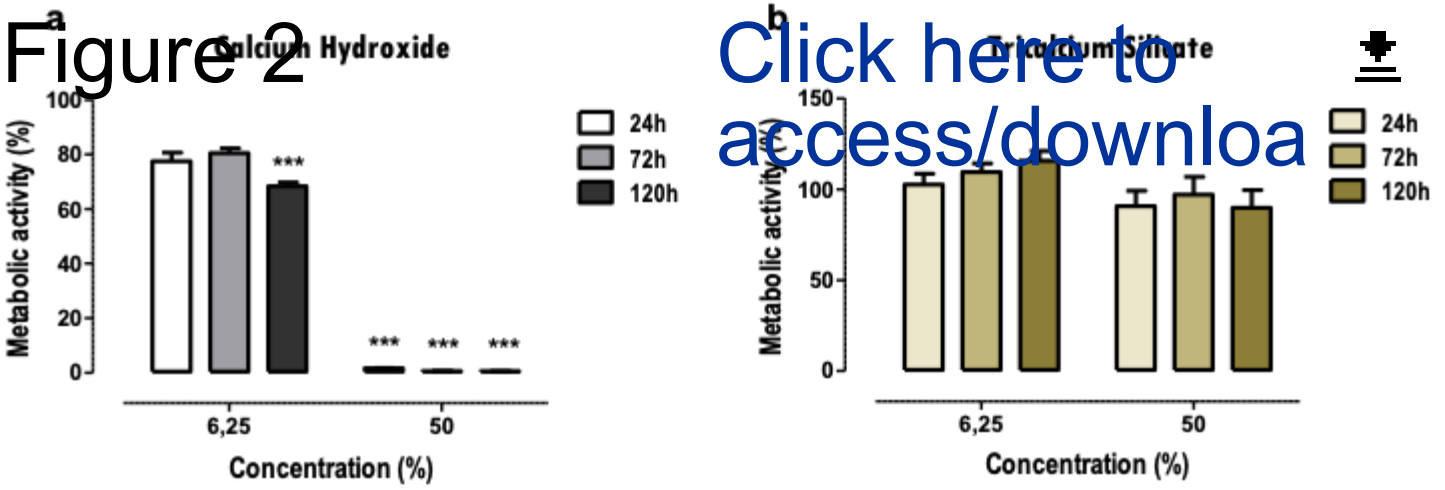
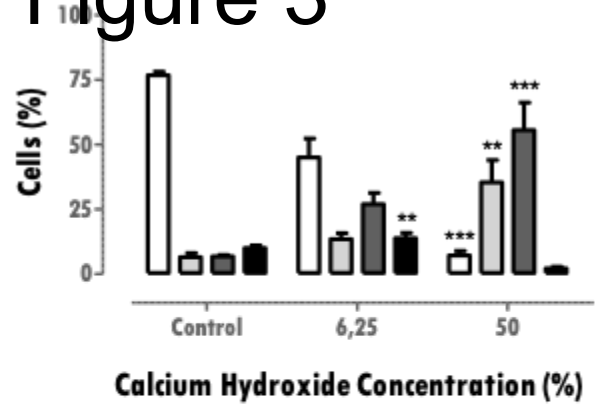
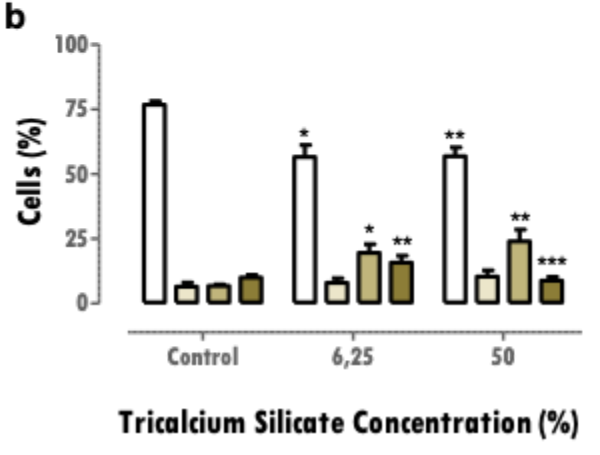


Figure 3





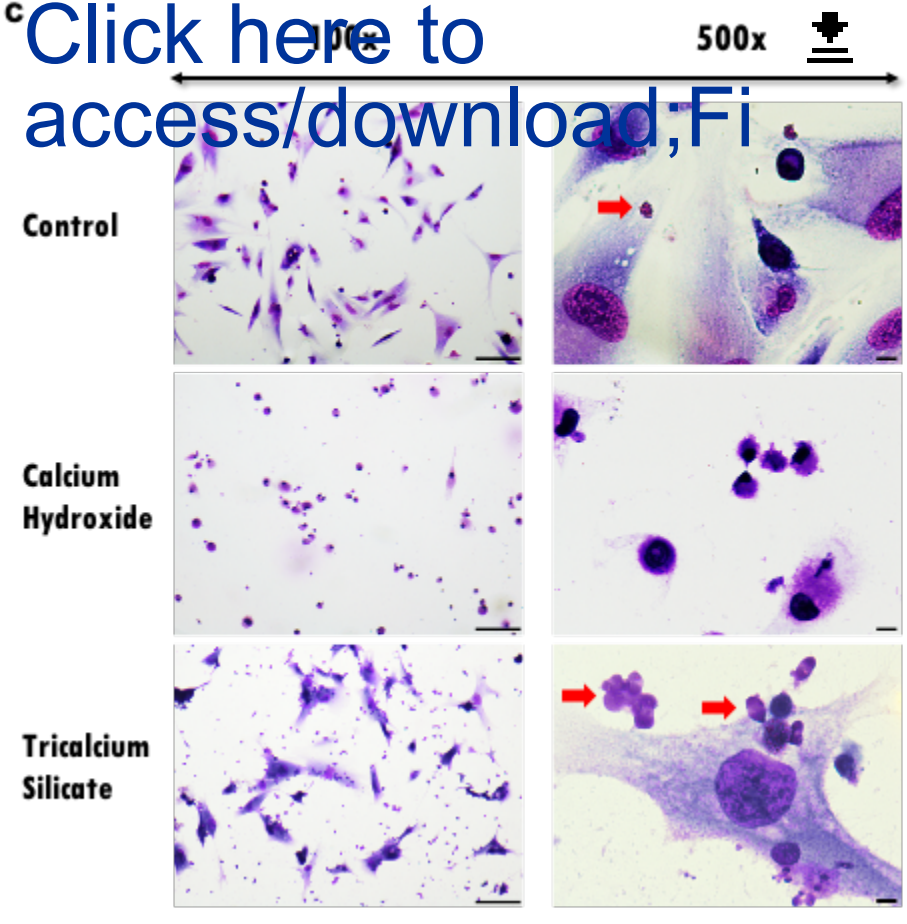


Figure 4

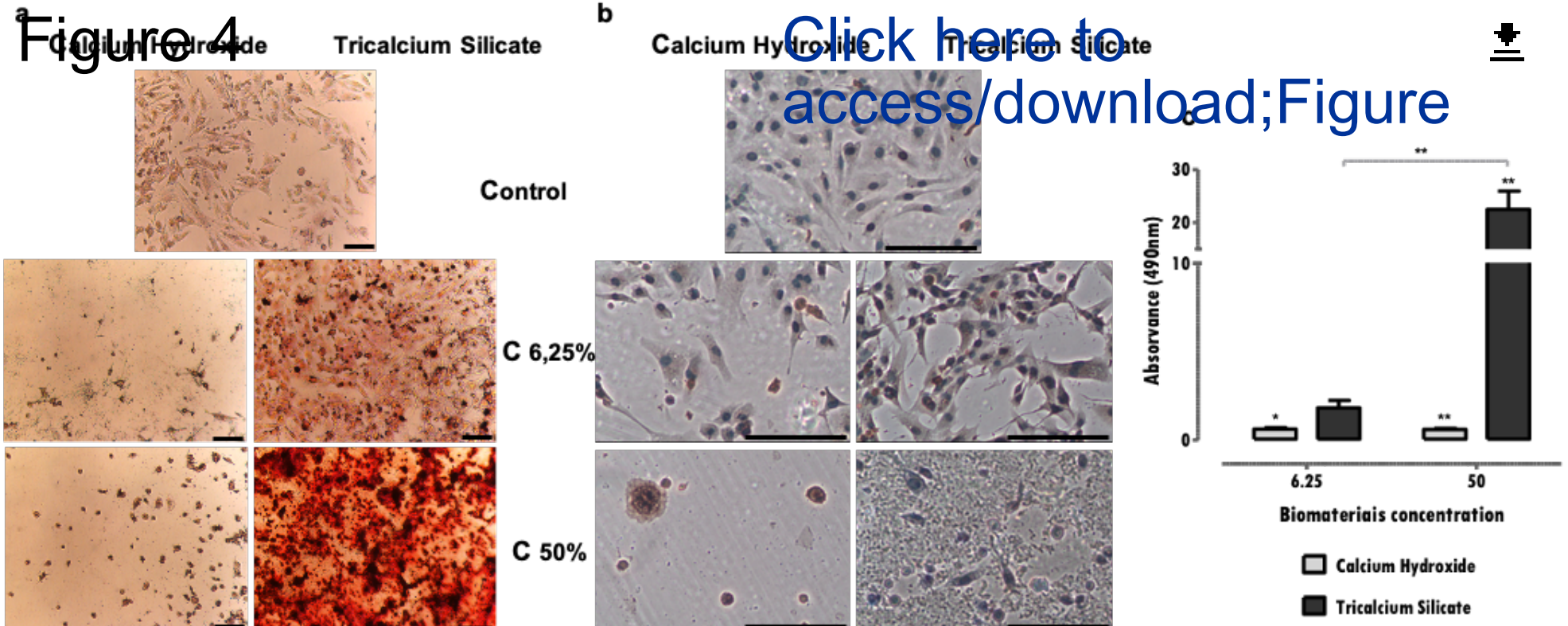


Table 1

Gene or Protein	Method	References
Alkaline Phosphatase (ALP)	Colorimetric	47 48
	Immunocytochemistry	20 49
	Northern Blot	50
	RT-PCR	51 52
Decorin (DCN)	Colorimetric ELISA	53
	Immunocytochemistry	54 55
	RT-PCR	53 56
Dentin Matrix Protein 1 (DMP-1)	Flow cytometry	57
	Immunocytochemistry	58 59
	Northern Blot	50 60
	RT-PCR	47 49
	Western Blot	50 60
Dentin Matrix Protein 2 (DMP-2)	Immunocytochemistry	60 61
	RT-PCR	50 62
	Northern Blot	60
	Western Blot	62
Dentin Phosphoprotein (DPP)	Immunocytochemistry	63
	Northern Blot	63
Dentin Sialoprotein (DSP)*	Immunocytochemistry	20 60
	Northern Blot	60 63
	RT-PCR	50
	Western Blot	64 65
Dentin Sialophosphoprotein (DSPP)	Flow cytometry	57
	Immunocytochemistry	66 54
	RT-PCR	47 49
	Northern Blot	67 68
	Western Blot	64 62
Enamelysin/Matrix Metalloproteinase-20 (MMP-20)	Northern Blot	68

	RT-PCR	49 68
Nestin	Immunocytochemistry	54 69
	RT-PCR	70 71
	Western Blot	72
Osteoadherin (OSAD)	Immunocytochemistry	73 74
	Northern Blot	73
	RT-PCR	75
	Western Blot	73 74
Osteopontin (OPN)	Immunocytochemistry	76
	Northern Blot	50
	RT-PCR	66 51
	Western Blot	77
Osteocalcin (OCN)	Immunocytochemistry	52
	Northern Blot	50
	RT-PCR	51 52
	Western Blot	77 78
Osterix (OSX)/ Transcription factor Sp7 (Sp7)	Immunocytochemistry	54 58
	RT-PCR	78
	Western Blot	78 79
Phosphate-regulating gene with homologies to endopeptidases on X-chromosome (Phex)	Northern Blot	68
	RT-PCR	49 68
	Western Blot	79
Runt-related transcription factor 2 (Runx2)	Immunocytochemistry	66 52
	RT-PCR	66 70
	Western Blot	62 77

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Absolute ethanol	Merck Millipore	100983	
Accutase	Gibco	A1110501	StemPro Accutas Cell Dissociation Reagent
ALDH antibody	Santa Cruz Biotechnology	SC166362	
Annexin V FITC	BD Biosciences	556547	
Antibiotic antimycotic solution	Sigma	A5955	
BCA assay	Thermo Scientific	23225	Pierce BCA Protein Assay Kit
Bovine serum albumin	Sigma	A9418	
CaCl2	Sigma	10035-04-8	
CD133 antibody	Miteny Biotec	293C3-APC	Allophycocyanin (APC)
CD24 antibody	BD Biosciences	658331	Allophycocyanin-H7 (APC-H7)
CD44 antibody	Biolegend	103020	Pacific Blue (PB)
Cell strainer	BD Falcon	352340	40 µM
Collagenase, type IV	Gibco	17104-019	
cOmplete Mini	Roche	118 361 700 0	
DAB + Chromogen	Dako	K3468	
Dithiothreitol	Sigma	43815	
DMEM-F12	Sigma	D8900	
DNAse I	Roche	11284932001	
DSP (M-20) Antibody, 1: 100	Santa Cruz Biotechnology	LS-C20939	
ECC-1	ATCC	CRL-2923	Human endometrium adenocarcinoma cell line
Epidermal growth factor	Sigma	E9644	
Hepes 0.01 M	Sigma	MFCD00006158	
Fibroblast growth factor basic	Sigma	F0291	
Giemsa Stain, modified GS-500	Sigma	MFCD00081642	
Glycerol	Dako	C0563	
Haemocytometer	VWR	HERE1080339	
HCC1806	ATCC	CRL-2335	Human mammary squamous cell carcinoma cell line
Insulin, transferrin, selenium Solution	Gibco	41400045	
May-Grünwald Stain MG500	Sigma	MFCD00131580	
MCF7	ATCC	HTB-22	Human mammary adenocarcinoma cell line
Methylcellulose	AlfaAesar	45490	
NaCl	JMGS	37040005002212	
Polyclonal Rabbit Anti-goat immunoglobulins / HRP, 1:	Dako	G-21234	
Poly(2-hydroxyethyl-methacrylate	Sigma	P3932	
Putrescine	Sigma	P7505	
RL95-2	ATCC	CRL-1671	Human endometrium carcinoma cell line
Sodium deoxycholic acid	JMS	EINECS 206-132-7	
Sodium dodecyl sulfate	Sigma	436143	
Substrate Buffer	Dako		926605
Tris	JMGS	20360000BP152112	
Triton-X 100	Merck	108603	
Trypan blue	Sigma	T8154	
Trypsin-EDTA	Sigma	T4049	
β-actin antibody	Sigma	A5316	

Rebuttal letter

Dear Editor,

After consideration of the comments and suggestions of the last round of reviews to the manuscript 61512, we point the following items:

1. The title was altered according to the suggestions translation a more broad approach than the previous one, considering dental biomaterials only. Moreover, a few alterations were made through the text to reflect this change.

2. It was necessary to alter the affiliation section due to the new guidelines of our institution.

3. The introduction was deeply revised and reorganized to include the aspects pointed in the comment:
"Please revise the Introduction to include all of the following: a) A clear statement of the overall goal of this method b) The rationale behind the development and/or use of this technique c) The advantages over alternative techniques with applicable references to previous studies d) A description of the context of the technique in the wider body of literature e) Information to help readers to determine whether the method is appropriate for their application."

4. Regarding the protocol section, **we have doubts about how to proceed**. We think the current comments are not in accordance with the previous revision round and JoVE instructions.

Several comments ask to be more specific regarding cell lines used, materials tested and so on, but this is not compatible with a protocol aiming to provide a guideline to evaluate biomaterials concerning several medical specialities.

We consider the version of the protocol we are submitting now to be following the title providing a way to evaluate biomaterials for medical use. Further specificities will limit the scope of the protocol.

5. Regarding the comment about lines 811-818 "*Significance with respect to biomaterials*?" we ask for clarification. What do you mean with this comment?

6. Limitations were included in the discussion section in the last round of reviews. Please see lines 924-927.

Dear Editor-in-chief

We are pleased to submit the revised version of the manuscript entitled “Evaluation of dental biomaterials cytotoxicity through soluble extracts” to be considered to publication in JOVE, as well as the answers to the questions posed by the editor and the reviewers.

We thank the questions and suggestions made, which allows us to substantially improve the manuscript, making it more complete and more explicit. The rebuttal letter refers to all the performed changes; besides, the main document is marked to easier visualization.

As we previously stated, we believe this protocol provides a comprehensive guide for dental biomaterials evaluation using the extracts methodology, allowing to determine its cytotoxicity and cellular effects in a reproducible and robust approach, which can be of great utility to all researchers working in the dental materials field.

We look forward to hearing from you and the reviewers, and we hope the manuscript now achieves the standards of Jove. If further improvements are needed, we are available for that.

Kind regards,

Anabela Baptista Paula