Cytotoxicity of fluconazole on canine dental pulp-derived stem cells

Paulo Henrique Utumi^a, Letícia Fracaro^b, Felipe Yukio Ishikawa Fragoso^b,
Dayane Mayumi Miyasaki^b, Paula Joly dos Santos^c, Lidiane Maria Boldrini-Leite^b,
Paulo Roberto Slud Brofman^b, José Ademar Villanova Jr.^a, Alexandra Cristina Senegaglia^{b,*}

^a Postgraduate Program in Animal Science, School of Life Sciences, Pontifícia Universidade Católica do Paraná (PUCPR), Curitiba, Paraná, Brazil

^b Core for Cell Technology, School of Medicine, Pontifícia Universidade Católica do Paraná (PUCPR), Curitiba, Paraná, Brazil

^c Undergraduate in Veterinary Medicine, School of Life Sciences, Pontifícia Universidade Católica do Paraná (PUCPR), Curitiba, Paraná, Brazil

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ABSTRACT

Objective: In order to use fluconazole as an antifungal in cell cultures, we evaluated its possible cytotoxic effects and its influence on the proliferation and viability of canine dental pulp-derived stem cells (cDPSCs).

Methods: Samples from permanent canine teeth were placed in a sterile tube with IMDM, penicillin-streptomycin, sodium heparin, and different concentrations of fluconazole. Dental pulp was digested (collagenase type II) and expanded *in vitro*. After 12 days of culture, enzymatic dissociation of the cDPSCs was performed to quantify, differentiate, and characterize the cells. Cytotoxicity was evaluated based on cell viability in response to fluconazole treatment using the 7-AAD dye.

Results: Characterization of the cDPSCs revealed that fluconazole had no influence on the immunophenotypic characteristics and differentiation of these cells. Cell proliferation assay revealed that fluconazole did not significantly interfere with the replication capacity of the cDPSCs. Cytotoxicity analysis revealed a loss of cell viability as the fluconazole concentration increased. Although there was an increase in cell mortality, the number of dead cells remained low. Though the higher concentration of fluconazole (240 µg/mL) resulted in a higher number of non-viable cells, it remained safe for use.

Conclusion: To prevent fungal contamination that causes a loss of samples during expansion of cDPSCs and to maintain minimal cell toxicity, we suggest adding 120 µg/mL of fluconazole to the teeth collection medium and cDPSCs culture.

1. Introduction

Stem cells (SCs) are undifferentiated cells that have the capability for self-renewal; they are also able to differentiate into multiple cell lineages.¹ Mesenchymal stem cells (MSCs) are adult multipotent cells that can differentiate into several cell types. They were first described by Friedenstein, Chailakhjan, and Lalykina² as fibroblast precursors isolated from the bone marrow.² MSCs have a perivascular and avascular origin and can be isolated from several sources, such as bone marrow, the placenta, the amnion, the umbilical cord and umbilical cord blood, dental pulp, and adipose tissue.^{3–7} These cells have the potential to differentiate into several other cell types, including adipocytes, chondrocytes, osteoblasts, myoblasts, and astrocytes.⁸ MSCs from the dental pulp (DPSCs) are being recognized as ideal candidates for regenerative medicine protocols.⁹ Sample collection is minimally

invasive, the biological material is discarded, following which the cells can be isolated, expanded *in vitro*, and characterized.^{10,11} Dental pulp contains stem cells with a perivascular location and similar potential compared to SCs from other sources; they also show higher proliferation rates relative to the bone marrow MSCs.¹² However, the difficulty with DPSCs arises from the condition of the oral environment, which leads to higher contamination rates compared to other sources.

The oral environment is naturally septic and may contain bacteria, fungi, and protozoans.¹³ Periodontal diseases are caused by several factors; however, bacterial plaque is the primary etiological agent responsible for most oral infections.¹⁴ Dental plaques are formed on the enamel surface and typically occur as a result of bad oral hygiene and food debris, which is a substrate for bacterial growth.¹⁵ The human oral microbiome is complex in nature and composed of bacteria and fungi.¹⁶ More than 500 different types of bacteria have been isolated from the

* Corresponding author. Pontifícia Universidade Católica do Paraná, Rua Imaculada Conceição, 1155, Prado Velho, Curitiba, Paraná, 80215-901, Brazil.

E-mail addresses: utumipr@hotmail.com (P.H. Utumi), leticiafracaro@gmail.com (L. Fracaro), yukio.ishikawa@gmail.com (F.Y.I. Fragoso), day_mayumi@hotmail.com (D.M. Miyasaki), paula_joly_ds@hotmail.com (P.J. dos Santos), lidiane.leite@pucpr.br (L.M. Boldrini-Leite), paulo.brofman@pucpr.br (P.R.S. Brofman), jose.villanova@pucpr.br (J.A. Villanova), alexandra.senegaglia@pucpr.br (A.C. Senegaglia).

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Fig. 1. Study design. A representation of this study that demonstrates the collection and isolation of teeth and the procedures performed with the samples.

Table 1

Antibodies used for immunophenotypic characterization by flow cytometry.

Antibodies	Fluorochrome	Brand	Clone	Target species
CD14	APC	BD Pharmingen (San Jose, CA, USA)	M5E2	Human, Rhesus, Cynomolgus, Baboon, Dog
CD29	PE	Abcam (Cambridge, UK)	MEM-101A	Dog
CD34	PE	eBioscience (San Diego, CA, USA)	1H6	Dog
CD44	Alexa Fluor 488	ABD Serotec (Kidlington, UK)	YKIX337.8.7	Dog
CD45	FITC	eBioscience (San Diego, CA, USA)	YKIX716.13	Dog
HLA-DR	PerCP	BD Pharmingen (San Jose, CA, USA)	TU36	Human, Rhesus, Cynomolgus, Baboon, Dog, Rabbit
7-AAD	–	BD Pharmingen (San Jose, CA, USA)	–	–

oral cavity, most of which are innocuous.^{17,18} In addition to bacteria, fungi constitute a major population of the oral microbiome (oral mycobiome). The oral cavity contains 74 culturable and 11 non-culturable fungal genera.¹⁹

Unlike humans, dogs do not have a proper oral hygiene routine; thus, bacterial plaques easily form on their teeth. Periodontal diseases, such as gingivitis and periodontitis, are the most common diseases in dogs (estimated prevalence of 95–100% and 50–70%, respectively).^{20,21} Dog teeth are thus exposed to several contaminant agents, which can hinder canine DPSC (cDPSC) expansion *in vitro*. Santin et al.²² found that *Malassezia pachydermatis* and *Candida* spp. are part of the normal microbiota in different regions of the canine oral cavity and can be isolated from animals affected by halitosis at a higher rate, suggesting that these fungi play an important role in compromising the oral health of dogs. Carreira et al.²³ identified the accumulation of bacterial plaques and a higher incidence of periodontitis—due to age-related reductions in the immune response—in older animals.

However, contamination of cell culture is a legitimate issue, especially in oral samples. Several strategies have been used to lower the contamination rates in cell culture.²⁴ Studies have employed amphotericin B, penicillin, and streptomycin or penicillin and streptomycin along with nystatin and amphotericin B to avoid contamination in cell culture.^{25–27} However, the use of fungicides in cell culture is not as common. Therefore, the standardization of a broad-spectrum antifungal agent that is not toxic and simultaneously does not interfere with the properties of cells would be extremely beneficial for avoiding contamination in cDPSC culture.

Fluconazole is a compound that is largely used alone or in combination with other drugs to treat fungal diseases as it is a broad spectrum antifungal compound.²⁸ It is a fungicide that inhibits ergosterol synthesis during the final steps of its biosynthesis and thus can be used in tooth collection as well as cDPSC culture.²⁹ Lombardi et al.³⁰ studied

the *in vitro* susceptibility of to this antimycotic drug to evaluate whether the minimal inhibitory concentration (MIC) of fluconazole was comparable to that of amphotericin B. The results showed a tenfold lower average MIC for amphotericin B than for fluconazole, suggesting that fluconazole may serve as a valid alternative to amphotericin B in the treatment of fungal infections caused by *Malassezia* spp. and *Candida* spp.³⁰ Relative to other fungicides that are commonly used in cell culture, fluconazole shows the same efficacy against fungi of the *Candida* genus with fewer adverse effects.^{31,32} However, few studies have employed fluconazole or defined doses for its use in stem cell culture. Therefore, evaluation of the influence of fluconazole on standardized methods of cDPSC collection and isolation to avoid fungal contamination is necessary.

Aiming to use a more efficient and easily available antifungal agent in hospitals and veterinary clinics, we evaluated whether different concentrations of fluconazole in the collection medium of canine teeth and during expansion of cDPSCs are toxic or affect the proliferation and viability of these cells.

2. Materials and methods

2.1. Sample collection

This study was approved by the Ethics Committee in Animal Use at Pontifícia Universidade Católica do Paraná, Curitiba, Brazil (registry number 01211/2018). Teeth were extracted after each animal owner signed consent forms. Three permanent canine teeth were obtained from each dog for a total of nine samples. The samples were collected from young adult mongrel dogs right after death. We only used teeth in which the dental pulp was not damaged and from dogs that did not have endocrine or neoplastic diseases or infections of the oral cavity aside from mild periodontitis in their medical history.

Fig. 2. Time lapse of cDPSC culture. Development of cDPSC colonies collected without fluconazole (WFC) or with different concentrations of fluconazole (F120 or F240) over time (3, 7, and 12 days). On day three after isolation, a few cells and significant cell debris were observed. On day seven, lesser amount of cell debris and the first colonies of cells initiating expansion were observed. By day 12, the cells were found to have great proliferative activity. cDPSCs: canine dental pulp stem cells; WFC: control without fluconazole; F120: 120 µg/mL fluconazole group; F240: 240 µg/mL fluconazole group.

Canine teeth were extracted with dental surgical instruments and washed with 0.12% chlorhexidine gluconate (Periogard® Colgate, São Paulo, Brazil). With a dental bur, the teeth were cut in half by the veterinarian to expose the dental pulp, and the pieces were placed in a falcon tube with Iscove's Modified Dulbecco's Medium (IMDM) (Gibco Invitrogen, Carlsbad, CA, USA), 1% penicillin-streptomycin (Gibco Invitrogen), sodium heparin (5000 U/mL; Hemofol, Cristália, São Paulo, Brazil), and different fluconazole concentrations (Isofarma, Eusébio, Brazil).

Two different concentrations of fluconazole (Isofarma) were used in sample collection media to assess its influence on cell viability (120 µg/mL (F120) and 240 µg/mL (F240); and a control without fluconazole (WFC); Fig. 1).

2.2. Cell isolation and expansion

Before pulp collection, the teeth were washed twice in phosphate-buffered saline (PBS; Gibco Invitrogen) containing 1% penicillin-streptomycin. Fragments of dental pulp were collected with an endodontic file, and canine dental pulp stem cells (cDPSCs) were isolated by enzymatic digestion with collagenase type II (0.048 g/10 mL; Gibco Invitrogen) under constant stirring at 37 °C for 1 h. The cDPSCs were seeded in a 25 cm² culture flask containing IMDM, 1% penicillin-streptomycin, 20% fetal bovine serum (FBS; Gibco Invitrogen) and fluconazole treatments. The cells were maintained in an incubator supplied with a humidified atmosphere of 5% CO₂ at 37 °C. The culture media was exchanged every three days.

2.3. Characterization of the cDPSCs

For cell characterization, the cDPSCs were differentiated into three cell lineages (osteogenic, adipogenic, and chondrogenic) and immunophenotypically characterized by flow cytometry.

2.4. Adipogenic and osteogenic differentiation

For adipogenic and osteogenic differentiation, the cDPSCs (n = 3, passage 3) were seeded in triplicate (20,000 cells/cm²) in 24-well plates (TPP, Trasadingen, Switzerland) on glass cover slips and cultured with the Adipogenic Differentiation BulletKit (Lonza, Basel, Switzerland) and

Osteogenic Differentiation Basal Medium (Lonza), respectively. Media exchanges were performed three times per week. For the control, the cells were cultured with IMDM supplemented with 15% FBS without differentiation factors or inducers. After 21 days of adipogenic differentiation, the samples were stained with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA), to evaluate the presence of lipid vacuoles. After seven days of osteogenic differentiation, the samples were stained with Alizarin Red S (Sigma-Aldrich) to evaluate the presence of calcium crystals.

2.5. Chondrogenic differentiation

Micromass culture was performed for chondrogenic differentiation. In this process, 1.0×10^6 cDPSCs (n = 3, passage 3) were centrifuged in a 15 mL conical tube to form the micromass and cultured for 21 days with Chondrogenic Differentiation Basal Medium (Lonza). For the control, the cells were cultured with IMDM supplemented with 15% FBS without differentiation factors or inducers. The culture media was exchanged three times per week, and the cellular aggregate was fixed and stained with toluidine blue (Sigma-Aldrich) to identify the presence of mucopolysaccharides in the extracellular matrix.

2.6. Immunophenotypic characterization

Immunophenotypic characterization was performed by flow cytometry. Commercial antibodies were used for analyzing the expression of cell surface markers as characteristics of cDPSCs (Table 1). For this process, approximately 1.0×10^6 cells were washed with PBS and incubated with the antibodies in the dark for 30 min at room temperature (approximately 23 °C). The cells were washed with PBS, and 500 µL of formaldehyde (1%) was added. IgG1 isotypic antibodies were used as controls. The samples were acquired (approximately 100,000 cells) from a BD FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) and analyzed by FlowJo software v8.0.2 (Tree Star, Ashland, OR, USA).

2.7. Cell proliferation assay

Cell counts are important for monitoring cell health and proliferation rates. On the 12th day after isolation, a cell proliferation assay was

Fig. 3. cDPSC characterization. (A) Osteogenic, chondrogenic, and adipogenic differentiation of cDPSCs with their respective controls. The blue arrow represents calcium crystal formation in the osteogenic differentiation of cDPSCs. The black arrow represents lipid vacuoles inside the cDPSCs following adipogenic differentiation. The red arrow represents the deposition of proteoglycans and gaps around the young chondrocytes after chondrogenic differentiation of cDPSCs. (B) Immunophenotypic characterization of cDPSCs using flow cytometry of samples that were collected with (F120 or F240) or without fluconazole (WFC). The red histograms represent the isotype control (negative) of the antibodies, and the blue histograms represent the percentage of the positive population for each antibody. IMDM: Iscove's Modified Dulbecco's Medium; cDPSCs: canine dental pulp stem cells; WFC: control without fluconazole; F120: 120 μ M fluconazole group; F240: 240 μ M fluconazole group.

performed to analyze whether fluconazole affected the normal growth of these cells. Enzymatic dissociation of the cDPSCs was performed using 0.25% trypsin/ethylenediaminetetraacetic acid (EDTA; Gibco Invitrogen), and manual-counting using a hemocytometer (Neubauer chamber) was performed.

2.8. Cell toxicity analysis of fluconazole

For this analysis was used 7-aminoactinomycin D (7-AAD) (BD Biosciences), a fluorescent dye with high affinity for DNA. Cells with compromised membranes are stained with 7-AAD; this enables the identification of non-viable cells, thereby making 7-AAD an important dye for cytotoxicity assays. Shortly, after cellular dissociation, 7-AAD was added to the cells and incubated for 30 min at room temperature

Table 2

cDPSCs counted on a hemocytometer. cDPSCs: Canine dental pulp-derived stem cell; WFC: control without fluconazole; F120: Fluconazole group 120 µg/mL; F240: Fluconazole group 240 µg/mL.

	WFC	F120	F240
Sample 1	9.5×10^5	3.5×10^5	3×10^5
Sample 2	5.5×10^5	6.5×10^5	6.5×10^5
Sample 3	3.5×10^5	2.5×10^5	2×10^5

Fig. 4. Proliferation assay. The graph shows the means and standard deviations (SDs) of cell counting after 12 days of culturing cDPSCs that were collected with (F120 or F240) or without fluconazole (WFC). cDPSCs: canine dental pulp stem cells; WFC: control without fluconazole; F120: 120 µg/mL fluconazole group 120 µg/mL; F240: 240 µg/mL fluconazole group.

(approximately 22 °C). The samples were acquired on a BD FACSCalibur Flow Cytometer (BD Biosciences) and analyzed by FlowJo software v8.0.2 (Tree Star).

2.9. Statistical analysis

Data were presented as averages and standard deviations (SDs). Levine's test followed by a one-way analysis of variance (ANOVA) and Tukey's multiple comparison test were used for statistical analyses. A significance level of 5% ($p < 0.05$) was established. All calculations were performed using GraphPad Prism software v5.0 for Windows (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Cell isolation and expansion

One of the three samples in the WFC group was contaminated in the first 24 h and needed to be replaced by a new sample. None of the samples collected with fluconazole, regardless of concentration, were contaminated.

On the third day after cell isolation, small colonies of cDPSCs were observed, and the culture still showed significant cellular debris. By the seventh day, following changes in the culture medium, the culture appeared much cleaner and free of cellular debris. On day 12, larger and more developed colonies were observed (Fig. 2). As expected, all culture groups presented fibroblast-like cell morphology and adhesion to plastic. None of the fluconazole concentrations (0, 120, and 240 µg/mL) tested showed qualitative differences in their morphology or time of passage implementation.

3.2. cDPSCs characterization

After the induction of adipogenic differentiation, it was possible to

visualize the lipid vacuoles within the cells. In the cells cultured with Osteogenic Differentiation Basal Medium (Lonza), we observed the presence of calcium crystals. For chondrogenic differentiation, we noted the presence of mucopolysaccharides in the cartilaginous matrix and gaps around the young chondrocytes. However, we did not observe differentiation in the cells cultured with control medium (IMDM + 15% FBS) (Fig. 3A).

With respect to immunophenotypic characterization, the cDPSC samples were positive for CD29 (99.7%) and CD44 (60.1%), and negative for CD14 (0.11%), CD45 (1.45%), CD34 (1.31%), and HLA-DR (0.45%) (Fig. 3B).

None of the fluconazole concentrations (0, 120, and 240 µg/mL) tested had an influence on the cellular characterization.

3.3. Cell proliferation assay

From the cell proliferation analysis, we verified that fluconazole did not interfere with the proliferation of cDPSCs (Table 2) as there was no significant difference between the WFC group and the F120 and F240 groups ($p = 0.5142$) (Fig. 4).

3.4. Cellular toxicity analysis of fluconazole

Analysis of the effect of fluconazole on cell viability using 7-AAD dye showed that the WFC (1.51%), F120 (2.27%), and F240 (2.38%) groups had low cell mortality rates (Fig. 5A). Following statistical analysis, we verified that the F240 group presented a significantly higher number of non-viable cells than the WFC group. However, the F120 group did not exhibit any significant differences compared to the other two groups (Fig. 5B). The comparisons between WFC vs. F120 ($p = 0.1199$) and F120 vs. F240 ($p = 0.2104$) did not exhibit any significant differences, but WFC vs. F240 ($p = 0.0354$) did.

4. Discussion

Fluconazole, a triazole antifungal agent with fungistatic activity against many *Candida* species, is one of the most frequently prescribed antifungal drugs. *Candida* species exist as commensals on the skin, mouth, and gastrointestinal tract. The growth and spread of *Candida* species are kept in check by the coexisting microbial flora, intact epithelial barriers, and the innate immune system.³³ In humans, *Candida* species can be found in oral, skin and gastrointestinal mycobiota.^{19,33,34} In dogs, *Candida* species are commonly found in the gastrointestinal mycobiome.³⁵ Frias et al.³⁶ identified yeast fungi belonging to the genera *Candida* and *Malassezia* in the oral mucosa of dogs. During tooth collection, the pulp canal needs to be exposed to access the tooth pulp, so the pulp is exposed to contaminants in the environment. This makes an antifungal agent, such as fluconazole, necessary to prevent contamination of the cell cultures and therefore an excellent option for this study. Likewise, this methodology can be extended to future studies on human DPSCs.

In this study, the protocol for human dental pulp isolation was used for cDPSC isolation.³⁷ For characterization, cells from passages 3 and 4 were used to avoid any signs of senescence, especially as the culture is already homogeneous and free of other cell types in these passages.

The three minimum criteria standardized by the International Society for Cellular Therapy (ISCT) for the categorization of a particular cell as MSC are as follows: adhesion to plastic, differentiation into at least three cell types (osteoblasts, chondroblasts, and adipocytes), and the expression and non-expression of some specific cell surface markers.³⁸ It was observed in the present study that the cDPSCs exhibited adhesion to plastic and differentiated into the three proposed cell types, thereby exhibiting the same characteristics as human stem cells.³⁹

For osteogenic differentiation, we began to observe calcium crystal formation after seven days using Alizarin Red S. This calcium crystal accumulation in the cDPSCs after seven days of osteogenic

A **B**

Fig. 5. cDPSC viability. (A) Graph of 7-aminoactinomycin D (7-AAD) analysis showing the viability of cDPSCs that were collected with (F120 or F240) or without fluconazole (WFC). The red histograms represent the isotype control while the blue histograms represent the number of non-viable cDPSCs. (B) Graph representing the means and SDs of non-viable cDPSCs that were collected with (F120 or F240) or without fluconazole (WFC). The comparisons of WFC vs. F120 ($p = 0.1199$) and F120 vs. F240 ($p = 0.2104$) did not exhibit significant differences, but WFC vs. F240 ($p = 0.0354$) did. cDPSCs: canine dental pulp stem cells; WFC: control without fluconazole; F120: 120 $\mu\text{g/mL}$ fluconazole group; F240: 240 $\mu\text{g/mL}$ fluconazole group; SDs: standard deviation.

differentiation induction can be explained by the high rate of phosphatase alkaline (ALP) expression in these cells.⁴⁰ ALP is an enzyme and osteogenic marker, and studies have shown that it is possible to observe the activity of this enzyme even before the induction of osteogenic differentiation in DPSCs.⁴¹ Regarding adipogenic differentiation, we identified lipid vacuoles inside the cDPSCs, but the number and size of these vacuoles was small. Dissanayaka et al.⁴⁰ demonstrated that the adipogenic potential of cDPSCs is lower than that of human DPSCs. In our study, it was possible to notice the deposition of proteoglycans and gaps around the young chondrocytes after chondrogenic differentiation. This finding corroborated those of Dissanayaka et al.⁴⁰ who also observed chondrogenic differentiation in cDPSCs.

Due to the lack of specific markers for dogs, it was not possible to test for all the markers suggested by the ISCT.³⁸ However, we observed that the cDPSCs were positive for CD29 and CD44 and negative for CD14, CD45, CD34, and HLA-DR. CD14 is a characteristic marker of monocytes and macrophages, CD34 is a marker of hematopoietic progenitors and endothelial cells, and CD45 is a common leukocyte antigen.⁴² These results showed that the cells present in the culture were not blood-derived cells, which could determine a more heterogeneous

cell population. CD44 is a cell matrix marker while CD29 is an adhesion marker (integrin) and both showed a positive immunophenotypic profile, corroborating the findings of another study that used human DPSCs.⁴³ With respect to HLA-DR antigen expression (MHC class II cell surface receptor), previous studies have demonstrated that MSCs express a small percentage of these antigens, which was also observed in this study.⁴⁴

The cell proliferation assay results showed that fluconazole did not alter the normal proliferation of cDPSCs. De Logu et al.⁴⁵ similarly found that high doses of fluconazole (1000 mg/L) for 72 h did not alter the metabolic activity in a kidney cell line, demonstrating that both the viability and proliferation of these cells were not altered. We verified the results of cell viability using 7-AAD and found that the number of non-viable cells increased as the fluconazole concentration increased; this suggested that higher concentrations may be toxic to cells. Although the F240 group showed a significant decrease in viable cells compared to the WFC group; this decrease in cell viability did not damage the culture once the cDPSCs exhibited a mean viability of 97%.

All procedures involving the isolation and expansion of stem cells are extremely expensive. Therefore, procedures that avoid wasting of

culture samples are critical to allow for the further development of this technology to benefit a larger number of patients. The contamination of one of our study samples collected without fluconazole compared to no contamination in the samples with varying fluconazole concentrations demonstrates the necessity of using this fungicidal.

5. Conclusion

This study presented positive results regarding the use of fluconazole for fungal control in the collected samples. Although there was an increase in cell mortality when compared to control group, the number of dead cells remained low. While the higher concentration of fluconazole (240 µg/mL) resulted in an increased number of non-viable cells compared to the samples that were not exposed to fluconazole, it was still safe for use. Thus, in order to prevent contamination that causes sample loss during expansion with low toxicity to cells, we suggest adding 120 µg/mL of fluconazole to the teeth collection medium and cDPSCs culture.

Ethics statement

This study was approved by the Ethics Committee in Animal Use at Pontifícia Universidade Católica do Paraná under the registry number 01211/2018.

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Declaration of competing interest

The authors declare no conflicts of interest.

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