Cell-Based Regenerative Endodontics for Treatment of Periapical Lesions: A Randomized, Controlled Phase I/II Clinical Trial

C. Brizuela, G. Meza, D. Urrejola, M.A. Quezada, G. Concha, V. Ramírez, I. Angelopoulos, M.I. Cadiz, R. Tapia-Limonchi, and M. Khoury

SUPPLEMENTAL APPENDIX MATERIAL

Appendix Table 1. Inclusion and exclusion criteria for patient selection.

Inclusion and Exclusion criteria	
Inclusion	Exclusion
Patient: • Age: 16 - 58 years old. • Signed the informed consent. • Systemically healthy patients or with chronic controlled disease	Patient: • Patients without a phone number for contact during the study. • Subjects not available for follow up period (12 months). • Patients who are or will undergo orthodontic treatment over the next 12 months. • Patients with an allergy to any material or drug used in the study. • Patients who are pregnant or lactating. • Patients heavy smokers. • Patients with a history of systemic diseases that alter immune function, such as diabetes mellitus, immunodeficiency, leukemia, Addison's disease, and Cushing. • Patients who have used immunosuppressive drugs or chemotherapy, 3 months before the study.
Tooth: • Maxillary or mandibular incisors/canines and mandibular premolars with mature apex, pulp necrosis and apical radiographic evidence of apical periodontitis (PAI ≥2 and CBCTPAI ≥1). • Teeth non-responsive to both electrical and thermal pulp test • Teeth restorable (as defined by Class A or Class B using Samet and Jotkowitz classification) without the need of a stainless steel crown	Tooth: • Endodontically treated teeth • Teeth with signs of severe root resorption. • Teeth with mobility class III or Dens invaginatus. • Teeth with avulsion history. • Teeth with clinical and / or radiographic evidence of root fracture. • Teeth that cannot be absolutely isolated with a rubber dam. • Teeth with more than one root or root canal.

Umbilical cord mesenchymal stem cells (UC-MSC) production

Umbilical cords were obtained from full-term human placentas delivered by cesarean section after informed consent, from healthy donors, and processed within 4 hours after birth. To elaborate the end product, UC-

MSCs were cultured from the Master Cell Bank (MCB) of Cells for Cells S.A. and cryopreserved in aliquots of $1x10^6$ cells in FBS + 10% DMSO.
For quality control of the MCB and end cellular product, UC-MSCs were evaluated for identity and purity for the following surface markers: CD105, CD90, CD73, CD34, CD45, CD19 and HLA-DR by Flow Cytometry in a FACS Canto II Flow Cytometer (BD Biosciences) and analyzed with FlowJo analysis software. Furthermore, sterility of the product was evaluated by Hemoculture, Fast Gram Test, Endotoxin (Endosafe PTS, Charles River, Wilmington, MA, USA) and Mycoplasma (MycoAlert, detection kit, Lonza, Basel, Switzerland). Finally, cell count and viability were determined by Trypan Blue exclusion assay. In addition, MCB was evaluated for tri-differentiation potency and tumorigenicity.

Appendix Table 2. Quality control for batches of umbilical cord mesenchymal stem cells used in clinical trial.

Quality Control for Batches of Umbilical Cord-MSC			
Attributes of Quality	Specification	Batches	
		374-5	**745-3**
Identity	CD90	99,8	99,9
	CD73	99,9	99,8
	CD105	99,3	99,4
Purity	CD45	0,07	0,19
	CD34	0,04	0,33
	CD14	0,02	0,09
	CD19	0	0,1
	HLA-DR	0,03	0,04
Sterility	Hemoculture	Negative	Negative
	Fast Gram Staining	Negative	Negative
	Mycoplasm Quick Test	Negative	Negative
	Endotoxin	< 2 EU/mL	< 2 EU/mL
Stability	Cell Viability	98%	95%
	Cell Number	1,03	1,02

Appendix Table 3. Quality control for batches of platelet poor plasma (PPP) used in clinical trial.

Quality control for batches of platelet poor plasma (PPP)			
Specification	Platelet poor plasma batch		
	847	850	857
Number of platelets (per ul)	1000	2000	1000
Prothrombin (%)	177	103	114
Prothrombin time (s)	10.3	13.3	12.7

Fast gram staining	Negative	Negative	Negative
VEGF	126 pg	150 pg	143 pg
FGF	119 pg	157 pg	101 pg
DMP1	112 pg	131 pg	133 pg

Platelet-Poor Plasma (PPP) storage and UC-MSCs encapsulation

At 48 hours before treatment, cells were thawed and seeded in a T150 flask with □MEM supplemented with 5% Plasma AB+. MSCs were then harvested using TrypLE Express 1X (Walthan, MA, USA) and washed twice with PBS 1X and resuspended in 175uL de saline solution. The resuspended cell was then mixed with 760uL of freshly thawed PPP, 15uL of Tranexamic acid and 50uL of a 2% solution CaCl2 in a 1,5ml microfuge tube. All the components were mixed by pipetting and the suspension was incubated at 37°C for 5 minutes. After checking the gelification, the end-product was stored and transported at 4°C for transplantation (Figure 1C). See supplementary material table 6 histological analysis.

Histological analysis

For histological analysis purposes, PPP-UC-MSC scaffolds were fixed in 10% formalin solution and processed for histological analysis. The scaffolds were stained for hematoxylin and eosin (Sigma, USA). In order to evaluate the ultra-structural analysis, at different time points, Scanning Electron Microscopy (SEM) was used to investigate the structure, morphology of the PPP scaffolds with or without cells. The 5mm punches of PPP scaffolds described previously were fixed with 2.5% gluteraldehyde (Sigma, USA), dehydrated in a progressive series of ethanol before being mounted on an aluminum stub using silver paint. Samples were coated with gold/palladium before examination under a JSM-7500F scanning electron microscope (JEOL, USA).

Cell viability and stability of platelet poor plasma (PPP)-umbilical cord mesenchymal stem cells (UC-MSCs)

Cell viability was evaluated using AlamarBlue™ reagent (Invitrogen, Carlsbad, CA, USA) after 1, 3, 7 and 14 days. PPP-UC-MSCs was generated in a 100mm petri dish and several punches were generated using a 5 mm diameter biopsy punch (Dolphin Medical, Chile). Each punch was placed separately in a 96-well plate, covered with 200 µl of culture medium and 20 µl of AlamarBlue™ reagent and incubated for 2 hours at 37°C. After the incubation period, 100 µl of the supernatant was transferred to a fresh 96-well plate to measure absorbance at 570/600 nm. In parallel, punches of PPP scaffolds for each time point (1, 3, 7 and 14 days) were fixed in 10% formalin solution and processed for histological analysis. The scaffolds were stained with hematoxylin and eosin (Sigma, St. Louis, MO, USA).
Stability of the gelified end-product was determined after preservation at 4°C at 24, 48, 72, 96 and 120 hours. Matrices were taken from a 1.5 tube and after checking that maintains consistency and gelation properties, the surface area was measured and analyzed for densitometry using Image J software.

Clinical procedure for ENDO and REP groups

The first session was the same for every patient. Tooth sensitivity (cold, heat and electric) was assessed. Tooth vitality was tested using a Laser Doppler flowmetry (LDF) (Moor VMS-LDF; Moor Instruments Limited, Axminster, UK). Then intervention was started by the administration of local anesthesia (2% lidocaine hydrochloride with epinephrine 1:100,000; Septodont, Saint-Maur-des-Fosses, France). The access cavity was prepared using sterile high-speed round burs, size 018 (Dentsply Sirona, Ballaigues,

Switzerland). In all cases, rubber dam isolation was maintained (Hygienic; Coltene/Whaledent AG, Altstätten, Switzerland). A glide file was established with a size 10 hand K-file (Dentsply, Ballaigues, Switzerland). The working length to the apical constriction was confirmed using an electronic apex locator (Root ZX; J Morita, Tokyo, Japan) and verified radiographically. All root canals were enlarged using Reciproc® (VDW, GmbH, Munich, Germany) selected according to manufacturer's recommendation. All teeth were irrigated with 20 ml 2.5% sodium hypochlorite and Endoactivator system (Dentsply Tulsa Dental Specialties, Tulsa, OK) was used for final sonic activation. Finally, calcium hydroxide (Hertz Pharmaceutical, Santiago, Chile) was used as an intracanal medication and closed with glass ionomer (Vitrebond; 3M ESPE, St Paul, MN, USA). After three weeks in the second session, the medication was removed by irrigation with 20 ml 17% EDTA and canal dried with sterile paper points. Subsequently, each tooth was randomized and assigned to each group following a specific protocol. ENDO Group: canals were filled using gutta-percha cones (Reciproc® VDW, GmbH, Munich, Germany) and Topseal® sealer (Dentsply Sirona, Switzerland) through a continuous wave condensation technique with an Elements™ Obturation Unit (Sybronendo, Orange County, CA, USA). REP Group: apical bleeding was induced by using a size 8 stainless steel hand K-file (Dentsply Sirona) 3 mm beyond the apex. Once the apical portion of the root canal was filled with blood, PPP-UC-MSCs was implanted and absorbable gelatin sponge hemostats (Gelita-Spon® GmbH, Eberbach, Germany) was used to contain the Biodentine™ (Septodont, France) filling material. Final restorations in both groups were done with resin (Filtek™ Z350 XT Universal Restorative; 3M ESPE, St Paul, MN, USA). Patients were blinded to treatment group. All clinical procedures were performed under an operating microscope (OPMI® Pico; Carl Zeiss AG, Göttingen, Germany).

Appendix Table 4. Demographic and clinical patient's characteristics by treatment groups.

Variable		Treatment group	
		ENDO (n = 18)	REP (n = 18)
Age, median (IQR)		28.0 (24.0 - 43.0)	27.0 (22.0 - 39.0)
Sex			
	Female	13 (72%)	12 (67%)
	Male	5 (28%)	6 (33%)
Tooth type			
	Incisive	14 (78%)	16 (89%)
	Canine	2 (11%)	1 (6%)
	Premolar	2 (11%)	1 (6%)
Pulp status			
	Symtomatic	4 (22%)	7 (39%)
	Asymtomatic	9 (50%)	6 (33%)
	Acute abscess	1 (6%)	0 (0%)
	Chronic abscess	4 (22%)	5 (28%)

ENDO: Conventional endodontic treatment REP: Regeneration IQR: interquartile range

UC-MSCs safety results in previous preclinical and clinical trials

The effect of transplanted MSC circulating in the blood stream was addressed in a previous clinical trial published by our group in heart failure. UC-MSCs were produced under the same manufacturing condition and were injected intravenously at a high dose of 106/ kg of body weight, (Bartolucci et al. 2017), Briefly,

no acute adverse events associated with the infusion of allogenic UC-MSCs was observed. None of the tested individuals developed alloantigen-directed antibodies post-infusion. Our data confirm the absence of humoral immune reaction to UC-MSCs. The safety concerns were also addressed in a separate trial were cells were injected locally in the joint articulation. A 20 fold higher cell dose was used in comparison with the number of cells transplanted in the root canal (Matas et al. 2019). Again, no serious adverse events, deaths, permanent disability, neoplasia, or septic arthritis cases were registered during the trial. The carcinogenic and tumorigenic potential of UC-MSCs were evaluated through the subcutaneous injection of 5x106 cells in NOD mice. Gross examination of the mouse was performed 12-15 weeks after injection of UC-MSCs revealed normal skin, mucous membranes, and lymph nodes. Results demonstrate that the UC-MSCs batch is negative for the presence of lesions or tumors in the skin and organs of NOD mice.

The safety concerns were also addressed in a separate trial were cells were injected locally in the joint articulation (20x higher cell dose in comparison with the number of cells transplanted in the root canal in the frame of this trial) (Matas et al. 2019) Again, no serious AEs, deaths, permanent disability, neoplasia, or septic arthritis cases were registered during the trial. The most common adverse event related to intra-articular injection was acute synovitis. One week after first injections, mild to moderate symptomatic knee effusion was present more often in cell-treated groups than controls, but with no significant differences. Pain was the second most frequent AE without reaching statistical difference between groups. Both AEs were transient and responsive to rest and oral acetaminophen. No cases or controls required hospitalization or arthrocentesis.

The carcinogenic and tumorigenic potential of UC-MSCs were evaluated through the subcutaneous injection of 5x106 cells in NOD mice. An in vivo assay is performed by the animal husbandry service of Cells for Cells S. A., which consists of external and internal macroscopic inspections of the mice skin and organs respectively. Gross examination of the mouse was performed 12-15 weeks after injection of UC-MSCs revealed normal skin, mucous membranes, and lymph nodes. The skin had a normal smooth and pale pink skin appearance without continuity lesions or mucous protrusions. Organs, skin, and mucosa were brightly colored and evidenced normal characteristics for the species, with no signs of abnormal lymph nodes or other gross abnormalities. Results demonstrate that the UC-MSCs batch is negative for the presence of lesions or tumors in the skin and organs of NOD mice. Following gross anatomic evaluations, the skin and organs (liver, lung, and kidney) were sampled and sent to an independent Anatomic and Molecular Pathology Lab for further microscopic and histological examination. Macroscopic examination was performed using a piece of right-side murine skin (15 x 5 x 2 mm), left-side murine skin (12 x 5 x 3 mm), liver (30 x 25 x 10 mm), pulmonary tissue fragments (together 20 x 10 x 5 mm), and symmetrical kidney samples cut to preserve architecture (10 x 8 x 5 mm each). No evidence of tumor or focal lesions were identified in any tissues examined. Further histological examination showed preserved architecture with no evidence of tumors or cellular abnormalities. Hepatic tissues evidenced preserved architecture, central veins, portal spaces and hepatocytes of preserved morphology without tumors. Pulmonary tissues evidenced moderate capillary congestion and preserved architecture, without evidence of tumor, and samples also were shown to contain esophagus fragments which contained no evidence of tumor. The kidney parenchyma fragments evidenced good medullary cortical relationship and mild congestion in glomerular and peritubular chapels, without evidence of tumor or focal lesions.

Appendix Figure. Flow diagram of the progress of the parallel randomized trial.