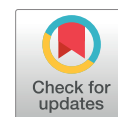## Clinical Investigation

Safety and Efficacy of Mesenchymal Stem Cells for Radiation-Induced Xerostomia: A Randomized, Placebo-Controlled Phase 1/2 Trial (MESRIX)

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Summary

Salivary gland hypofunction and xerostomia are major complications to head and neck radiotherapy. This

Background: Salivary gland hypofunction and xerostomia are major complications to head and neck radiotherapy. This trial assessed the safety and efficacy of adipose tissue-derived mesenchymal stem cell (ASC) therapy for radiation-induced xerostomia.
Patient and Methods: This randomized, placebo-controlled phase 1/2 trial included 30 patients, randomized in a 1:1 ratio to receive ultrasound-guided transplantation of ASCs

randomized, placebo-controlled phase 1/2 trial assessed the safety and efficacy of adipose tissue-derived mesenchymal stem cell (ASC) therapy for radiation-induced xerostomia, and 30 patients were randomized in a 1:1 ratio to receive ultrasound-guided transplantation of ASCs or placebo to the submandibular glands. No adverse events were detected. ASC therapy was safe and significantly improved salivary gland functions and patient-reported outcomes.

or placebo to the submandibular glands. Patients had previously received radiotherapy for a T1-2, N0-2A, human papillomavirus-positive, oropharyngeal squamous cell carcinoma. The primary outcome was the change in unstimulated whole salivary flow rate, measured before and after the intervention. All assessments were performed one month prior (baseline) and one and four months following ASC or placebo administration.

Results: No adverse events were detected. Unstimulated whole salivary flow rates significantly increased in the ASC-arm at one (33%; $P = .048$) and four months (50%; $P = .003$), but not in the placebo-arm ($P = .6$ and $P = .8$), compared to baseline. The ASC-arm symptom scores significantly decreased on the xerostomia and VAS questionnaires, in the domains of thirst (-22%, $P = .035$) and difficulties in eating solid foods (-2%, $P = .008$) after four months compared to baseline. The ASC-arm showed significantly improved salivary gland functions of inorganic element secretion and absorption, at baseline and four months, compared to the placebo-arm. Core-needle biopsies showed increases in serous gland tissue and decreases in adipose and connective tissues in the ASC-arm compared to the placebo-arm ($P = .04$ and $P = .02$, respectively). MRIs showed no significant differences between groups in gland size or intensity ($P < .05$).

Conclusions: ASC therapy for radiation-induced hypofunction and xerostomia was safe and significantly improved salivary gland functions and patient-reported outcomes. These results should encourage further exploratory and confirmatory trials. © 2018 Elsevier Inc. All rights reserved.

Background

The subjective feeling of dry mouth (xerostomia) may be present with or without reduced whole saliva secretion, although generally, it is perceived when the unstimulated whole saliva flow rate is reduced by more than 40% to 50% (1). Salivary gland hypofunction severely impacts quality of life; for example, it often leads to an increase in dental decay and impairments in eating, sleeping, and interacting socially (2, 3). The three main causes of severe xerostomia and hyposalivation are medication side effects, Sjögren's syndrome, and radiation therapy for head and neck cancer (2).

Head and neck cancer is the sixth most common malignancy worldwide. The majority of patients are at an advanced disease stage at presentation, and therefore, they are treated with chemotherapy, radiotherapy, or both (4). Radiation therapy increases tumor control and the chance of survival. However, despite advanced methods, a significant proportion of radiation is deposited in normal tissues, including the salivary glands, which leads to gland deterioration and hypofunction (5). Current strategies to improve salivary gland function after radiation therapy are aimed at the symptoms; thus, they merely stimulate the function of residual salivary glands or provide short-term lubrication. The methods used to reduce the incidence of xerostomia after radiotherapy include prevention, radio-protective agents, and a recently proposed strategy of stem cell-sparing radiation therapy (6). Accordingly, no treatment options are currently available for restoring gland function, per se.

Stem cells have been identified as a potential treatment modality for a wide variety of cell degenerative disorders by their ability to differentiate into diverse specialized cell types. Most of the early work on stem cells was performed in embryonic stem (ES) cells and induced pluripotent stem cells (iPS cells). However, clinical use of iPS cells as therapeutic agents is limited, due to histocompatibility problems, their potential ability to form malignant teratomas, and the risk of uncontrollably differentiating into cells derived from all three primary germ layers. Multipotent mesenchymal stem cells (MSCs) from adult connective tissues have a more limited ability to differentiate and have gained substantial attention in recent years, because they are relatively easy to isolate, culture, characterize, and apply clinically (7).

MSCs exhibit several interesting characteristics; they have angiogenic, anti-inflammatory, and tissue-regeneration properties (8). Notably, MSC transplantation therapy has shown promising results in preclinical studies for the treatment of xerostomia, including radiation-induced xerostomia (9).

MSCs were first discovered in the bone marrow, and most previous clinical studies have been performed by use of BM-MSCs. However, adipose tissue has been shown to be an abundant and accessible source of MSCs and thus increases the feasibility of using multipotent stromal cells in a clinical setting. Moreover, for clinical applications adipose-tissue derived MSCs seem to be as effective as BM-MSCs exhibiting equivalent immune modulatory, angiogenic, anti-inflammatory, and tissue-regeneration properties (10).

This trial aimed to assess the safety and efficacy of a local injection of autologous, adipose tissue-derived, mesenchymal stem cells (ASCs) for treating radiation-induced salivary gland hypofunction and xerostomia.

Patients and Methods

The trial protocol has been published elsewhere (11). Briefly, prior to participation in any trial procedures and after a full explanation of the trial from the principal study investigator (CG), all patients signed an approved written informed consent form. Eligibility criteria included previous radiotherapy, with or without concomitant chemotherapy, for a human papilloma virus-positive, T1-T2, and N0, N1, or N2A oropharyngeal squamous cell carcinoma; (12) two years of follow-up without disease progression; an unstimulated whole saliva flow rate in the range of 0.05 to 0.20 ml/min, corresponding to either hyposalivation (<0.10 ml/min) or subnormal (<0.20 ml/min) saliva flow rates; and xerostomia grades 1 to 3 (13). Patients were excluded if diagnosed with any cancer during the previous two years, were taking xerogenic medication, had any disease of the salivary glands (eg, sialolithiasis), were pregnant or planned a pregnancy, were breastfeeding, and when we failed to expand the ASCs to at least 50% of the calculated amount needed. The primary outcome was a change in the unstimulated whole salivary flow rate. Secondary outcomes were safety, patient-reported outcome measures, flow-rate induced changes in the composition of inorganic saliva components, changes in the stimulated whole salivary flow rate, changes in unstimulated and stimulated submandibular salivary flow rates, and changes in submandibular gland morphology, based on contrast-induced magnetic resonance imaging (MRI) and core-needle tissue samples. The assessments were performed at baseline (one month prior to treatment) and at one and four months after the administration of ASCs or placebo (Fig. 1).

This study was conducted according to the Helsinki Declaration and approved by the Danish National Scientific Ethics Committee (1406653), the Danish Medicines Agency (2014-004349-29), and the Danish Data Protection Agency (30-1452). The trial was monitored by the Good Clinical Practice Unit (2014-724). This trial was registered with ClinicalTrials.gov, number NCT02513238, EUDRACT number 2014-004349-29, and is completed.

Salivary flow rate assessments

The unstimulated whole saliva flow rate (ml/min) was determined with the drooling method. Each subject drooled for 15 minutes into a disposable plastic cup. The cups were weighed on a precision scale before and after collection, and the flow rates were calculated, presuming that 1 g saliva was equivalent to 1 ml (14). Stimulated whole saliva flow rates were obtained chewing 1 g of paraffin wax for a period of 5 minutes, and the collected saliva volume was similarly converted into the flow rate. Submandibular saliva was obtained with the swab method. We used commercially available saliva collection swabs positioned beneath the tongue, which corresponded to the openings of the Wharton ducts (14). After collection, all whole saliva samples were stored at -80°C, together with the swabs.

Inorganic saliva composition

The main inorganic elements, excluding chloride and bicarbonate, were determined in all whole saliva samples with inductively coupled plasma (ICP) spectrometry. In brief, 250 µl of the unstimulated whole saliva sample was diluted 20-fold in Millipore water, acidified with 2% Suprapure 65% HNO_3. After dilution, the samples were centrifuged at 1500 × g for 5 minutes. Samples, standards, and quality controls were prepared and processed in the same way and on the same day.

Patient-reported outcome measures

At baseline and at the one- and four-month visits following the intervention, participants completed questionnaires centered on xerostomia—that is, the Visual-Analogue-Symptomatic (VAS) questionnaire (15) and the Xerostomia Questionnaire (16). Both questionnaires were translated to Danish by forward- and back-translation by an English translator with Danish as her mother-tongue and familiar with the terminology of the field. An expert panel of three Oral Medicine professionals evaluated expressions and interpretation of the Danish translation and discrepancies of the English back-translation compared to the original version.

Magnetic resonance imaging (MRI) assessments

MRI scans were performed at baseline and at one and four months following the intervention. The scans were acquired
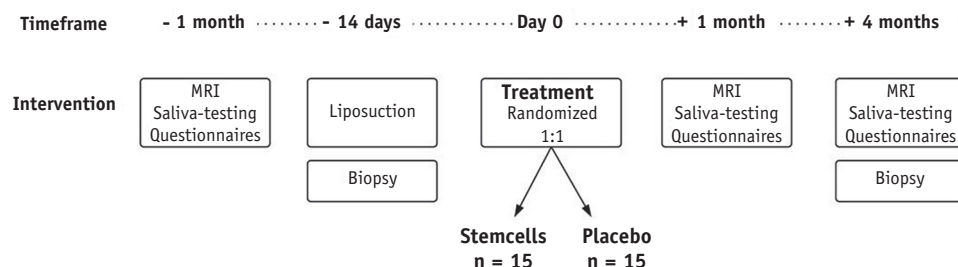
Fig. 1. Timeframe and trial design. *Abbreviation:* MRI = magnetic resonance imaging.

on a 3T Siemens Verio. The scans consisted of the following sequences: (1) EP2D DIFF, a single direction diffusion scan with b = 0 and b = 800, at a resolution of $0.98 \times 0.98 \times 4.4$ mm, from which the Apparent Diffusion Coefficient (ADC) was derived; (2) T1 MPRAGE $0.49 \times 0.49 \times 4.4$ mm and T2-blade sequences at a resolution of $0.98 \times 0.98 \times 4.4$ mm. The T1 scan was used to measure the size of the glands. The T1 and T2 scans were analyzed for signal intensity. The diffusion scans were corrected for eddy currents with the eddy-correct FSL tool. The submandibular glands were manually contoured by the principal investigator (CG).

Core-needle biopsy assessments

Core-needle biopsies were performed at baseline and four months. Participants were randomized in a 1:1 ratio to undergo the tissue biopsies from either the left or the right submandibular gland. Biopsies were taken from the same gland on each visit. Head and neck expert pathologists (assessor 1 and 2) evaluated tissue samples on hematoxylin-eosin (H&E)-stained slides. Changes in fibrosis, inflammation, and gland structure were reported. The pathologists were blinded to the times of tissue sample collection and the patient treatment allocations.

Tissue analyses

Initially, all sets of periodic acid-Schiff (PAS) and Pan Cytokeratin AE1/AE2 (CKAE1/AE2) stained serial tissue sections were automatically aligned with the TissueAlign functionality in Visiopharm Software (Copenhagen, Denmark). To assure consistent alignment, tissue regions were aligned individually (Fig. 2). First, the outer boundary of the glandular area was outlined by thresholding the brown feature (HDAB-DAB) of the CKAE1/AE2 slide. Artifacts, such as areas with tissue folds, were removed manually. Then, the final glandular ROI (blue dotted line in Fig. 2) was transferred to the PAS stained specimen.

Glandular areas within each ROI were classified by thresholding the intensity of the CKAE1/AE2 slide, which provided a ratio of glandular tissue (green in Fig. E1; available online at www.redjournal.org) to connective/adipose tissue. Finally, the ROIs in the PAS slide were classified into mucous acini, serous acini, adipose tissue, and connective tissue or ducts. The classification of serous acini, adipose tissue, and connective tissue or ducts was based on the red intensity; the mucous acini were classified based on an H&E-stain feature (Fig. E2; available online at www.redjournal.org). The areas of mucous acini, serous acini, adipose tissue, and connective tissue/ducts were calculated from these classifications.

Statistics

For salivary flow rates, patient-reported outcome measures, and tissue type analysis we evaluated the changes from baseline to the one- and four-month follow-up visits. Within-group comparisons were performed with the Wilcoxon signed-rank test, and between-group comparisons were performed with the Mann-Whitney U test. We chose to use non parametric statistics as the data were not normally distributed, evaluated by Shapiro-Wilks tests.

For MRI scans, the acquired images of each visit were registered with MATLAB and mutual information. In the aligned T1 images of each participant, the manual contours of both submandibular glands were designated regions of interest (ROIs). Then, averages were computed. The visible tissues in the T1 and T2 images were normalized by scaling across time, excluding the submandibular glands. In the T1 and T2 weighted scans, we compared the ADCs and the intensity changes across time, within each subject for the left and right sides. Furthermore, we compared the sizes of the glands across time and between groups. We used the two-sided t test to assess group differences and the linear discriminant analysis (LDA) to classify the two groups.

For salivary gland function, we determined whether the inorganic whole saliva composition behaved as physiologically
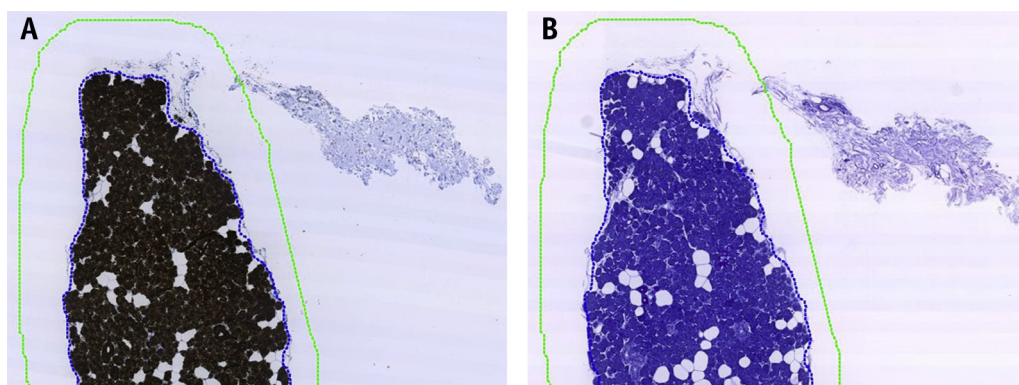

Fig. 2. Assessment of gland tissue in micrographs of serial sections. A: CKAE1/AE2-stained tissue section. B: PAS-stained tissue section. The alignment region is outlined in green; the glandular region of interest (ROI) is outlined with a blue dotted line. Scale: 2.5x. (A color version of this figure is available at www.redjournal.org.)

expected, upon stimulation. Thus, unstimulated concentrations of sodium, potassium, phosphorus, calcium, and magnesium were compared to the corresponding stimulated values for the same ions. We expected that sodium would increase upon stimulation and that potassium, phosphorous, calcium, and magnesium would decrease within this relatively short time of stimulation [17]. "Physiologically normal" was defined as a flow-dependent, significant change in the expected direction for the element in question. "Physiologically subnormal" was defined as saliva in which one or more ions behaved physiologically abnormally. "Physiologically abnormal" was defined as saliva in which more than half the ions behaved physiologically abnormally. Pearson's χ^2 test was used to evaluate the percentage distribution of ions that behaved physiologically normally versus those that behaved physiologically abnormally in the two groups.

Procedures and preparation of adipose tissue-derived mesenchymal stem cellsAdipose tissue was harvested under local anesthesia from the abdomen of each participant with standard sterile liposuction techniques. ASCs were expanded in culture, with good manufacturing practice (GMP)-grade reagents in a certified laboratory, approved by the Danish National Board of Health for clinical stem cell expansion, as previously described [11, 18]. In brief, mononuclear cells were isolated from adipose tissue digested in 60 ml collagenase (N002880, Serva). The cells were seeded in 5-layer cell culture flasks (PFHYS1008, Merck) at $10 \times 10^6/cm^2$ cell density in Dulbecco's modified Eagle's media (22320022, Gibco) supplemented with 10% pooled human platelet lysate (produced, as previously described, [18]), 2 IE/ml heparin (741827, Amgros I/S), and 1000 U/ml penicillin-streptomycin (15140-122, Gibco). Cell culture flasks were placed in humidified tissue culture incubators at 37°C, 5% CO_2. Plastic-adherent ASCs were expanded for 14 to 16 days. Cell culture supernatants were replaced every 4 to 5 days. On the day of harvest, the ASCs (P_0) were visually inspected for signs of deterioration, such as granularity, cytoplasmic vacuoles, and detachment from the plastic surface. When visually cleared, cells were washed with PBS without calcium or magnesium (14190-169, Gibco) and harvested with Tryple Select (12563-029, Gibco). Following neutralization with media, cells were washed twice with isotonic NaCl (0.9 mg/ml) supplemented with 1% human albumin (109697, CSL Behring) by pelleting ($300 \times g$ for 5 minutes). Cell count and viability were assessed with an NC-100 Mammalian Cell Counter. Only preparations that exhibited >50% of the calculated cell dose and >85% viability were processed further. Cell doses that corresponded to 2.8×10^6 ASCs × the volume of the gland (cm^3) were loaded into 1 ml syringes, which were subsequently masked for blinding purposes.

During development of the ASC manufacturing protocol, ASCs were characterized for transplantation suitability, in accordance with the criteria set by the joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) [19]. ASCs were released for transplantation, according to the following criteria: (1) Absence of pathogen contamination in a sample taken one week prior to harvest; (2) ASC viability greater than 85%; and (3) morphology characteristic of ASCs, based on observations with a reverse microscope. The cell dose or placebo (isotonic NaCl supplemented with 1% human albumin) was injected into the submandibular glands with ultrasound guidance.

Randomization

Participants underwent randomization in a 1:1 ratio (www.randomization.com). Only specified personnel that handled the cell expansion procedure in the Cell Therapy Facility, Department of Clinical Immunology, Rigshospitalet, were allowed access to the randomization table. Therefore, these individuals handled the blinding. All other personnel (eg, the P.I.) were blinded to the treatment allocation until study completion.

Sample size calculation

The sample size determination was based on a non-paired t test. We aimed to determine a minimum of 50% change in salivary flow rate. The standard deviation (SD) of a salivary flow rate measurement could be minimized to about 20%, with the spitting method, in healthy participants and under completely standardized conditions. Because the SD can vary greatly, we used an SD value based on published medical literature [20], measured in individuals with reduced unstimulated whole salivary flow rates (from 0.00 to 0.20 ml/min) and/or hyposalivation (<0.10 ml/min); among these groups, the relative SD was 58%. For this study, we were interested in comparing the change in scores (change from baseline to after treatment) between the intervention and placebo groups. Therefore, we applied the rule of thumb that the SD of the change score should equal $SD/2^{0.5}$, or approximately 41%. We set the power $(1-\beta)$ to 90%. We set the significance level (α) to 0.05. This trial evaluated a continuous response variable from independent control and experimental research units, with one control per experimental research unit. For a true difference of 50% in the mean change scores between experimental and controls, we needed to include 15 participants in both the experimental intervention and control groups. This sample size was sufficient to reject the null hypothesis that the population means of the experimental and control groups were equal, with a study power of 90%. The Type I error probability associated with this test of the null hypothesis was 0.05.

Results

Between 1 May 2015 and 7 April 2017, 87 participants were screened for eligibility to enter the study (Fig. 3). Of
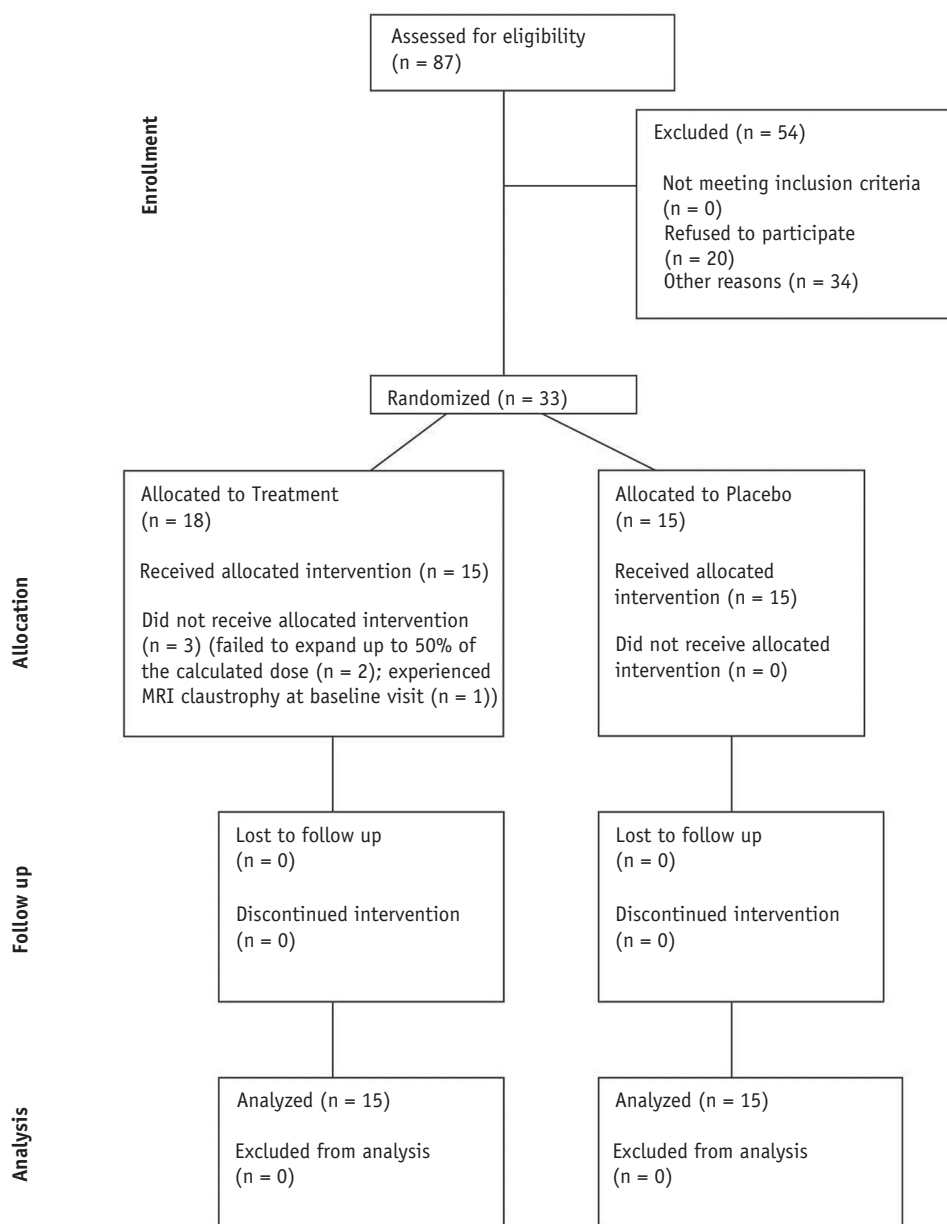
Fig. 3.   CONSORT flow-diagram of patient selection and allocation.

these, 33 participants met the inclusion and exclusion criteria. Eighteen participants were allocated to the ASC-arm and 15 to the placebo-arm. Three participants from the ASC-arm were withdrawn from the trial before intervention, due to technical constraints or lack of compliance (Fig. 3). Thus, 30 participants entered this trial, with a median age of 60.4 years (male:female, 19:11) and a median interval of 4.1 years (SD 1.1 years) after completing radiation treatment. Twenty-seven participants had previously received radio- and chemotherapy, and three had received radiotherapy alone. Baseline patient characteristics are reported in Table 1. No adverse events occurred within the first four months following administration. Nine of the 15 participants in the ASC-arm were followed for at

least one year with no adverse events. The remaining six participants in the ASC-arm are currently awaiting the one-year follow-up visit (to be completed within the next four to seven months). No participants have been lost to follow-up.

Thirty-three participants entered the trial. Three participants failed to complete (Fig. 3). One patient was excluded due to claustrophobic issues during the MRI-procedure. One patient had an insufficient number of MNC/ml isolated, leading to very low cell yield at the scheduled day of harvest. For the last patient, premature cell growth arrest occurred combined with a subtle change of morphology toward a more flattened and granulated state. In both instances, no deviation from standard protocol was registered, and contamination status was negative. In addition, the age

of both patients was well within the age range of all enrolled patients. Thus, as such we have no discernible explanation for the ASC isolation/expansion failure, which could be related to previous disease, treatment, or both.

Salivary flow rates

At baseline, the ASC-arm had a mean unstimulated whole saliva (UWS) flow rate of 0.12 ml/min (95% CI 0.08-0.17); the placebo-arm had a mean UWS of 0.16 ml/min (95% CI 0.12-0.21) (Table E1; available online at www.redjournal.org). In the ASC-arm, the UWS increased by 33% at one month ($P = .04$) and 50% at four months ($P = .003$; Figs. 4A and 4B) compared with baseline. In the placebo group, the UWS decreased by 5% at 1 month and increased by 0.5% at four months ($P = .76$ and $P = .68$, respectively; Figs. 4A and 4B). The net scores for UWS were similar between the two groups ($P = .81$). The other measures of salivary flow rates were unchanged from baseline to four months.

Assessment of patient-reported outcomes

We also evaluated whether the scores on the questionnaires changed significantly. In the ASC-arm, the patient-reported VAS questionnaire scores did not change significantly, except for the domain regarding thirst, which decreased by 22% at four months ($P = .035$; Figs. E3 and E4; available online at www.redjournal.org). Also, in the ASC-arm, the xerostomia questionnaire score of oral dryness decreased by 2% for the domain addressing solid foods ($P = .008$; Fig. E5; available online at www.redjournal.org). In the placebo-arm, no significant changes in the oral dryness scores were observed in either the VAS scores or the xerostomia questionnaire.

Inorganic saliva composition

At baseline, 40% of participants in the ASC-arm exhibited a physiologically normal secretory function, compared to 60% in the placebo-arm. At the four-month visit, 100% of participants in the ASC-arm exhibited normalized secretory function, based on the inorganic saliva composition, compared to 80% in the placebo-arm ($P < .001$; Fig. E6; available online at www.redjournal.org).

Magnetic resonance imaging (MRI) assessment

Neither the ADC nor the T1 and the T2 sequences were significantly different between the ASC-arm and the placebo-arm. The LDA classification produced a random classification (50/50) in the leave-one-out cross-validation.

Histologic assessment

Nineteen core-needle samples taken at baseline and at the four-month visit were evaluable by the blinded head and neck pathologists (assessors 1 and 2). The remaining eleven biopsies were not suitable for evaluation (eg, due to lack of gland-tissue). In Table 1, the assessors' evaluations are available.

Tissue staining analysis

When assessing staining intensity on CKAE1/AE2 slides of tissue samples, at baseline and four months, we found a significant increase in serous gland tissue in the ASC-arm compared to the placebo-arm ($P = .038$). We also found a significant decrease in the amount of connective and adipose tissues in the ASC-arm compared to the placebo-arm ($P = .02$; Figure 5 and Fig. E7; available online at www.redjournal.org). We found no significant difference between the ASC- and placebo-arms in the fraction of mucinous to serous tissue, the fraction of mucinous tissue, the fraction of glandular tissue, or the fraction of adipose tissue.

Discussion

To our knowledge, this is the first randomized-controlled trial to assess the safety and efficacy of stem cell transplantation for treating radiation-induced salivary gland hypofunction and xerostomia. No adverse events were found in the primary study period. In the ASC-arm, we observed a significant increase in the unstimulated whole saliva flow rates and improvements in patient-reported outcomes, gland morphology, and saliva inorganic composition.

In the ASC-arm, the combination of improving saliva flow by 50% and enhancing gland function might explain the improvement in patient-reported outcomes. These improvements might contribute to reducing the incidences of oral infections, mucositis, and to some extent also dental decay, which increase rapidly at subnormal saliva flow rates (17).

The improvements in the inorganic saliva composition were more evident than the improvements in the saliva secretion rate, when the two arms were compared. In the salivary glands, the secretion rate is determined solely by acinar cells, and the ductal cells determine the inorganic saliva composition. It is unlikely that ASCs should affect ductal cells more than acinar cells; however, perhaps due to vascular impairment following radiation therapy, the effect on the ducts became more apparent than the effect on the acini. When blood passes through the salivary glands, it becomes the source of primary saliva formation in the acinar lumen. Radiation therapy-induced reductions in flow of blood to the affected regions, including blood vessels in neighboring and surrounding regions, may

Table 1   Characteristics of study participants, treatment allocation, and doses

ID	Sex	Age at intervention	Primary cancer treatment (modality)	Time from radiation-therapy to study intervention (years)	Submandibular gland size (cm^3)		Dates of current study interventions	
					Left	Right	Liposuction	ASC/placebo
1	M	55.4	RTC	5.2	11.8	4.6	05-08-15	17-08-15
2	M	63.6	RTC	4.7	8.9	4.7	09-09-15	21-09-15
3	M	63.0	RTC		4.0	6.8		
4	M	68.5	RTC	6.0	10.0	3.5	04-11-15	17-11-15
5	M	60.4	RTC	6.5	4.5	5.3	25-11-15	07-12-15
6	F	46.1	RTC	6.0	3.0	2.5	02-12-15	15-12-15
7	M	70.9	RTC					
8	F	60.1	RTC	4.1	4.3	3.2	09-12-15	22-12-15
9	F	58.9	RTC	5.5	2.5	2.0	12-01-16	27-01-16
10	M	55.5	RTC	4.4	17.2	7.4	16-02-16	02-03-16
11	M	61.0	RTC	4.1	10.8	2.8	05-01-16	20-01-16
12	F	55.6	RTC	4.9	6.0	12.8	23-02-16	08-03-16
13	M	61.8	Only RT	4.1	8.9	11.4	01-03-16	16-03-16
14	M	67.8	RTC	3.3	15.5	9.9	29-03-16	13-04-16
15	M	71.1	RTC	4.0	15.9	2.5	05-04-16	20-04-16
16	F	56.5	Only RT	3.9	2.3	4.5	12-04-16	27-04-16
17	M	55.8	RTC	3.6	6.0	4.6	03-05-16	18-05-16
18	F	65.7	RTC	3.0	9.0	6.0	25-05-16	07-06-16
19	M	66.7	RTC	2.8	12.0	n/a	11-05-16	24-05-16
20	M	42.5	RTC	2.7	5.0	9.5	15-06-16	28-06-16
21	M	58.1	RTC					
22	F	61.6	RTC	2.9	4.5	8.5	11-08-16	26-08-16
23	M	67.5	RTC	2.7	9.0	5.5	18-08-16	30-08-16
24	M	69.3	RTC	5.4	9.0	14.0	26-08-16	09-09-16
25	M	59.2	Only RT	3.3	23.0	12.0	31-08-16	13-09-16
26	M	66.5	RTC	3.0	19.0	11.0	16-09-16	28-09-16
27	F	68.6	RTC	2.9	6.0	8.0	27-09-16	11-10-16
28	M	58.6	RTC	3.2	16.0	9.0	12-10-16	25-10-16
29	F	49.4	RTC	4.4	10.0	16.0	26-10-16	08-11-16
30	F	47.7	RTC	4.2	4.0	5.5	07-11-16	21-11-16
31	M	65.2	RTC	5.6	8.0	6.0	14-11-16	29-11-16
32	F	50.7	RTC	4.6	5.5	10.0	23-11-16	06-12-16
33	M	55.4	RTC	3.0	6.3	10.0	30-11-16	13-12-16

Abbreviations: ASC = adipose-derived mesenchymal stem cells; M = male; F = female; RTC = radio- and chemotherapy; Only RT = radiotherapy as single modality; P = placebo.

account for a maximal volume of saliva to be secreted, regardless of the effect of the stem cells. Another possibility is that stem cells preferentially migrate to the ductal compartment of the gland rather than the acinar compartment. It has been reported that the ductal compartment contains stem cells, and therefore the microenvironment surrounding the ducts may support stem cell migration toward the ducts (21). Nonetheless, treatments targeted at increasing the blood flow to the affected areas, including hyperbaric oxygen treatment, could potentially be used in combination with the stem cells in future studies.

Table 1　Characteristics of study participants, treatment allocation, and doses (*continued*)

ASC isolated ×10⁶	Left submandibular gland Mean Gy	Max Gy	Right submandibular gland Mean Gy	Max Gy	Site of core-needle biopsy	Pathologist assessment of HE-tissue slides Pathologist #1	Pathologist #2
P	23.6	49.9	64	68.4	Right gland	Not suitable for evaluation	
52	66.7	70.2	31.7	40.2	Right gland	Not suitable for evaluation	
Excluded						Not suitable for evaluation	
46	57.2	64.8	66.3	68.7	Left gland	No improvement	No improvement
P	67.6	70.3	69.2	71	Left gland	No improvement	No improvement
3.63	64.8	67.2	62.1	67.2	Left gland	Not suitable for evaluation	
Excluded						Not suitable for evaluation	
59	53.3	62.7	69.4	71.2	Left gland	Decrease in fibrosis	Decrease in fibrosis
33.3	63	68.6	69.3	71.3	Right gland	Not suitable for evaluation	
55.6	33.7	45.6	68.1	69.8	Left gland	No improvement	Decrease in fibrosis
P	25.6	33	66.8	69.8	Left gland	Not suitable for evaluation	
53.9	65.3	69.7	20.3	35.5	Right gland	Increase in gland tissue and decrease in fibrosis	No improvement
35.6	53.4	62.6	68.1	83.2	Right gland	Increase in gland tissue and decrease in fibrosis	Increase in gland tissue and decrease in fibrosis
P	21.9	35.4	60.6	69.5	Right gland	Decrease in inflammation	Increase in gland tissue and decrease in fibrosis
P	51.4	54.9	68.3	70.9	Left gland	Increase in gland tissue and decrease in fibrosis	Increase in gland tissue and decrease in fibrosis
P	66.9	69.2	41	53.1	Right gland	Increase in gland tissue and decrease in fibrosis	No improvement
P	56.2	68.3	68.5	70.5	Right gland	Increase in gland tissue and decrease in fibrosis	No improvement
P	49.5	52	no gland	no gland	Left gland	No improvement	No improvement
13.7	62.1	67.6	48.3	61.3	Right gland	No improvement	Not suitable for evaluation
P	25.5	39.1	64.3	68.2	Right gland	No improvement	No improvement
Excluded						Not suitable for evaluation	
P	36.7	56.2	66.7	68.7	Left gland	Not suitable for evaluation	
32.75	11.5	26	67.2	70.4	Right gland	No improvement	No improvement
P	64.9	66.5	35.4	43.6	Right gland	No improvement	No improvement
82	11.4	29.1	55.2	68.4	Left gland	No improvement	No improvement
P	31	42.5	61.8	67.7	Left gland	No improvement	No improvement
P	56.8	67.2	65.7	68.4	Left gland	Increase in gland tissue and decrease in fibrosis	No improvement
P	25.5	36.6	62	67.1	Left gland	No improvement	No improvement
55	64.8	68.7	21.5	50.7	Right gland	Increase in gland tissue and decrease in fibrosis	No improvement
70	65.5	68	57.2	67.6	Left gland	Not suitable for evaluation	
53	65.1	68.4	65.1	68.1	Right gland	Not suitable for evaluation	
P	66.7	68.9	64.7	68.7	Left gland	Not suitable for evaluation	
51	66.5	68.3	50.4	63.7	Right gland	Not suitable for evaluation	

We strove to standardize saliva testing by requiring patients to fast for 2 hours prior to all visits; moreover, all visits were performed between 2 PM and 3 PM, and supervised by the same individual (CG). The participants were tested for a total of 26 minutes, which was probably too extensive for the majority of patients; thus, all tests performed after 15 to 20 minutes were considered less controlled (eg, submandibular tests). Consequently, the specific submandibular test results were considered to be of questionable value, and this assumption was supported by the low flow rates recorded. The setup may have introduced some confounding, because cotton swabs were placed under the tongue. For example, the collection of saliva from the sublingual glands might have been confounded, to some
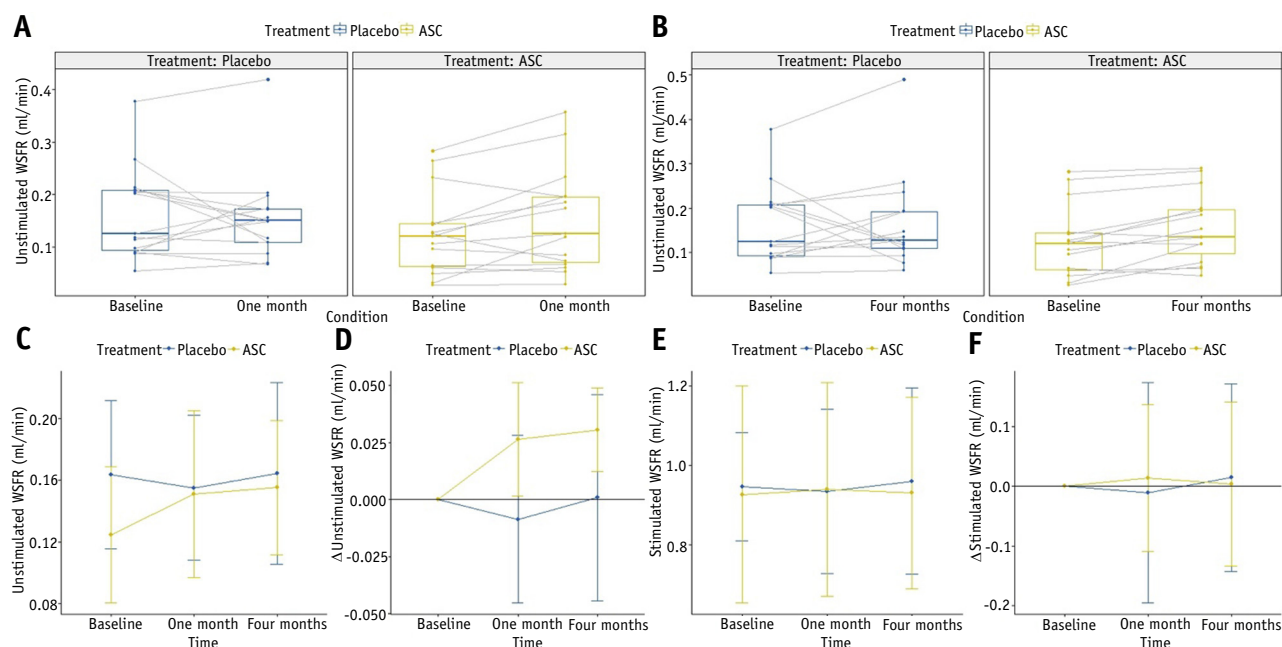
Fig. 4. Salivary gland flow rates in participants with radiation-induced xerostomia. Groups were treated with injections of adipose-tissue derived mesenchymal stem cells (ASCs, orange) or placebo (blue), and rates were compared (A) before treatment (baseline) and after one month and (B) at baseline and after four months. Mean saliva flow rates are shown, measured without (C-D) and with (E-F) stimulation, and mean changes (Δ) over time are plotted. WSFR: whole salivary flow rate, including all glands. (A color version of this figure is available at www.redjournal.org.)

extent, by the collection procedure (11). Notably, gland-invasive saliva collection is not recommended for this patient population, due to the vulnerable mucosa. Our study design with 30 study participants lacks power to perform a multivariate analysis of which factors determine outcome —for example, effect of treatment when adjusted for age, gender, size of salivary gland, etc. This issue is, however, especially important before the possible implementation of this treatment for a broader patient group.

The results from this trial were promising and should encourage future exploratory research. Several adjustments might improve the outcomes of future confirmatory trials. The 2-hour fasting window prior to testing should preferably be reduced to 1 hour to accommodate participants with hyposalivation. Furthermore, due to the significant variation in UWS, more precision could be achieved by increasing the number of flow-rate tests. Preferably, trials should include two baseline tests, and saliva tests should be conducted each month following the intervention, until month four. Also, in future trials, the number of ASCs per cm^3 gland could be increased. An important observation in the present study was that we observed no injection site
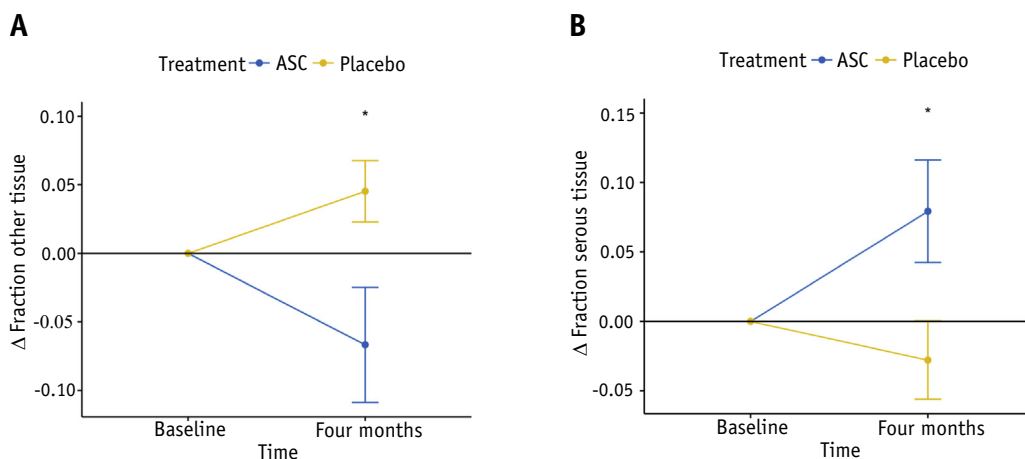

Fig. 5. Tissue analyses, based on PAS and CKAE1/AE3-staining. (A) Fraction of other tissue (connective tissue). (B) Fraction of serous tissue. * = Significant different.

reactions or short-term adverse events (up to 1 year). Also, it is well known that the submandibular gland is responsible for approximately two thirds of the unstimulated whole saliva flow rate, and therefore, it is the most important gland for decreasing morbidity associated with hypo-salivation. The administration of ACSs to the parotid glands, in addition to the submandibular glands, could further improve total saliva production. During stimulation, the parotids become much more active, and their saliva contribution is equal to that contributed by the subman-dibular glands. Finally, to enhance the effect of ASCs, cell administration may be repeated after three months.

MRI scans are useful for assessing gland morphology, but morphology is merely a surrogate marker for gland function. Instead, it might be relevant to include scintig-raphy to address salivary gland function (22, 23) as well as measurement of the intra glandular arterial blood flow by Doppler ultrasound to ensure that the glands receive suffi-cient blood. Our MRI protocol could be optimized to improve the resolution of the acquired images, both spatially and directionally. The resolution and extreme degree of anisotropy in the voxel size may introduce excess variability in the signal, due to partial volume effects and a single diffusion direction. Our suspicion that we lacked a clear signal in the data was supported by the LDAs, which were inadequate for distinguishing between the two groups. Of note, the manner of performing the biopsy might also influence the image data. Interestingly, we found a signif-icant reduction in connective tissue in the ASC-arm compared to the placebo-arm. This suggested that the ef-fect of ASC treatment might be due to an improved envi-ronment surrounding the glands—for example, a reduction in fibrous tissue.

Salivary gland tissue responds to radiotherapy promptly, and cell apoptosis occurs at even low radiation doses. Multiple pathways could lead to salivary gland dysfunction following irradiation (24); acute damage occurs likely due to damage to the plasma membrane of the secretory cells, depressing stimulated watery secretion (25, 26), and late damage to glands is likely due to apoptosis of the pro-genitor cells and thereby the inability to replace secretory cells (27). Although little is known on the progenitor/stem cells in salivary glands, studies have indicated that the basal cells may contain the potential progenitor/stem cell for the striated and excretory ducts whereas acinar cells and intercalated duct cells undergo self-proliferation (27). Additionally, late effects might also compromise the extracellular environment preventing proper cell func-tioning; here amongst decreased blood flow and hampering of relevant growth factors, cytokines, extracellular- and anti-apoptotic proteins (27). The possible mechanism of action of ASCs on these damages is most likely through a supportive and paracrine function exhibiting anti-apoptosis, immunomodulation, angiogenesis, anti-scarring, and sup-port of growth and differentiation of stem and progenitor cells (28). Whether or not ASCs actually engraft or act as a hit-and-run phenomenon is of great debate (29). A

standardized proper in vivo surveillance, however, has yet to be defined. In this respect, the ASC defining surface markers are less useful since they are defined as a co-existence of three markers (as well as absence of hemato-poietic markers), of which none are specific for ASCs and found on a variety of other cells. Thus, demonstration of only one surface marker, as would be possible in a biopsy, would not prove the presence of ASCs. In addition, a negative finding could reflect either sample variation or differentiation into mature cells without ASC markers. Therefore, we chose not to include these analyses in the present study.

In conclusion, this randomized, placebo-controlled trial assessed the safety and efficacy of stem cell transplantation for treating radiation-induced salivary gland hypofunction and xerostomia. We found that, in a small population, ASC treatment was safe and exhibited promising efficacy on both objective and patient-reported outcomes.

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