Case Report

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Retrieval of a periodontally compromised tooth by allogeneic grafting of mesenchymal stem cells from dental pulp: A case report

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Abstract

Objective: To report a case of successful allogeneic grafting of mesenchymal dental pulp stem cells (DPSCs) as preliminary findings in a patient with periodontal disease enrolled into clinical trial ISRCTN12831118.

Methods: Mesenchymal stem cells from the dental pulp of a deciduous tooth from a 7-year-old donor were separated from the pulp chamber and processed via enzymatic digestion and centrifugation. DPSCs were passaged and cultured on a 35 × 13 mm culture dish in minimum essential medium-alpha, without supplementation. After reaching 80% confluency, 5 x 10^6 allogeneic DPSCs in 250 μl phosphate buffered saline were seeded onto a dry scaffold of lyophilized collagen-polyvinylpyrrolidone sponge placed in the left lower premolar area of a 61-year-old patient with periodontal disease. Surgical access to the lower premolar area was achieved using the flap technique.

Results: At 3 and 6 months following allogeneic graft, the patient showed no sign of rejection and exhibited decreases in tooth mobility, periodontal pocket depth and bone defect area. Bone mineral density had increased at the graft site.

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Conclusions: Regenerative periodontal therapy using DPSCs of allogeneic origin may be a promising treatment for periodontal disease-induced bone defects.

Keywords
Periodontitis, tissue regeneration, periodontal treatment

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Introduction

Periodontal disease is a chronic inflammatory condition that is highly prevalent in elderly patients.[1] Disease progression is marked by the destruction of underlying support structures of the teeth, which eventually leads to definitive tooth loss.[1] Periodontal disease has negative effects on digestive, nutritional and glycaemic control in patients with diabetes, and is also associated with an increased incidence of Alzheimer's disease, rheumatoid arthritis and heart disease.[2] Moreover, periodontal disease is common in pregnant females and may lead to premature birth and a low birth weight.[3] Conventional periodontal disease treatments include changes in hygiene and dietary habits, scaling and root planing, and even flap periodontal surgery. In general, however, these treatments control only the acute phase of disease and do not restore the tissues damaged by periodontal disease.[4] Thus, recent research has focused on regenerative approaches, including mesenchymal stem-cell grafting, which has promising applications. Mesenchymal stem cells are involved in growth, wound healing and replacing cells that have been lost through either daily exfoliation or pathological conditions.[5]

Traditionally, bone marrow has served as the main source of mesenchymal stem cells for basic research and therapeutic use; however, there are mesenchymal stem cells in almost all tissues, including teeth. Because of their accessibility and ability to differentiate into multiple lineages, mesenchymal dental pulp stem cells (DPSCs) are increasingly recognized as a viable source for the development of effective cell-based therapies.[6,7]

Although patient trials are limited, multiple groups view mesenchymal stem cells as a promising and safe treatment, regardless of human leukocyte antigen (HLA) compatibility.[8,9] In this sense, there is evidence that allogeneic mesenchymal stem-cell infusion appears to be well tolerated. Most trials have reported only mild or transient effects, and some have even reported the absence of adverse reaction to allogeneic mesenchymal stem cells.[10] Of patients with osteogenesis imperfecta treated with mesenchymal stem cells, none exhibited clinical symptoms of an autoimmune response to allogeneic cells.[11] Furthermore, an intravenous infusion of allogeneic mesenchymal stem cells in patients with aplastic anaemia has been shown to be safe.[12] More recently, myocardial regeneration was safely achieved using human allogeneic umbilical cord matrix-derived mesenchymal stem cells.[13,14] Thus, the present authors hypothesized that an infusion of allogeneic DPSCs would similarly induce periodontal tissue regeneration in adult patients with periodontal disease. This case report presents clinical, radiographic and surgical evidence of successful periodontal

regeneration following the therapeutic application of allogeneic DPSCs, as preliminary findings in one patient enrolled into trial ISRCTN12831118, which is a pilot trial aimed at standardizing techniques for periodontal regeneration.

Case report

A 61-year-old male patient was referred to a dental clinic at the National Autonomous University of Mexico to receive treatment for periodontal disease. The patient first presented at the clinic in March 2016, at which time oral diagnosis was confirmed, as were supra- and subgingival dental calculus, bleeding upon probing, grade II mobility (graded by holding one side of the tooth with a metal instrument and the other side with the index finger and moving in buccolingual and vertical directions, as previously described)[15] and a periodontal pocket depth of 6.5 mm in the second left lower premolar (No. 35). Radiological analysis showed a radiolucent zone on the mesial side of the root of the same tooth. No deep caries, changes in tooth mobility, or root involvement were detected in any other teeth.

The patient reported a history of systemic arterial hypertension since the age of 35, which he controlled by taking metoprolol and nifedipine every 24 h. The mother had a history of systemic arterial hypertension and diabetes, and the father had no history of pathology. The patient provided written informed consent for treatment and publication of the case and was enrolled into trial ISRCTN12831118. The surgical protocol followed the principles of the Helsinki Declaration and was approved by the Bioethics and Biosafety Committee of the Research Committee of the Faculty of Higher Studies Zaragoza, National Autonomous University of Mexico, ref: 25/11/SO/3.4.1.

Mesenchymal stem cells were obtained from the dental pulp of a 7-year-old male donor under aseptic conditions and under the strict criteria of good manufacturing practices, using reagents that were free of products of animal origin. The dental pulp was gently removed from the teeth and was immersed in a digestive solution (3 mg/ml type I collagenase plus 4 mg/ml dispase in Minimum Essential Medium [MEM]-α [Life Technologies; Grand Island, NY, USA]) for 1 h at 37 °C. Once digested, the dental pulp was dissociated and centrifuged at 497 *g* for 5 min. After centrifugation, the dissociated tissue was resuspended in MEM-α and seeded at 2×10^4 cells/ml into a 35 mm × 13 mm dish, then incubated at 37 °C until 80% confluence was achieved. The cells were expanded by serial passage into 35 mm dishes, and at the third passage, cells were cultured to 80% confluence for analyses by flow cytometry and for use in treating the recipient patient. Cell phenotype was analysed using BD antibodies to human cell antigens and a BD FACSCalibur™ flow cytometer with BD Pro v.5.1.1 software (all BD Biosciences, San José, CA, USA), according to the manufacturer's instructions. Cells were found to be positive for cluster of differentiation (CD)105 (85.81%), CD73 (99.81%), and CD90 (99.54%) and negative for CD45 (0.06%), CD34 (0.09%), CD31 (0.20%), CD14 (0.04%), CD11b (0.04%) and HLA–D related (DR) (1.06%). In addition, differentiation assays (Figure 1) were performed for osteogenic, adipogenic and chondrogenic lineages as described previously.[16,17] The present findings were consistent with the International Society for Cellular Therapy criteria for mesenchymal stem cells.[18]

The recipient patient's blood chemistry, blood cytometry, prothrombin time and activated partial thromboplastin time were all within normal parameters (Table 1). Cone beam volumetric tomography was performed

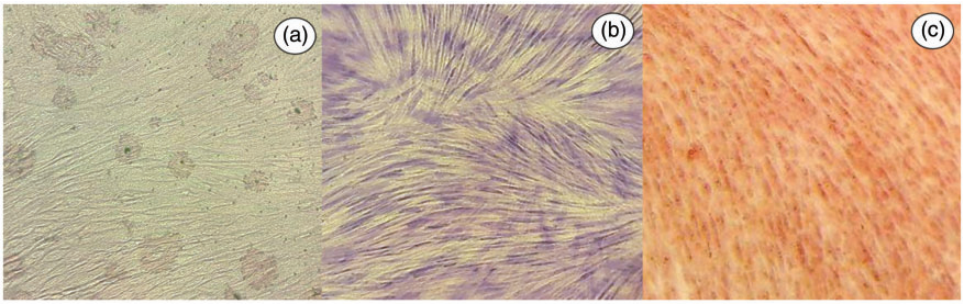

Figure 1. Representative images showing *in vitro* multi-lineage differentiation of dental pulp stem cells obtained from a 7-year-old donor: (a) oil red O staining showing lipid deposits (arrows), indicative of adipogenic lineage; (b) alcian blue staining showing glycosaminoglycans deposits (arrows), indicative of chondrogenic lineage; and (c) alizarin red staining showing more densely stained areas with mineral deposits (arrows), indicative of osteogenic lineage; (all images, original magnification × 40).

Table 1. Blood chemistry, blood cytometry, prothrombin time and activated partial thromboplastin time before and after allogeneic dental pulp stem cell grafting in a 61-year-old male patient with periodontal disease.

	Assessment time-point		
Blood parameter	Pre-surgery	6 months post-surgery	Normal range
Haematocrit, %	48.0	47.5	38–54
Haemoglobin, g/dl	15.8	15.6	12–16
White cell count, $\times 10^3/mm^3$	8.5	8.3	5.0–10.0
Red cell count, $\times 10^6/mm^3$	5.23	5.11	4.2–6.2
Platelet count, $\times 10^3/mm^3$	172	162	150–400
MCHC, %	31	32	26–32
MCH, %	29	30.5	27–32
MCV, fl	94	93	82–98
Lymphocytes, %	22	21	20–40
Neutrophils, %	67	67	40–70
Eosinophils, %	1	0	0–1
Monocytes, %	8	7	2–8
Basophils, %	0	0	2–4
Biochemistry			
Glucose, mg/dl	101	100	70–100
Urea, mg/dl	50.3	49	10–50
Creatinine, mg/dl	0.95	0.89	0.50–1.20
Uric acid, mg/dl	5.0	5.0	2.40–5.4
Cholesterol, mg/dl	160	150	150–200
Triglycerides, mg/dl	89	83	50–160
Bilirubin, mg/dl	0.25	0.10	0–0.3
C-reactive protein, mg/l	0	0	0–10
Coagulation			
PT, s	12.8	12.0	10–15
aPTT, s	33.1	32.1	27–45

MCHC, mean corpuscular haemoglobin concentration; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; PT, prothrombin time; aPTT, activated partial thromboplastin time.

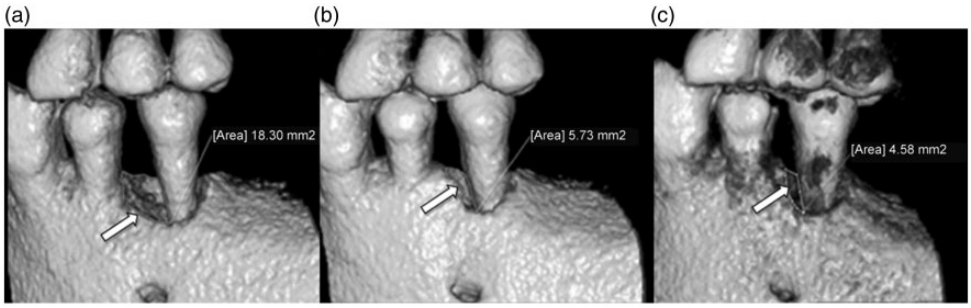

Figure 2. Cone beam volumetric tomography images of the left lower premolar area of a 61-year-old male patient with periodontal disease, showing: (a) boundary of the initial bone defect area of 18.30 mm^2 (arrow) at baseline; (b) bone defect area reduced to 5.73 mm^2 (arrow) at three months following dental pulp stem cell graft; and (c) bone defect area reduced to 4.58 mm^2 (arrow) at six months following dental pulp stem cell graft.

Table 2. Clinical evaluation before and after allogeneic dental pulp stem cell grafting in a 61-year-old male patient with periodontal disease, showing baseline and post-intervention (3- and 6-month) results.

	Study time-point		
Parameter	Baseline	3 months	6 months
Depth of periodontal defect, mm	6.5	3.6	3.5
Tooth mobility, grade	II	I	I
Bone mineral density			
Distal area, Hounsfield Units	916	1111	1328
Proximal area, Hounsfield Units	612	880	1058
Middle area, Hounsfield Units	163	381	929

Tooth mobility grade: I, normal; II, slight mobility.[15]

to calculate the size of the periodontal defect and the bone mineral density (Figure 2a; Table 2) using OnDemand3D™ Project Viewer software (Cybermed Inc., Seoul, Korea). Subsequently, phase I periodontal therapy (non-surgical) was performed, comprising the removal of supra- and subgingival calculus using a Tigon Piezo Scaler (W&H Impex, Windsor, OH, Canada), root scaling/root planing, replacement of restorations, occlusal adjustment and instructing the patient in proper oral hygiene. After 12 weeks, the amount of biofilm detected was minimal, and the periodontal defect remained unchanged, with a depth of 6.5 mm in the mesial, middle and distal areas.

Prophylactic antibiotic therapy, using 500 mg metronidazole and 300 mg clindamycin, orally, every 8 h, was initiated three days prior to the surgical phase. Before proceeding to the surgical phase, a rinse was performed with chlorhexidine 0.12% for 2 min, and the area was cleaned with benzalkonium chloride. Loco-regional anaesthesia was applied (1:50 000 lidocaine and adrenaline).

Surgical access to the left lower premolar area was achieved using the flap technique. The root surfaces were smoothed using an

ultrasonic reamer (W&H Impex, Windsor, OH, Canada), the granulation tissue was removed and the area was irrigated with 0.9% NaCl in injectable water. After cleaning the surface, a dry scaffold of lyophilized collagen-polyvinylpyrrolidone sponge (Fibroquel; Aspid, Mexico City, Mexico) was placed on the bone defect in 0.5 cm^2 fragments using an Adson calliper (Hu-Friedy, Chicago, IL, USA) filled with chlorhexidine gel. Following three passages, a total of 5 x 10^6 DPSCs in 250 µl of phosphate buffered saline were seeded onto the scaffold.[19] A Teflon-coated titanium membrane (Cytoplast® Ti-250/Ti-150 non-resorbable barrier membranes; Osteogenics Biomedical, Lubbock, TX, USA) was applied, and the flap was repositioned with absorbable suture points. Surgical cement was added to the area to protect the intervention. The intervention was further protected with metronidazole and clindamycin (as above) supplemented with 100 mg nimesulide, orally, every 12 h for 5 days to control oedema and pain (Figure 3).

Following surgical intervention, the patient was instructed to rinse with Oxoral® solution for 2 min twice daily for two weeks. In addition, the patient was instructed to use Tebodont® toothpaste and to perform dental cleaning with a Curaprox Surgical brush. Weekly observations were performed to monitor the healing process.

The surgical cement was removed at 7 days following surgery, and cone beam

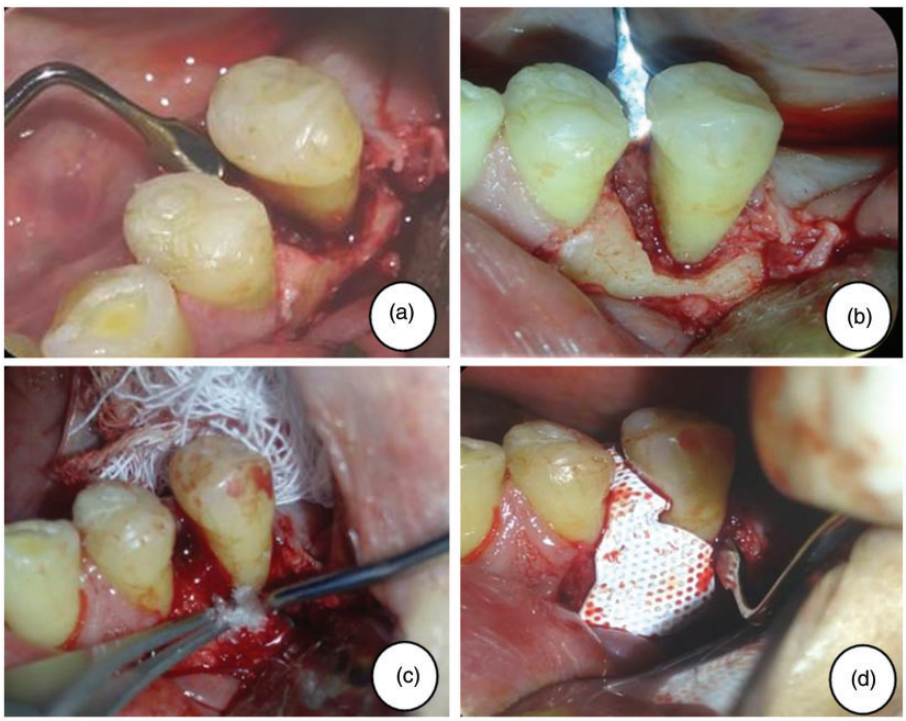

Figure 3. Representative clinical views of the left lower premolar area of a 61-year-old male patient with periodontal disease, showing: (a) the mesial circumferential bone defect during periodontal surgery; (b) debridement and collocation of a dry scaffold of lyophilized collagen-polyvinylpyrrolidone sponge; (c) seeding of dental pulp stem cells; and (d) adaptation of non-resorbable membrane.

volumetric tomography was performed at 3 and 6 months following surgery (Figure 2b and 2c). The patient will be examined monthly up to 1 year following the surgical intervention and every 6 months for 3 years after the intervention to confirm that the defect has filled with bone tissue and that the new tissue remains stable over time.

At 3 and 6 months following surgical intervention, the patient showed no signs or symptoms of rejection. Throughout this time, the gingiva showed no signs of inflammation, and depth of the periodontal pocket and dental mobility both decreased compared with initial baseline findings (Table 2). Furthermore, the patient's blood chemistry and blood cytometry parameters at 6 months postoperatively (white blood cell count, red blood cell count, the percentage of neutrophils, the percentage of lymphocytes, blood bilirubin and C-reactive protein) were comparable with values at 2 weeks prior to surgery (Table 1), suggesting that there was no immunological rejection, as also noted in a previously published study.[20]

The clinical results were in agreement with the findings of cone beam volumetric tomography and bone densitometry assays. Cone beam volumetric tomography revealed that an initial damaged area of 18.30 mm^2 was reduced to 5.73 and 4.58 mm^2 at 3 and 6 months, respectively, following the DPSC graft (Figure 2). Densitometry assays revealed an increase in bone mineral density in the walls of the defect at 3 and 6 months post-treatment, which is suggestive of bone tissue regeneration (Table 2).

Discussion

Tissue engineering using stem cells is an approach that might address the deficiencies of more conventional therapeutic options by regenerating living and functional periodontal structures.[21] For many years, bone marrow has been the main source of stem cells but harvesting mesenchymal stem cells from bone marrow is difficult and painful. DPSCs represent an ideal stem cell source, as they are easily accessible, can be harvested using a non-invasive protocol, and are rapidly expandable *in vitro*. Animal studies have suggested that the application of DPSCs could promote the growth of human dental tissues *in vivo*. When transplanted into mice and immunodeficient rats, DPSCs isolated from human third molars differentiate into cementoblast-like cells and can form complex structures, such as pulp-dentin and a periodontal ligament-cementum root.[22–25]

In the present study, collagen scaffold was chosen because it is the main protein of undifferentiated mesenchymal tissues and because scaffolds with a collagen base have been reported to support stem cells during the first weeks of differentiation by facilitating initial cell attachment and cell differentiation and, consequently, the formation of calcified tissue.[26] Collagen is radiolucent with high periodontal tissue compatibility and degradability.[27] Furthermore, the pore structure of a collagen sponge is ideal for the colonization of seeded cells and enhances bone formation by promoting the differentiation of osteoblasts,[28] thus providing a suitable scaffold for the engineering of tooth tissue.

Compared with other adult tissue sources, DPSCs are an easily accessible source of adult dental stem cells.[29] The supply of pulp tissue decreases with age, however, due to narrowing root canals, physiological secondary dentin formation, and pathological tertiary dentin formation and mineralization.[30] Thus, older patients' teeth contain fewer DPSC colonies than the teeth of younger patients.[31] There is some conflicting evidence, as some groups have reported no such age-related change, but the majority of studies find an age-related decrease in differentiation/regeneration capacity of

cells derived from dental pulp. Human DPSCs obtained from older donors are thought to lose their proliferative activity and differentiation capability, and become senescent, after fewer passages than cells derived from younger donors.[32–34] Thus, the present authors believe that minimal manipulation of DPSCs in culture will increase the possibility of achieving tissue regeneration.

Allogeneic DPSCs were obtained from a 7-year-old donor patient in the present study, following good manufacturing practices, to graft onto a periodontal defect in an elderly male patient. A previous study, published in 2009, showed that mesenchymal stem cells are only weakly immunogenic in humans and validated the clinical use of mesenchymal stem cells from HLA-mismatched donors.[35] Results from the same study indicated that mesenchymal stem cells could be transplanted successfully into allogeneic recipients across HLA barriers, with little evidence that the mesenchymal stem cells would be rejected or would sensitize the recipient to other cells of the same HLA type.[35] Since then, many procedures have been performed with allogeneic HLA-mismatched mesenchymal stem cells without evidence of immune rejection.[36]

These and other experimental data present strong evidence that DPSCs have the potential to induce the formation of human dental tissues *in vivo*; however, clinical data were available for only a few human cases.[37] Thus, the present case report examined whether the transplantation of human DPSCs contributed to periodontal repair in a patient with a periodontal disease-induced infrabony defect.

The combination choice of metronidazole and clindamycin for pre-surgical prophylactic therapy in the present case is supported by an investigation by Sigusch *et al* (2001)[38] that reported the use of metronidazole and clindamycin in treating periodontal disease. In addition, these antibiotics have been used in combination to treat other infectious diseases.[39] Furthermore, the present authors have used the combination of metronidazole and clindamycin as a prophylactic treatment for surgical intervention and achieved good results.

The patient described here exhibited clinical improvement at 3 and 6 months following DPSC treatment, as indicated by a decreased depth of periodontal defect, normal gingiva characteristics, decreased tooth mobility, and observation of bone-like tissue in both tomographic and bone densitometry assays. To the best of the authors' knowledge, the present case provides the first evidence that allogeneic DPSC transplantation in humans is capable of inducing bone tissue regeneration in a periodontal disease-induced infrabony defect. The findings are consistent with those of a previous study that provided the first evidence that an autologous human DPSC transplant could induce the restoration of mandibular bone tissue in patients with third molar extraction,[40] and are consistent with another study demonstrating that DPSCs deposited in a collagen scaffold are able to successfully repair bone.[41] In the present case, a collagen scaffold was used to generate bone tissue, however, it is possible to transplant DPSCs and obtain a remodelled and highly vascularized bone tissue without the use of scaffolding.[42] Thus, the present authors would like to explore this less invasive possibility in future studies.

A possible mechanism behind the bone defect repair observed in the present study is that conditions in the oral microenvironment facilitated DPSC differentiation. Evidence suggests that local factors can modulate the physiology of mesenchymal stem cells to promote tissue regeneration in injured organs and tissues.[43] Negative signals in the aged periodontium can send distress signals to the grafted allogeneic mesenchymal stem cells, thereby inducing

the mesenchymal stem cells to secrete a diverse repertoire of factors, such as growth factors, cytokines, mRNA and extracellular matrix, that support cell survival. Components of the mesenchymal stem cell secretome might therefore rescue injured cells, reduce tissue damage, and accelerate repair.[43,44]

In addition to bone formation, the complete regeneration of periodontal tissue requires the formation of periodontal ligament and root cement.[45] Further research should, therefore, include analysis of a sample of neoformed tissue in terms of histological composition, to confirm the kinds of tissue formed other than bone.

Regenerative periodontal therapy using allogeneic DPSCs did not present evidence of immunological rejection in the current case, and it was shown to help reduce the periodontal pocket and drive the formation of bone-like tissue to actively repair the periodontal disease-induced infrabony defect.

The present work represents a pilot proposal for a periodontal disease treatment protocol, and a control case is not presented for comparison. A split-mouth model could be considered in future research, in which the patient simultaneously serves as control and treatment and both sites are exposed to the same internal and external factors that could affect the outcome. In addition, a randomized clinical study is required to corroborate the present findings in a larger patient population.

A further limitation was that fact that due to the treatment protocol, it could not be determined whether the number of cells seeded onto the scaffolding was maintained on the scaffolding. Thus, it is suggested that the cells be transferred and allowed to attach to the scaffold before surgery, so that when the surgery is performed, the likelihood of a successful therapy is increased.

In conclusion, the findings of the present case report suggest that DPSC treatment promotes periodontal regeneration. Further research should include a randomized clinical trial to verify the findings.

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Declaration of conflicting interests

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