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Research Article

A 3D Osteoblast *In Vitro* Model for the Evaluation of Biomedical Materials

Luciana Restle,[1] Daniela Costa-Silva,[2] Emanuelle Stellet Lourenço,[1] Rober Freitas Bachinski,[2,3] Ana Carolina Batista,[2] Adriana Brandão Ribeiro Linhares,[2] and Gutemberg Gomes Alves[2,4]

[1]*Post-Graduation Program on Dentistry, Fluminense Federal University, 22245-100 Niterói, RJ, Brazil*
[2]*Clinical Research Unit, Antônio Pedro Hospital, Fluminense Federal University, 22245-100 Niterói, RJ, Brazil*
[3]*Post-Graduation Program on Science and Biotechnology, Fluminense Federal University, 22245-100 Niterói, RJ, Brazil*
[4]*Department of Molecular and Cell Biology, Institute of Biology, Fluminense Federal University, 22245-100 Niterói, RJ, Brazil*

Correspondence should be addressed to Gutemberg Gomes Alves; gutemberg_alves@id.uff.br

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Biomedical materials for bone therapy are usually assessed for their biocompatibility and safety employing animal models or *in vitro* monolayer cell culture assays. However, alternative *in vitro* models may offer controlled conditions closer to physiological responses and reduce animal testing. In this work, we developed a 3D spheroidal cell culture with potential to evaluate simultaneously material-cell and cell-cell interactions. Different cell densities of murine MC3T3-E1 preosteoblasts or human primary osteoblasts (HOb) were used to determine the ideal procedure of spheroidal cultures and their adequacy to material testing. Cells were seeded on 96-well plates coated with agar and incubated in agitation from 1 to 7 days. Aggregate morphology was qualitatively evaluated considering the shape, size, repeatability, handling, and stability of spheroids. Higher cell densities induced more stable spheroids, and handling was considered appropriate starting from 2×10^4 cells. Confocal microscopy and Scanning Electron Microscopy indicate that most cells within the aggregate core are viable. Exposure to positive controls has shown a dose dependent cell death as measured by XTT assay. Aggregates were stable and presented good viability when employed on standardized testing of metallic and polymer-based biomaterials. Therefore, osteoblast spheroids may provide a promising tool for material screening and biocompatibility testing.

1. Introduction

Currently, there is a wide range of products and processes that involve direct or indirect contact with human tissues, with possible impact on the health of users. This risk is generally increased in the case of biomaterials and grafted medical/dental devices and may extend to both the healthcare professional and end users (patients) [1]. Materials with intimate and prolonged contact with body tissues include those employed in the therapy of degenerative diseases and orthopedic or maxillofacial bone traumas, which often require the preservation or restoration of tissue functions through complex processes that are faced with the need for bone-substitute materials [2]. In this context, the need to establish appropriate methodologies for assessing the quality of biotechnology products and processes aimed at bioengineering and development of medical/dental biomaterials becomes clear [3]. Moreover, the importance of assessing biomaterials biocompatibility is highlighted by the development and publishing of specific international standards for the evaluation of biocompatibility, such as ISO 10993-1:2009 [4].

In this context, *in vitro* tests for the evaluation of biocompatibility of materials allow for the assessment of the biological response of specific cell lines when conditioned to the presence of the products or processes in question, with recognized efficacy and within the principles of bioethics, through indicator parameters such as cytotoxicity, genotoxicity, and immunogenicity [5]. However, the *in vitro* evaluation of biocompatibility of materials is often controversial, due to the myriad of analytical methods available, whose suitability

is highly dependent on characteristics of the assessed material [6].

Bidimensional (2D) monolaminar cellular models certainly brought major contributions to the understanding of cellular physiology and to the advancement of basic sciences, as well as for the testing and screening of novel biocompatible materials [7]. However, such models have inherent limitations in predicting tissue responses since, even in cocultures, cell-cell interactions are impaired due to their two-dimensional cell disposition, which only slightly correlates with cellular interactions in living tissue [8]. These limitations have led to the development of three-dimensional culture models (3D) that can better mimic the microenvironment in tridimensional tissues, including cell communication via solubilized factors in the interstitial fluid, adhesion between cell-cell and cell-extracellular matrix, or mechanisms involving mechanical stress in the core layers of the tissue [9]. Tridimensional models are employed on cancer research since the 1970s, helping to identify different environments in cell cultures and resulting cytophysiological changes [10, 11]. The clusters formed by these cells then showed a spherical morphology and were called multicellular spheroids [12]. Ever since, spheroidal cell models have been studied and developed for multiple applications, including bone therapy and material testing [13–16].

International standards for evaluation of medical and dental use materials (ISO 10993-1:2009; ASTM) recommend the use of control materials of recognized biocompatibility (negative controls) or well-known cellular toxicity (positive controls of cytotoxicity) [17]. In addition, it is recommended by ISO 10993-5:2009 [18] that testing should be performed during the late log phase of cell growth, attaining approximately 80% confluence. The parameters recommended by these same standards to determine the cytotoxicity of biomedical materials may be grouped as follows: (i) determining morphological changes, generally associated with a decrease in cell adhesion, cell lysis, or effects on the integrity of membranes or (ii) assessment of impacts on cell growth, cell damage, and specific cell metabolism (through analyses of cell number, total protein, reducing, and uptake of vital dyes) [18].

However, three-dimensional models based on cell spheroids do not easily fit on recommendations by these standards, since their cell conformation does not allow the analysis of confluence and it is not known if these models enable even distribution of the dyes or their elution throughout the cellular barrier, being possible for traditional tests to under- or overestimate the toxicity. Moreover, the literature lacks detailed descriptions of such models, including parameters such as ease of handling, uniformity, and repeatability of results, in order to insure the effectiveness of this new methodology as a standardized predictive tool for the clinical success of medical materials. Therefore, in this preliminary work, we aimed to develop an *in vitro* tridimensional spheroidal osteoblast model for the evaluation of the biological properties of bone therapy materials, ensuring their better efficacy and safety, employing conditions which are more similar to the actual physiological exposure to materials.

2. Materials and Methods

2.1. Materials. In this study, materials of broad clinical use and recognized cytocompatibility or cytotoxicity were employed, in order to standardize and validate the use of the spheroids. As representatives of negative, biocompatible controls, we employed smooth surface machined titanium (SIN Implants, Brazil) and dense beads of polystyrene (INT, Brazil). Amber latex tubes obtained commercially were used as positive controls, since they are known for their increased cytotoxicity [17].

2.2. Cell Culture. In order to investigate the adequacy of the spheroidal cell culture with diverse cell models, we employed either cells of murine preosteoblast lineage MC3T3-E1 or human bone cells in primary culture (HOb), at #4th passage, from the Collection at the Clinic Research Unit of the University Hospital Antonio Pedro, UFF (UPC-HUAP-UFF). These cells were cultured in DMEM (Dulbecco's modified Engle's medium) supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 0.1% nonessential amino acids, and 1% streptomycin/penicillin and incubated in 5% CO_2, at 37°C.

2.3. Development of Cellular Spheroids. In order to produce spheroids, cells kept in monolaminar culture were trypsinized and counted with the help of a hemocytometer, and different cell densities (5, 10, 20, 30, 40, 50, 75, 90, and 110 × 10^3 cells/well) were seeded along with 200 μL medium supplemented with 10% FBS in 96 round bottom microwell plates, precoated with 40 μL of sterile agar-agar (2% in DMEM), and incubated for up to 7 days in stirring with the help of a microplate mixer (Genie, Scientific Industries, USA), until the formation of aggregates. The morphology and size of the spheroids were analyzed by phase contrast microscopy and registered with an Axio A1 Observer Microscope (Zeiss, Germany).

2.4. Aggregate Quality Assessment. The spheroids produced by the agar method (to avoid adherence to culture dish) and agitation (to ensure spheroidal shape) were evaluated qualitatively to determine the best cell density in terms of different parameters related to handling and quality of cell clusters (Table 1).

2.5. Ultrastructural Analysis. For the Scanning Electronic Microscopy (SEM), spheroids were fixed in Karnovsky fixer solution for 30 minutes, followed by washing three times for 5 minutes in cacodylate buffer. After postfixation process, the samples were dehydrated in a series of ethanol solutions (15%–100%) and were treated with 1 : 1 ethanol and hexamethyldisilane (HMDS) for 10 minutes, followed by pure HMDS for 10 minutes. After complete drying of the samples, spheroids were mounted on aluminum stubs and coated with 20 nm thick layer of gold. The samples were examined with a JEOL 5310 scanning electron microscope (JEOL, Japan).

Whole aggregates were stained for apoptosis using CellEvent Caspase 3/7 Green Probes and Hoechst 33342 (1 : 5000) (Life Technologies, USA). Incubation was done directly in 6-well plates containing living aggregates, for 30 minutes, at

Table 1: Parameters and scores for the qualitative assessment of the spheroids.

	Score			
	1	2	3	4
Regularity of shape	Mostly amorphous	Mostly irregular	Mostly regular spheroids	Completely spheroidal
Size	Too small	Small	Medium	Big
Repeatability	Strong variation in shape, size, and number	Variation in one or two parameters	Most parameters are similar	Very similar in all parameters
Handling	Very difficult	Difficult	Easy	Very easy
Stability	Always disintegrate	Disintegrate on a considerable proportion (after 2-3 days)	Most remain stable	All remain stable after 2-3 days

the same conditions as those for maintenance of aggregate cultures. Aggregates were then collected to a 15 mL tube and washed 3 times with PBS, to remove the free fluorophores, and fixed in paraformaldehyde 4% for 15 minutes at 4°C. Aggregates were kept in PBS at 4°C and observed on a fluorescence confocal microscope (DMI 6000, Leica, USA).

2.6. Development of Conditioned Media for Cytocompatibility Testing. The extracts were prepared as described in ISO 10993-12:2008 [19], which establishes mass ratios and volume between the test material and the amount of extraction medium (nonsupplemented culture medium, DMEM). Extract media were exposed to each sample on a proportion of 200 mg/mL and incubated by 24 hours at 5% CO_2, 37°C.

2.7. Cytotoxicity Assays. For the assessment of cytotoxicity, spheroids were collected with a micropipette on a volume of 10 microliters and individually transferred to wells containing 190 microliters of each extract or unexposed culture medium (control). Cells were exposed by 24 hours (5% CO_2, 37°C) and viability was determined by the XTT assay (In Cytotox, Xenometrix, Germany). It consisted of the addition of XTT reagent (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), with incubation and measurement of the resulting soluble formazan salts by their Optical Density (OD) at 480 nm, using a Microplate Reader (Synergy II Biotek Inst., USA). The ideal time of incubation was determined by performing subsequent readings at 30-minute intervals, until maximal stable readings were attained. For the spheroidal culture, it was reached at 3 hours after addition of XTT. Also, readings were performed in the presence and absence of spheroids in the well, without any alteration observed.

2.8. Statistical Analysis. Results from the cytotoxicity assay were tested for normality, and a Kruskal-Wallis nonparametric analysis of variance was performed with a Dunn posttest comparing all the experimental groups, with an alpha error of 5%. The analysis was performed with the help of the software GraphPad Prism 5.0 (GraphPad, USA).

3. Results and Discussion

Biomedical materials for bone therapy are usually assessed for their biocompatibility and safety employing animal models or *in vitro* monolayer cell culture assays [20]. However, alternative *in vitro* models may offer controlled conditions which are closer to physiological responses than monolaminar *in vitro* systems. In this work, we developed a 3D spheroidal cell culture with potential use for cytocompatibility assessment of materials intended for biomedical uses.

There are many different methodologies for aggregate production, including the use of microfluidics, spinner and pellet culture, and hanging-drop techniques [21]. In the present study, the simple method proposes the use of agar coating to avoid cell adhesion on the culture-plate bottom, providing the conditions to cell aggregation. In fact, it is considered that osteoblast differentiation and survival are dependent on cell adhesion on the extracellular matrix (ECM) via integrin and, in the absence of contact, cells generally come into anoikis [22]. However, a fundamental concept for the formation of cellular aggregates in general is the idea that cellular interaction via e-cadherins can suppress cell sensitivity to anoikis [23]. Therefore, if we rapidly induce the approximation and interactions between preosteoblast cell in an environment where the same do not connect preferentially (e.g., agar-agar), we may support the survival of those cells that were capable of aggregating and establishing cell-cell contacts via cadherin, with the subsequent production of matrix proteins within the aggregate.

Nevertheless, several parameters of culture might affect the outcome of spheroid formation, such as the initial cell density seeded on each well. In this regard, Figure 1 shows that when producing such spheroids, while initial lower densities induced lower times of formation, most groups had already formed aggregates between the 2nd and 3rd day after seeding. Higher densities formed more stable spheroids, that is, with a longer duration before disintegration and cell separation. The handling (measured by the ability to be easily seen and moved from one plate well to another) was considered ideal in densities equal to or greater than 30,000 cells.

It was also observed that, for densities above 75,000 cells, irregular patterns were present in most spheroids, as well as an increased number of multiple spheroids in the same well. It can be concluded from the results of the qualitative analysis, presented in Table 2, that the density of 30,000 cells is ideal for further tests, as the spheroids produced in such conditions performed comparatively better on all major items evaluated.

When the evaluation was carried out with the spheroids obtained by primary culture, an interesting phenomenon was

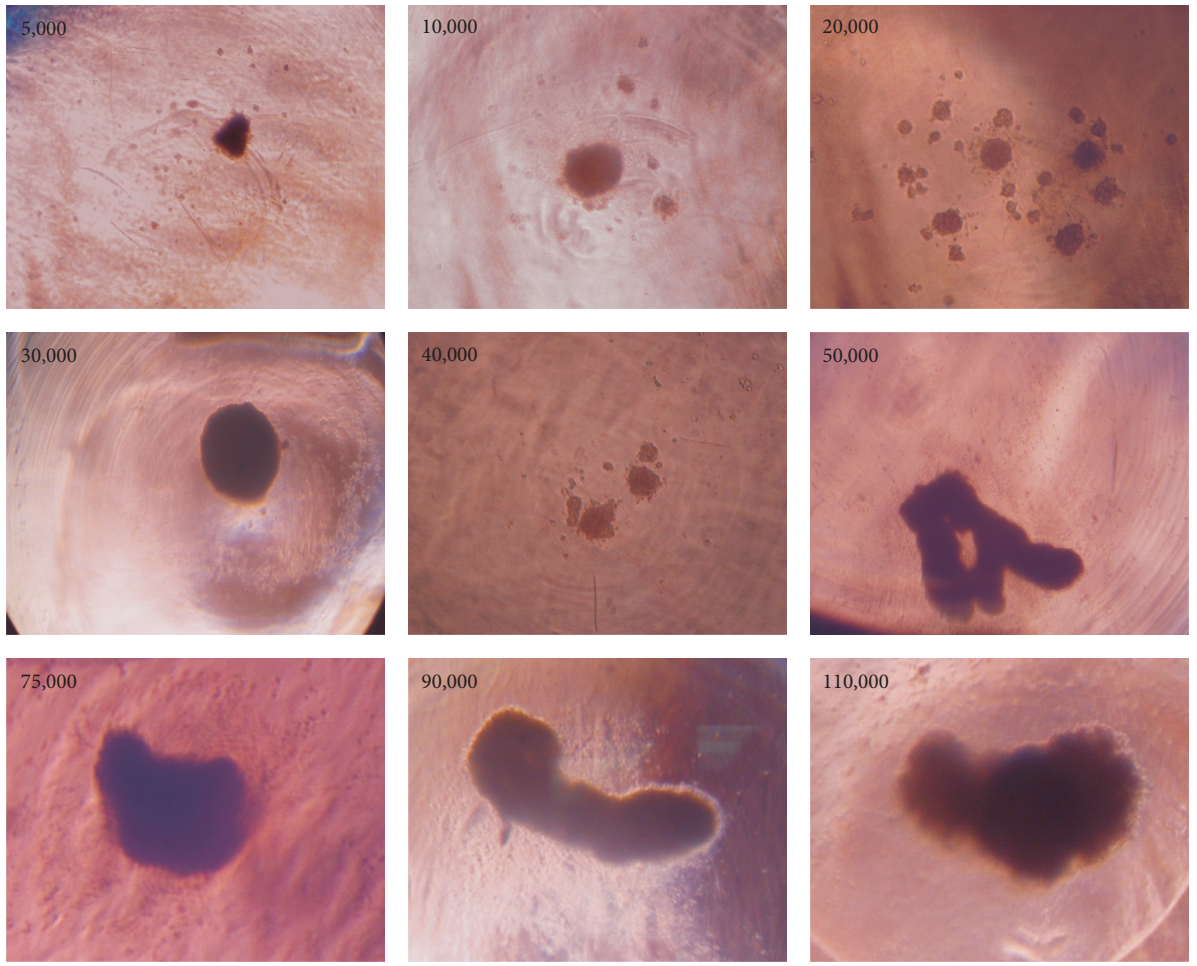


Figure 1: Optical microscopy of MC3T3-E1 aggregates, at day 7, developed with different cell densities (20x objective).

Table 2: Scores obtained in the qualitative assessment for the different cell densities.

Initial cell density (per well)	Regularity of shape	Size	Repeatability	Handling	Stability
5,000	3	1	3	1	1
10,000	3	2	3	1	2
20,000	2	1	2	2	2
30,000	**4**	3	3	3	3
40,000	3	2	2	3	2
50,000	2	3	2	3	3
75,000	3	**4**	2	3	3
90,000	1	**4**	3	**4**	**4**
110,000	1	**4**	3	**4**	**4**

observed: experimental times to spheroids formation were higher, indicating a slower process of activation and cell to cell adhesion, perhaps primary cells not themselves transformed/immortalized. However, formation of spheroids was consistent 7 days after plating, and their average was superior stability, remaining joined for periods close to 30 days of cultivation. Table 3 shows the average times of formation of spheroids of primary culture and murine lineage. It is important to notice that transformed cells are usually more prone to aggregate than primary cells, as described in several works on cancer research [24, 25]. Therefore, it could be expected, as observed in the present work, that immortalized cell lines should be less affected by phenomena such as anoikis and might demand lower times for spheroidal culture, as confirmed by the present results.

Another important observation was the fact that the primary cell spheroids had great uniformity and low multiplicity at densities from 30,000 to 50,000 cells, while at densities

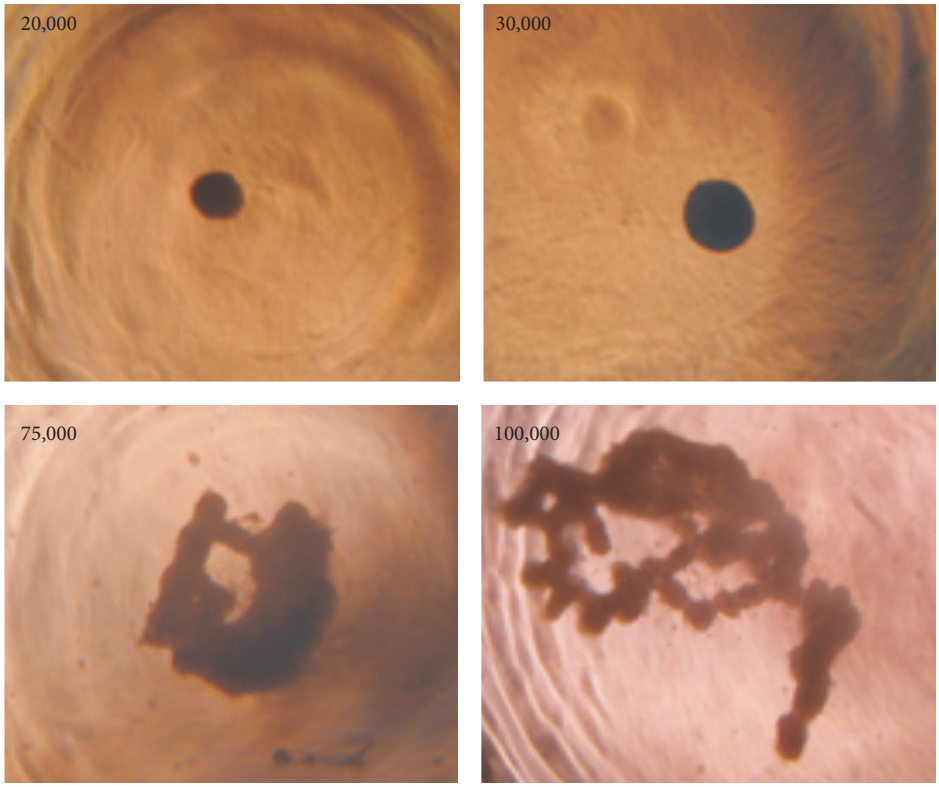
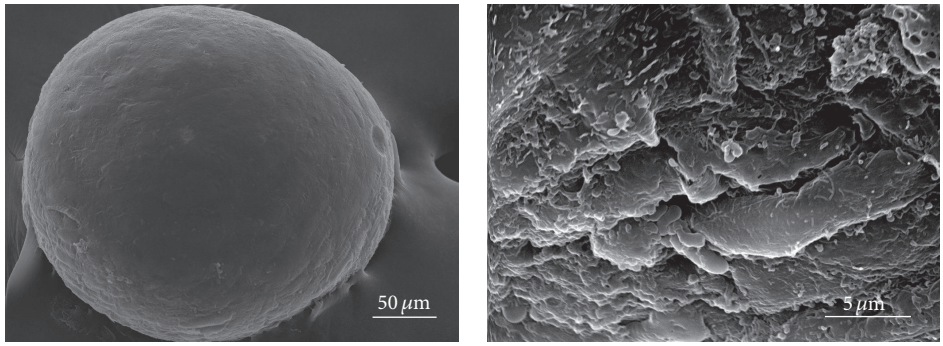


Figure 2: Spheroids obtained from primary human bone cells in three-dimensional culture, at day 7, developed with different cell densities (20x objective).



(a) (b)

Figure 3: Electron micrographs of spheroids of MC3T3-E1 cells at 30,000 cells' density, obtained by Scanning Electron Microscopy at different magnifications.

Table 3: Time of formation and stability of spheroids produced with primary human or murine line cells, at an initial density of 20,000 cells ($n = 20$).

Cell type	Average time of aggregate formation	Limit of stability on culture
Primary human bone cells	7.1 ± 2.2 days	35 ± 5.5 days
MC3T3-E1	2.0 ± 0.6 days	20.6 ± 5.4 days

greater than 75,000 cells all major parameters evaluated in the qualitative analysis were strongly altered (Figure 2). The multiplicity was apparently higher in primary cells when compared to the spheroids of murine lineage.

Bone cells spheroids at 30,000 cells presented a very regular and compact surface when observed by Scanning Electron Microscopy (SEM), as shown in Figure 3(a). Imaging at higher magnification (Figure 3(b)) confirms strong interactions between cells from the outer layer, with an elongated morphology very similar to previous reports on osteoblast aggregation [13]. It can also be observed that in spite of the compact nature of the spheroids, some spaces may be available for the passage of nutrients to the inner layers of

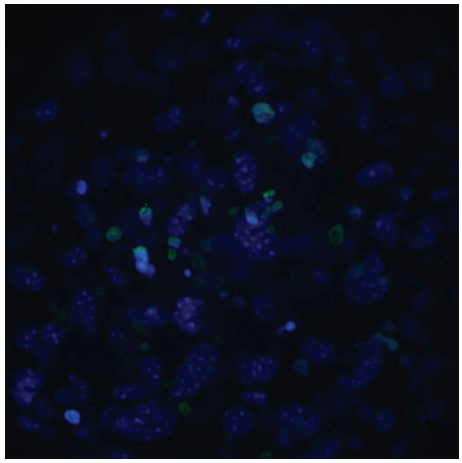


Figure 4: Confocal microscopy image of the middle section (center) of a 30,000 cells' spheroid obtained from MC3T3-E1 cells, stained for caspases 3/7, indicating apoptosis (green).

the aggregate, which might contribute to cell survival in the core of the cell mass.

In fact, the possibility of reduced transit of molecules and nutrients from the media to the innermost portions of aggregates is of special concern on studies of 3D models [13, 14]. Indeed, 3D models are helping to understand how cells on a tridimensional physiological environment respond to the spatial organization, on parameters such as proliferation and survival, including possible apoptotic pathways [26].

Figure 4 shows the assessment of cell viability on an inner layer of the aggregate, as observed by confocal microscopy. The caspase 3/7 probes indicate the presence of diffuse apoptotic cells surrounded by viable cells inside the spheroids. Even though it was not sufficient to cause hollow spaces on the spheroid core on the experimental times hereby studied, it may contribute to a reduction on aggregate mean size, similar to that described on previous works for differentiated mesenchymal stem cell aggregates [14]. It may also impose a limit to maximum viable size of bone cell spheroids, on the range of 300–400 micrometers, in which most of the aggregates of this study pertained. Also, some authors have proposed and investigated the production of aggregates in coculture with endothelial cells on collagen scaffolds for cell therapy (and not material testing, as hereby proposed), aiming to contribute on vascularization, with interesting results [27].

To assess the suitability of the murine spheroids produced for the performance of medical materials cytotoxicity tests, we conducted an assay according to ISO 10993-5:2009, using the metabolic activity parameter (XTT test). While this approach is not sufficient for a complete understanding of the biological response of bone cells to a novel material, this kind of extract (conditioned media) based assay corresponds to the first step of screening of novel biocompatible materials and is among the most common practices on the initial *in vitro* assessments of medical materials and is usually accepted/recommended for regulatory purposes [4]. Therefore, aiming to verify if the proposed aggregates behave as expected and appropriately during material testing, we have chosen this methodology as it is simple, reproducible, standardized, and widely applied, with well-known results in literature.

Figure 5(a) confirms, as expected, that surgical grade titanium and polystyrene are detected as being not cytotoxic, while the latex showed high cytotoxicity, in a dose dependent manner, as expected. However, we can see that the toxicity levels observed for the positive control were much lower than those usually observed in two-dimensional culture, which attain, in the same conditions, cytotoxicity approximately 3 times higher for MC3T3-E1 cells [17]. This lower toxicity may reflect results closer to the clinic, where latex gloves, tourniquets, probes, and tubes are used constantly in direct contact with the body of patients without causing necrosis or actual levels of relevant chronic toxicity or allergy, when not associated with powder [28, 29].

The present results also show that the sensitivity of spheroids may be affected by cell model and initial cell density. When the test is repeated with human spheroids (Figure 5(b)) at a density of 50,000 cells (the larger spheroids density obtained which still retains morphological uniformity), we realize that the 100% latex extract is perceived as much less cytotoxic, even though with significant difference from polystyrene and the control group ($p < 0.05$). Also, an increase in the standard deviation of the test is observed. A possible explanation for this lower toxicity may be connected to alterations on surface/volume ratio. With the increase in volume of 3 and a power of the surface area of 2, larger spheroids have an increased proportion of cells in the protected inner area of the cell mass. Thus, the outer layer cells possibly killed by exposure to the toxicant may be outweighed by the metabolic activity of cells of the inner layers.

Osteoblasts in spheroidal culture can interface with the biomaterial either indirectly, as assessed in the present study, or directly by the removal from agar and deposition or seeding of a controlled number of aggregates on the surface of the material. Cells from the outer layer may provide information on the direct cell-surface interactions, such as adhesion, or the presence of focal contacts, while cell-cell interactions with the inner layers may contribute with an insight on the effects of the material on tridimensional environments, such as biological tissues, and even assess the impact of penetration of liberated toxicants on the material. It is noteworthy that, in addition to being capable of binding to adherent surfaces, like any other cell culture, spheroids are also capable of fusing together, allowing for the formation of an "organotypic" layer of bone cells on the surface of a material for advanced studies. Such interactions might be observed in future studies with techniques such as confocal fluorescence microscopy.

Nowadays, great importance has been given to the applicability of the knowledge generated by basic research on material science, providing resources for major therapeutic advances. This implies that the search for new tools for biological evaluation of materials will directly affect the biotechnological advances in the field of bone biology [3]. In this context, the present results indicate that the development of tridimensional osteoblast culture of both primary human and transformed murine cells lines for the evaluation of

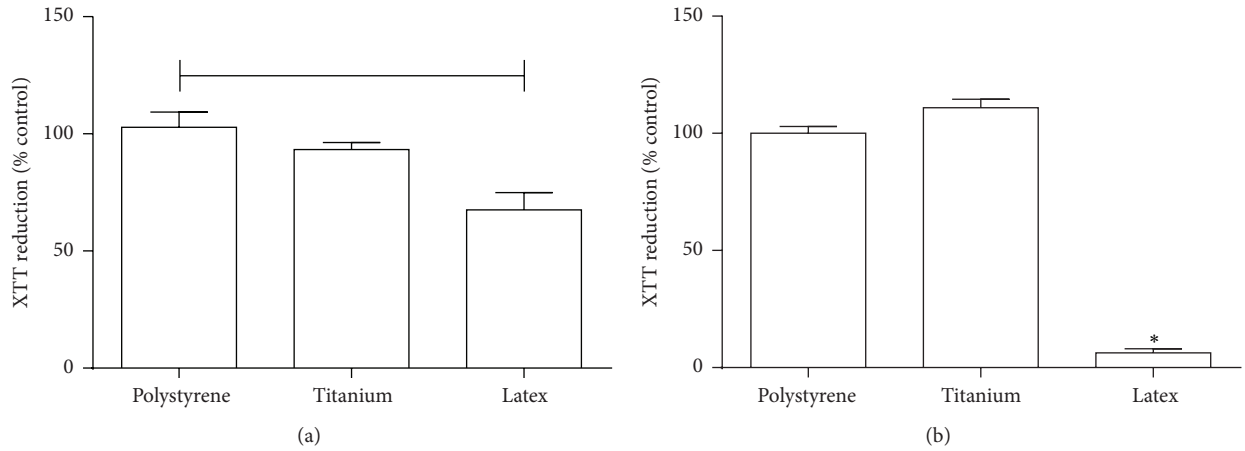


Figure 5: Cytocompatibility assay (XTT) performed with (a) MC3T3-E1 cells at 30,000 cells in three-dimensional culture or (b) HOb spheroids at 50,000 cells' density. A line between bars indicates significant difference between groups ($p < 0.05$). An asterisk indicates significant difference from all other groups ($p < 0.05$).

biocompatibility is feasible. More studies are still needed to ensure the development of tools based on these 3D models, which can be an impact on improving the quality of results from *in vitro* testing of biomedical materials, fulfilling their potential to offer test models closer to the physiological real conditions to which materials are exposed.

4. Conclusions

Aggregates from both human primary and animal line cell culture were stable and presented good viability when employed on a standardized testing of metallic and polymer-based biomaterials. Therefore, osteoblast spheroids may provide a promising tool for material screening and biocompatibility testing.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] J. M. Morais, F. Papadimitrakopoulos, and D. J. Burgess, "Biomaterials/tissue interactions: possible solutions to overcome foreign body response," *The AAPS Journal*, vol. 12, no. 2, pp. 188–196, 2010.

[2] P. C. Salgado, P. C. Sathler, H. C. Castro et al., "Bone remodeling, biomaterials and technological applications: revisiting basic concepts," *Journal of Biomaterials and Nanobiotechnology*, vol. 2, no. 3, pp. 318–328, 2011.

[3] W. F. Zambuzzi, P. G. Coelho, G. G. Alves, and J. M. Granjeiro, "Intracellular signal transduction as a factor in the development of 'Smart' biomaterials for bone tissue engineering," *Biotechnology and Bioengineering*, vol. 108, no. 6, pp. 1246–1250, 2011.

[4] International Organization for Standardization, "Biological evaluation of medical devices—part 1: evaluation and testing within a risk management process," ISO 10993-1:2009, International Organization for Standardization, 2009.

[5] C. T. Hanks, J. C. Wataha, and Z. Sun, "In vitro models of biocompatibility: a review," *Dental Materials*, vol. 12, no. 3, pp. 186–193, 1996.

[6] J. A. Cortês, E. Mavropoulos, M. Hausen, A. Rossi, J. M. Granjeiro, and G. G. Alves, "Methodological implications on quantitative studies of cytocompatibility in direct contact with bioceramic surfaces," *Key Engineering Materials*, vol. 493-494, pp. 325–330, 2012.

[7] P. Torricelli, M. Fini, G. Giavaresi, and R. Giardino, "In vitro models to test orthopedic biomaterials in view of their clinical application in osteoporotic bone," *The International Journal of Artificial Organs*, vol. 27, no. 8, pp. 658–663, 2004.

[8] F. Pampaloni, E. G. Reynaud, and E. H. K. Stelzer, "The third dimension bridges the gap between cell culture and live tissue," *Nature Reviews Molecular Cell Biology*, vol. 8, no. 10, pp. 839–845, 2007.

[9] N. T. Elliott and F. Yuan, "A review of three-dimensional in vitro tissue models for drug discovery and transport studies," *Journal of Pharmaceutical Sciences*, vol. 100, no. 1, pp. 59–74, 2011.

[10] C. M. Nelson and M. J. Bissell, "Modeling dynamic reciprocity: engineering three-dimensional culture models of breast architecture, function, and neoplastic transformation," *Seminars in Cancer Biology*, vol. 15, no. 5, pp. 342–352, 2005.

[11] R. M. Sutherland, "Cell and environment interactions in tumor microregions: the multicell spheroid model," *Science*, vol. 239, no. 4849, pp. 177–184, 1988.

[12] R. M. Sutherland, J. A. McCredie, and W. R. Inch, "Growth of multicell spheroids in tissue culture as a model of nodular carcinomas," *Journal of the National Cancer Institute*, vol. 46, no. 1, pp. 113–120, 1971.

[13] J. Neunzehn, S. Heinemann, and H. P. Wiesmann, "3-D osteoblast culture for biomaterials testing," *Journal of Developmental Biology and Tissue Engineering*, vol. 5, no. 1, pp. 7–12, 2013.

[14] A.-C. Tsai, Y. Liu, X. Yuan, and T. Ma, "Compaction, fusion, and functional activation of three-dimensional human mesenchymal stem cell aggregate," *Tissue Engineering Part A*, vol. 21, no. 9-10, pp. 1705–1719, 2015.

[15] J. S. Burns, P. L. Rasmussen, K. H. Larsen, H. D. Schrøder, and M. Kassem, "Parameters in three-dimensional osteospheroids of telomerized human mesenchymal (Stromal) stem cells grown on osteoconductive scaffolds that predict in vivo bone-forming potential," *Tissue Engineering—Part A*, vol. 16, no. 7, pp. 2331–2342, 2010.

[16] W. Wang, K. Itaka, S. Ohba et al., "3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells," *Biomaterials*, vol. 30, no. 14, pp. 2705–2715, 2009.

[17] E. S. Lourenço, J. Côrtes, J. Costa, A. Linhares, and G. Alves, "Evaluation of commercial latex as a positive control for *in vitro* testing of bioceramics," *Key Engineering Materials*, vol. 631, pp. 357–362, 2014.

[18] International Organization for Standardization, *Biological Evaluation of Medical Devices Part 5: Tests for In Vitro Cytotoxicity*, International Organization for Standardization, 2009.

[19] EN-ISO-10993-12, "Biological evaluation of medical devices—part 12: sample preparation and reference materials," ISO 10993-12:2008, vol. 3, 2008.

[20] P. E. Murray, C. García Godoy, and F. García Godoy, "How is the biocompatibilty of dental biomaterials evaluated?" *Medicina Oral, Patología Oral y Cirugía Bucal*, vol. 12, no. 3, pp. E258–266, 2007.

[21] T.-M. Achilli, J. Meyer, and J. R. Morgan, "Advances in the formation, use and understanding of multi-cellular spheroids," *Expert Opinion on Biological Therapy*, vol. 12, no. 10, pp. 1347–1360, 2012.

[22] M. Alcaide, M.-C. Serrano, J. Roman et al., "Suppression of anoikis by collagen coating of interconnected macroporous nanometric carbonated hydroxyapatite/agarose scaffolds," *Journal of Biomedical Materials Research Part A*, vol. 95, no. 3, pp. 793–800, 2010.

[23] J. L. Luebke-Wheeler, G. Nedredal, L. Yee, B. P. Amiot, and S. L. Nyberg, "E-cadherin protects primary hepatocyte spheroids from cell death by a caspase-independent mechanism," *Cell Transplantation*, vol. 18, no. 12, pp. 1281–1287, 2009.

[24] X. Zhang, L.-H. Xu, and Q. Yu, "Cell aggregation induces phosphorylation of PECAM-1 and Pyk2 and promotes tumor cell anchorage-independent growth," *Molecular Cancer*, vol. 9, article 7, 2010.

[25] A. F. Steuer and R. C. Ting, "Formation of larger cell aggregates by transformed cells: an in vitro index of cell transformation," *Journal of the National Cancer Institute*, vol. 56, no. 6, pp. 1279–1280, 1976.

[26] N. Zahir and V. M. Weaver, "Death in the third dimension: apoptosis regulation and tissue architecture," *Current Opinion in Genetics & Development*, vol. 14, no. 1, pp. 71–80, 2004.

[27] A. Stahl, X. Wu, A. Wenger, M. Klagsbrun, and P. Kurschat, "Endothelial progenitor cell sprouting in spheroid cultures is resistant to inhibition by osteoblasts: a model for bone replacement grafts," *FEBS Letters*, vol. 579, no. 24, pp. 5338–5342, 2005.

[28] L. Cormio, K. Turjanmaa, M. Talja, L. C. Andersson, and M. Ruutu, "Toxicity and immediate allergenicity of latex gloves," *Clinical and Experimental Allergy*, vol. 23, no. 7, pp. 618–623, 1993.

[29] B. L. Charous, C. Blanco, S. Tarlo et al., "Natural rubber latex allergy after 12 years: recommendations and perspectives," *Journal of Allergy and Clinical Immunology*, vol. 109, no. 1, pp. 31–34, 2002.