A Pilot Study to Assess the Salivary Gland Regenerative Potential of Bone Marrow Mesenchymal Stromal Cells from Treated Head and Neck Cancer Patients

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Research

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Abstract

Background: Salivary dysfunction is a significant side effect of radiation therapy for head and neck cancer (HNC). Preliminary data suggests that mesenchymal stromal cells (MSCs) can improve salivary function. Whether MSCs from HNC patients who have completed chemoradiation are functionally similar to those from healthy patients is unknown. We performed a pilot clinical study to determine whether bone marrow-derived MSCs (BM-MSCs) from HNC patients could be used for the treatment of RT-induced salivary dysfunction.

Methods: An IRB-approved pilot clinical study was untaken on HNC patients with xerostomia who had completed treatment two or more years prior. Patients underwent iliac crest bone marrow aspirate and BM-MSCs were isolated and cultured. Culture expanded BM-MSCs were stimulated with IFNγ and cryopreserved prior to reanimation and profiling for functional markers by flow cytometry and ELISA. BM-MSCs were additionally injected into mice with radiation-induced xerostomia and the changes in salivary gland histology were examined.

Results: A total of six subjects were enrolled. BM-MSCs from all subjects were culture expanded to >20 million cells in a median of 15.5 days (range 8-20 days). Flow cytometry confirmed that cultured cells from HNC patients were BM-MSCs. Functional flow cytometry demonstrated that these IFNγ-stimulated BM-MSCs acquired an immunosuppressive phenotype. IFNγ-stimulated BM-MSCs from HNC patients were found to express GDNF, WNT1, and R-spondin 1 as well as pro-angiogenesis and immunomodulatory cytokines. In mice, IFNγ-stimulated BM-MSCs decreased the loss of acinar cells decreased the formation of fibrosis.

Conclusions: BM-MSCs from previously treated HNC patients can be expanded for auto-transplantation and are functionally active. Furthermore, IFNγ-stimulated BM-MSCs express proteins implicated in salivary gland regeneration. This study provides preliminary data supporting the feasibility of using autologous BM-MSCs from HNC patients to treat RT-induced salivary dysfunction.

Trial Registration: NCT04007081, Registered July 5, 2019, https://clinicaltrials.gov/ct2/show/NCT04007081

Introduction

Salivary dysfunction is a side effect of radiation to the head and neck region that affects more than 40% of all patients with head and neck cancer (HNC)(1). Salivary dysfunction can be due to hyposalivation (decreased salivary production) or alterations in sialochemistry (pH, electrolyte, and protein content). Patients with radiation-induced salivary dysfunction often experience xerostomia (the subjective sensation of dry mouth). Salivary dysfunction in HNC patients leads to increased dental caries, impaired swallowing ability, difficulty speaking, and diminished taste. These complications can be severely detrimental to a patient's overall quality of life. Current treatment options for radiation-induced xerostomia are supportive in nature: carrying a water bottle, consumption of specially prepared food, use

of salivary substitutes, chewing gum, sugar-free mints, and pilocarpine (2–5). These supportive interventions do not reverse the causes of xerostomia and are palliative in nature highlighting the critical need for improved therapies.

Mesenchymal stromal cells (MSCs) have been shown to promote tissue healing and regeneration in a variety of injurious settings(6). These healing effects may derive from the MSC secretome, which has broad immunomodulatory and trophic activity(7, 8). WNT1 and glial cell line-derived neurotrophic factor (GDNF) have been shown to be key drivers of adult salivary gland stem cells, allowing for expansion and restoration of function(9, 10). Several groups have demonstrated in preclinical systems, that injecting MSCs into salivary glands results in a 32%-200% increase in salivary flow(11–16). There have been no preclinical studies on IFNγ stimulated BM-MSCs for radiation-induced xerostomia in the murine model. There has been one trial to date examining autologous adipose-derived MSCs to treat radiation-induced xerostomia in humans: the MESRIX study demonstrated increased salivary flow rates at early time points and improved patient reported salivary function (17).

The MESRIX trial did not characterize the secretome profile of the adipose-derived MSCs utilized, and no group has described the functionality of bone marrow-derived MSCs (BM-MSCs) from patients treated with radiation for HNC. Multiple clinical studies have demonstrated that the radiation and chemotherapy regimens given to patients with hematological malignancies can damage the bone marrow and affect MSC function; however, no studies have been done in patients treated for HNC(18–20). Additionally, the MESRIX trial investigated the effectiveness of adipose MSCs only at 1 and 4 months – as xerostomia is a chronic issue, later endpoints may be pertinent. Preliminary studies suggest that MSCs do not linger in the salivary glands, their tissue healing effects may instead be due to their secretome. Thus, multiple injections of MSCs may be needed to effectively treat chronic radiation-induced xerostomia. One way to accomplish this is by expanding MSCs and cryopreserving them until clinically needed, which can result in cellular injury, less effective immunomodulation, or senescence(21–23). Stimulating BM-MSCs with interferon gamma (IFNγ) prior to cryopreservation has been shown to prevent this immune dysfunctionality (22–24). We investigated this method of IFNγ stimulation for the BM-MSCs from HNC patients to inform a future phase I clinical trial. Additionally, we investigated the role of these IFNγ stimulated BM-MSCs in the murine model of xerostomia, providing preclinical support of a future phase I clinical trial.

In preparation for a first-in-human study utilizing IFNγ stimulated BM-MSCs, we performed this pilot study to verify the ability of BM-MSCs obtained from previously treated HNC patients to be ex vivo expanded and to assess the functionality and secretome of IFNγ stimulated BM-MSCs.

Methods And Materials

BM-MSCs Isolation and Culture

After institutional review board approval (UW HSB IRB#19009, ClinicalTrials.gov: NCT04007081) and informed consent, BM-MSCs were isolated from 10 to 25 mL of bone marrow aspirated from the iliac crest of patients previously treated for HNC with symptomatic xerostomia. Bone marrow from healthy adult control volunteers were purchased from a commercial source (AllCells, Alameda, CA). We had originally planned to enroll 15 patients: 5 patients with HNC who underwent chemoradiation, 5 patients with HNC who underwent radiation therapy alone, and 5 HNC patients who had not yet received treatment for their disease. After enrolling 6 patients, the COVID-19 pandemic caused a pause in trial enrollment. An unplanned interim analysis of the BM-MSCs from the 6 patients with HNC (4 chemoradiation, 2 radiation alone) did not reveal any significant differences between these BM-MSCs and the healthy control BM-MSCs and the protocol was amended to end enrollment.

BM-MSCs isolation and culture were undertaken as previously described(25). Briefly, bone marrow aspirates were diluted 1:2 with phosphate-buffered saline and layered onto a Ficoll density gradient. Cells were centrifuged at 400 xg for 20 minutes with no acceleration or brake, and the mononuclear cells were then collected and plated in complete human MSC medium (MSC NutriStem XF Basal Medium, 5% human platelet lysate (MillCreek PLT MAX), 1% GlutaMAX, 2U/mL heparin, and 100 U/mL penicillin/streptomycin) at 100,000 to 300,000 cells/cm^2. Non-adherent hematopoietic cells were removed by changing the medium after 3 days of culture, and BM-MSCs were allowed to expand for 7-15 days. Thereafter, cells were passaged 1-3 times weekly by treatment with TrypLE select and reseeded in fresh complete MSC medium at 3,000-5,000 cells/cm^2. BM-MSCs were counted at each passage using a Countess™ automated cell counter (Invitrogen™ Grand Island, NY). Doubling time was calculated by log2 × $\Delta t/\log(n_h) - \log(n_1)$.

IFNγ Stimulation

Recombinant IFNγ was reconstituted to 200 µg/mL and added to complete MSC medium to a final concentration of 1,200 IU/mL. When BM-MSCs reached the desired number for cryopreservation, complete MSC medium was aspirated from flasks and IFNγ-supplemented medium was added to all but the negative control flask. BM-MSCs were then cryopreserved 24 +/- 2 hours later.

Cryopreservation of BM-MSCs

BM-MSCs were counted and resuspended in Plasma-Lyte A at a concentration of $5x10^6$ cells/mL. Cryopreservation medium was prepared (40% Plasma-Lyte A, 40% human serum albumin, 20% DMSO) and chilled. Five hundred µL of cryopreservation medium and 500 µL of BM-MSCs were placed in each cryovial, and the cryovials were placed in a ThermoScientific™ Mr. Frosty™ Freezing container at -80°C for 24 hours. The cryovials were then transferred to vapor-phase liquid nitrogen storage.

Phenotyping BM-MSCs by Flow Cytometry

BM-MSCs were thawed from cryopreservation and plated in complete MSC medium for 24 hours. BM-MSCs were then harvested and resuspended at a concentration of 1 x 10^6 cells/mL and analyzed by flow cytometry for the expression of CD73, CD90, CD105, CD14, CD20, CD34, and CD45 with appropriate

isotype controls using the Miltenyi MSC Phenotyping Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). See Supplemental Table 1 for complete antibody details. All HNC MSC samples were run on an Attune NxT flow cytometer. Data are presented as normalized median fluorescent intensity compared with isotype control and as histogram overlay.

Functionality of BM-MSCs by Flow Cytometry

Both IFNγ stimulated and non-IFNγ stimulated HNC BM-MSCs were thawed from cryopreservation and plated in complete MSC media for 24 hours. BM-MSCs were then harvested and resuspended at a concentration of 1 x 10^6 cells/mL and analyzed by flow cytometry for the expression of MHC I, MHC II, IDO, ICAM-1, and PD-L1 (Miltenyi Biotec, Bergisch Gladbach, Germany; BioLegend, San Diego CA; eBioscience, San Diego CA), see Supplemental Table 1 for complete antibody details. All samples were run on an Attune NxT flow cytometer with the appropriate isotype controls (ThermoFischer Scientific, Waltham, MA). Data are presented as normalized median fluorescent intensity.

ELISAs and Cytokine Multiplex

Cryopreserved IFNγ BM-MSCs were thawed and plated at a concentration of 1 x 10^6 cells/well in 12 well plates with 1 mL complete MSC media. The supernatant from the BM-MSCs was collected at 24 hours. Enzyme-linked immunosorbent assays (ELISAs) of WNT1 (MyBioSource WNT1 ELISA Kit), GDNF (Human GDNF ELISA Kit, Invitrogen ThermoFisher Scientific), and R-spondin 1 (MyBioSource R-spondin 1 ELISA Kit) were run according to the manufacturer's instructions. The ELISAs were read on a SpectraMax i3 plate reader (Molecular Devices San Jose, CA), and the protein concentrations were interpolated from curves constructed from the protein standards and their respective median fluorescence intensity (MFI) readings. A multiplex immunoassay was used to determine the concentrations of 30 cytokines in the MSC supernatant (Human Cytokine Magnetic 30-Plex Panel, Invitrogen ThermoFisher Scientific) following the manufacturer's instructions. The multiplex was read on the MAGPIX System (Millipore), and the protein concentrations were interpolated from curves constructed from the protein standards and their respective median fluorescence intensity (MFI) readings (Milliplex Analyst, Millipore).

Mouse Care and Irradiation

Six- to eight-week-old male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME) and the animal study was approved by the Institutional Animal Care and Use Committee (IACUC) at University of Wisconsin-Madison.

Mice were irradiated on salivary glands with 15 Gy using Xstrahl Small Animal Radiation Research Platform (SARRP, Xstrahl, UK) one day before surgery. Mice were anesthetized in a chamber with 3-5% isoflurane at 1-2L/min O_2. Mice were then moved to a flatbed with a nosecone within the SARRP and maintained with 1-3% isoflurane for the duration of treatment. Using the MuriPlan software, a Cone-Beam CT image was acquired with the X-ray tube operating at 60 kV and 0.8 mA with aluminum filtration and a protocol was established for administering a total of 15 Gy split between two beams at 90 and -90

degrees to equally affect both salivary glands. Delivery of 15 Gy dose for irradiation was applied by operating at 220 kV and 13 mA with copper filtration. The dose rate was 2.68 Gy/min.

One day after radiation, mice were randomized to treatment groups, anesthetized with isoflurane, and a small incision was made in the skin of the ventral neck of each mouse followed by subcutaneous blunt dissection. The salivary glands were located and then human BM-derived MSCs ($1x10^6$ cells in 50 ul PBS) were injected into each gland for BM-MSC treated groups. Both control and radiation groups were injected with only PBS. The skin was closed using wound clips. Clips were removed in 14 days once the incision has healed. Submandibular salivary gland tissue was harvested at the end of study, 3 months after injection.

Immunohistochemistry and Staining

Salivary gland tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin blocks. Five-µm sections of salivary gland were stained with hematoxylin and eosin (H&E), alcian blue (ALB), and Massons trichrome (MTC) and imaged on an Olympus BX41 microscope (Olympus America, Inc). For ALB staining, formalin-fixed paraffin embedded samples were deparaffinized with Xylene and hydrated through graded solutions of ethanol up to distilled water. Deparaffinzed slides were stained in alcian blue solution (Acros Organics CAS# 33864-99-2) for 30 minutes and counterstained with 0.1% nuclear fast red solution (Acros Organics CAS# 6409-77-4) for 5 minutes. Slides were then dehydrated through 95% alcohol and xylene solutions and mounted with Cytoseal (Thermo Scientific #8312-4). For MTC staining, formalin fixed paraffin embedded slides were deparaffinized and rehydrated. Hydrated slides were fixed in Bouin's solution (RICCA #5860-16, Alfa Aesar #33314) for 1 hour at 56°C and rinsed in running tap water. Slides were then stained with Weigert's iron hematoxylin working solution (Electron Microscopy Sciences (EMS) #26109-1A, EMS # 26102-1B) for 10 minutes and rinsed in running tap water. Slides were then stained with Biebrich scarlet-acid fuchsin solution (EMS #26033-25) and washed with DI water. Slides were differentiated with phosphomolybdic-phosphotungstic acid solution (EMS #26364-01) for 15 minutes. Slides were then transferred directly into aniline blue solution (Acros Organics CAS# 28983-56-4), rinsed in DI water, and differentiated in 1% acetic acid solution for 3 minutes. Slides were washed in DI water, dehydrated through 95% alcohol and xylene, and mounted using Cytoseal (Thermo Scientific #8312-4).

In addition, each salivary gland was stained for a-Amylase (Ab125230, Abcam, 1:1000 dilution). For in vivo immunohistochemistry, 5 µm sections from formalin fixed paraffin embedded samples were deparaffinized with Xylene and hydrated through graded solutions of ethanol. Antigen retrieval was conducted in sodium citrate retrieval buffer (pH 6.0) followed by washing in running water. Slides were washed in PBS and then incubated with 0.3% hydrogen peroxide solution. Blocking was carried out using 10% goat serum in PBS and then incubated with the primary antibody, a-Amylase diluted in 1% goat serum in PBS containing 0.1% Triton X-100 overnight at 4°C. Slides were washed with PBS next day; secondary antibody was used (SignalStain Boost IHC Detection Reagent (HRP, Rabbit) CST #8114). Staining was detected using diaminobenzidine (Vector Laboratories, Inc. #SK-4100). The slides were counterstained with 1:10 hematoxylin (Termo Scientifc #TA-125-MH) solution for 2 min, then dehydrated

in ethanol and xylene solutions and sections were covered with coverslip with Cytoseal (Termo Scientifc #8312-4).

Statistical Analysis

Data are reported as mean +/- standard deviation or standard error, or median with range. Calculations were carried out using GraphPadPrism software (La Jolla, CA). Comparisons between groups were made by unpaired t-test between isotype and MSC using the False Discovery Rate approach with a two-stage step-up method and a Q=1%.

Results

We obtained bone marrow aspirates from six patients with HNC who had completed curative-intent therapy at least two years prior to enrollment. Four of the six patients underwent concurrent chemoradiation and two underwent radiation alone (Table 1). The patients had a median age of 65 (range 58-70). All six samples were obtained fresh (10 – 20 mL) with an average starting mononuclear cell count of 75 x 10^6 (range 7 x 10^6 - 180 x 10^6 cells). The BM-MSCs were expanded in culture prior to cryopreservation for a median of 16 days (range 8-20 days). Control BM-MSCs were purchased as frozen bone marrow from AllCells from 3 healthy adult donors, median age of 26. The BM-MSCs were isolated from an average starting mononuclear cell count of 94 x 10^6 cells and cultured as described above.

Table 1
Head and neck cancer diagnoses, curative therapies, and the treatment completion dates of the 6 patients enrolled in our study.

Subject	Diagnosis	Treatment	Treatment Completion
1	Stage IV (T3N2cM0) squamous cell carcinoma of the right base of tongue, p16+	Definitive chemoradiation 70 Gy with weekly cetuximab	2/2014
2	Stage IVA (T3N2cM0) squamous cell carcinoma of the right base of tongue, p16+	Definitive chemoradiation 70 Gy with weekly cisplatin	3/2014
3	Stage IVA (T1cN2cM0) squamous cell carcinoma of the right base of tongue, p16+	Definitive chemoradiation 70 Gy with weekly cisplatin	12/2014
4	Stage IVA (T3N2bM0) squamous cell carcinoma right tonsil and RMT, p16+	Definitive chemoradiation 70 Gy with weekly cisplatin	7/2014
5	Stage II (T2N0M0) squamous cell carcinoma right buccal mucosa	Adjuvant radiation 60 Gy	9/2014
6	Stage IVA (TxN2aM0) squamous cell carcinoma of unknown primary	Definitive radiation 64.8 Gy	7/2011

The BM-MSCs from HNC patients displayed the MSC phenotype ($CD73^+/CD90^+/CD105^+/CD14^-/CD20^-/CD34^-/CD45^-$) consistent with the minimal criteria for defining human MSCs by the International Society for Cellular Therapy (ISCT), with detectable expression of CD90, CD105, and CD73 ($p<0.05$) and lack of expression of CD14, CD20, CD34, and CD45. (Figures 1A-1B) compared to isotype control. Time from bone marrow harvest and plating to P0 was calculated and did not significantly differ between BM-MSCs from HNC patients and healthy control patients (Figure 1C). Doubling time of the HNC patients' BM-MSCs was 1.76 days.

After expansion, BM-MSCs from HNC patients were stimulated with IFNγ and cryopreserved for at least 5 days. The cryopreserved BM-MSCs were then thawed, and their growth curves were determined (Figure 2A). Each HNC patient's IFNγ-stimulated BM-MSCs were compared to their non-IFNγ stimulated cryopreserved BM-MSCs using flow cytometry. The IFNγ-stimulated BM-MSCs acquired an immunosuppressive phenotype with increased IDO, ICAM-1, PD-L1, MHC I, and MHC II compared to unstimulated BM-MSCs (Figures 2B). All markers except PD-L1 were significantly increased, $p<0.05$.

To assess the secretome of cryopreserved IFNγ stimulated BM-MSCs from HNC patients and healthy controls, we utilized ELISA assays. HNC BM-MSCs were found to express GDNF (mean 117.5 pg/mL, Figure 3A), WNT1 (mean 9.4 ng/mL, Figure 3B), and R-spondin 1 (mean 65.8 pg/mL, Figure 3C) at similar levels as BM-MSCs from healthy controls. Using a multiplex ELISA assay, cryopreserved IFNγ stimulated HNC and control BM-MSCs were also shown to express the pro-angiogenesis factors HGF, IL8 (CXCL8), FGF, and VEGF (Figure 3D), and immunomodulatory cytokines including IFNγ, IL12, MIG (CXCL9), and MCP1(CCL2) (Figures 3E and 3F).

To assess the pathohistological changes of IFNγ-stimulated BM-MSCs on radiated salivary glands, we injected IFNγ-stimulated BM-MSCs or PBS into the radiated salivary glands of mice. As expected, we found the salivary glands of mice injected with PBS after irradiation to have less amylase and mucin as well as increased fibrosis (Figure 4). In mice who were injected with IFNγ-stimulated BM-MSCs, there was less radiation damage observed both by H&E and decreased MTC staining of collagen fibrosis as compared to irradiated salivary glands. Additionally, injection of IFNγ-stimulated BM-MSCs allowed for increased levels of amylase and mucin expression – similar to unirradiated controls.

Discussion

In this study we verified that BM-MSCs of patients with HNC who underwent radiation (+/- chemotherapy) at least two years prior could be isolated, culture-expanded, cryopreserved, and culture-rescued. In addition, we confirmed that these BM-MSCs expressed key salivary stem cell morphogens and immunomodulatory cytokines consistent with a potential role in supporting salivary gland regeneration or downregulating a harmful inflammatory response. Our data demonstrate for the first time that functionally immunosuppressive BM-MSCs can be expanded from the bone marrow of patients with HNC who were treated with radiation (+/- chemotherapy), despite the potentially myelosuppressive effects of these treatments and the relatively advanced age of the average HNC patient.

We performed this study because several *in vitro* studies have demonstrated the damaging effects of chemotherapy and radiation on BM-MSCs (26, 27). Clinical studies have also shown the radiation and chemotherapy regimens given to patients with hematological malignancies can damage the bone marrow and affect BM-MSCs (18–20). In our study, we did not identify any negative effects of HNC treatment on BM-MSCs. This is likely due to the nature of the therapies for our patients. Radiation would have been directed at the head and neck regions, avoiding the majority of the MSC-producing marrow and the chemotherapy regimen was either cisplatin or cetuximab, both of which are much less myelosuppressive than the regimens typically used for patients with hematological malignancies. BM-MSCs from HNC patients were able to proliferate at a rate like those of healthy volunteers, despite having previously undergone curative treatments for HNC. Previous research by Copland et al. demonstrated the safety and potency of autologous MSCs from patients with acute graft versus host disease who had previously received high dose chemotherapy, resulting in FDA approval and clinical trial(28). This further supports the safety of autologous BM-MSCs in subjects previously exposed to cytotoxic chemotherapy.

We then undertook assays to examine the growth and function of these BM-MSCs after cryopreservation. Cryopreservation is a useful tool to allow the expansion of large numbers of BM-MSCs that can be frozen and thawed on an as-needed basis for clinical trials – particularly those that might require repeated MSC injections. There are some potential drawbacks to cryopreservation; the freeze/thaw cycle can result in cellular injury or senescence impairing the functional abilities of BM-MSCs(22, 23, 29). Prior studies have shown stimulation of BM-MSCs with IFNγ prior to cryopreservation can prevent cellular dysfunction(22–24). In our study we demonstrated that BM-MSCs from HNC patients can be successfully cryopreserved and culture-rescued while retaining appropriate function. BM-MSCs can exert their beneficial effects through modulation of the immune microenvironment and/or through the provision of tissue-promoting morphogens.

Interestingly, several studies on patients who underwent myeloablative chemotherapies for multiple myeloma found their MSCs to have impaired immunomodulatory activity(30, 31). Additionally, cryopreservation has been shown to result in less effective immunomodulation(21–23). Here we demonstrated that IFNγ stimulated BM-MSCs from HNC patients have an immunosuppressive phenotype with robust growth after thawing.

MSCs can also limit cell death and encourage repair and expansion of the tissue's resident stem cells through the release of tissue-promoting morphogens(6). We examined the paracrine factors expressed by BM-MSCs to determine if the BM-MSCs from treated HNC patients secrete restorative cytokines. We found production of GDNF, WNT1, and R-spondin 1 by the IFNγ stimulated BM-MSCs from HNC patients, suggesting these BM-MSCs can provide factors which are crucial to the function of adult stem cells in glandular tissues(9, 10, 32). The stem cell niche in gut epithelium utilizes WNT and R-spondin 1 signaling to allow for the renewal of adult stem cells(32–34). Maimets et al. has also shown WNT signaling to be a key driver of adult stem cells in the salivary glands(9). Further, GDNF has also been identified as an important chemokine in the adult salivary stem cell(10). Our research demonstrates that the secretome of IFNγ stimulated BM-MSCs from HNC patients contains key promoters for adult salivary stem cells. IFNγ

stimulated BM-MSCs additionally secreted pro-angiogenesis factors such as VEGF, FGF, HGF, and IL8 (CXCL8), supporting the pro-regeneration effects of the MSC secretome. Finally, we found IFNγ stimulated BM-MSCs from HNC patients to release immunomodulatory cytokines and chemokines, consistent with the known effects of BM-MSCs to influence their immune microenvironment to be pro-regeneration and anti-inflammatory, and suggestive of the potential for BM-MSCs to be used during radiation to prevent inflammatory damage(35–37). MSCs affect the innate immune system, including monocytes via MCP1/CCL2, which we found at high levels (36). Additionally, we found low production of MIG/CXCL9 which is pro-inflammatory, similar to the levels found in co-culture of MSCs and monocytes by Magatti et al, again supporting the anti-inflammatory effects of BM-MSCs(38).

This study was limited by the small number of HNC patients enrolled. Our goal was to ensure that the BM-MSCs from HNC patients met minimal criteria for phenotype and function per ISCT criteria, which was able to be met with our six biological replicates (21). Additionally, this study examined BM-MSCs, however the optimal tissue source for autologous cytokine-activated MSCs for salivary regeneration remains to be defined. Further research should be undertaken comparing the functionality and secretome of cryobanked bone marrow- and adipose-derrived MSCs. Our preclinical data examining the effects of IFNγ-stimulated BM-MSCs supports our hypothesis that these MSCs may restore salivary gland function after radiation.

The data presented here inform a clinical trial (NCT04489732) investigating the injection of autologous BM-MSCs into the salivary glands after radiation (+/- chemotherapy) as an innovative remedy to treat xerostomia and restore quality of life. This clinical trial will include a larger number of HNC patients which will allow us to better examine the immunosuppressive phenotype of IFNγ stimulated BM-MSCs and the secretome that these cells produce.

Conclusion

Based on these data we conclude that BM-MSCs from patients with HNC who underwent curative radiation (+/- chemotherapy) at least 2 years prior have similar expansion capabilities as BM-MSCs from healthy volunteers, express an immunosuppressive phenotype with IFNγ stimulation, and have an anti-inflammatory, pro-regeneration secretome. This work serves as a foundation for further studies examining the use of BM-MSCs from patients who underwent chemoradiation for HNC, including as a possible therapy for RT-induced salivary dysfunction.

Abbreviations

HNC – head and neck cancer

MSC – mesenchymal stromal cells

BM-MSC - bone marrow-derived MSCs

GDNF - glial cell line-derived neurotrophic factor

IFNg - interferon gamma

ELISAs - Enzyme-linked immunosorbent assays

MFI - median fluorescence intensity

IACUC - Institutional Animal Care and Use Committee

SARRP - Small Animal Radiation Research Platform

H&E - hematoxylin and eosin

ALB - alcian blue

MTC - Massons trichrome

Declarations

Approvals:

This study was approved by the University of Wisconsin Health Sciences Institutional Review Board (#19009). All animal work was approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Data Availability:

The datasets used during the current study are available from the corresponding author on reasonable request. The data are not publicly available due to privacy or ethical restrictions.

Consent for publication:

Not applicable.

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Author Contributions:

GCB was involved in all aspects of conception, design, collection and assembly of data, and manuscript writing. RJM was involved in collection of bone marrow aspirates. AP, RM, JG OG, and JG were involved in culture and analysis of MSCs. CP, AG, OG, JG, AMB, KPN, ZSM were involved in flow cytometry and cytokine analysis. TL, DJR, SLT, CP, AG, and KPN were involved in animal studies. RC was involved in conception, study design, and statistical analysis of the data. NMR and TAG were involved in patient accrual and study design. GCB, JG and RJK provided financial support. RJK was involved in all aspects of the work and manuscript writing. All authors provided final approval of the manuscript.

Conflicts of Interest:

RJK receives research support through the University of Wisconsin from Bridge Bio that is unrelated to the work presented here. The authors have no additional conflicts of interest to disclose.

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Figures

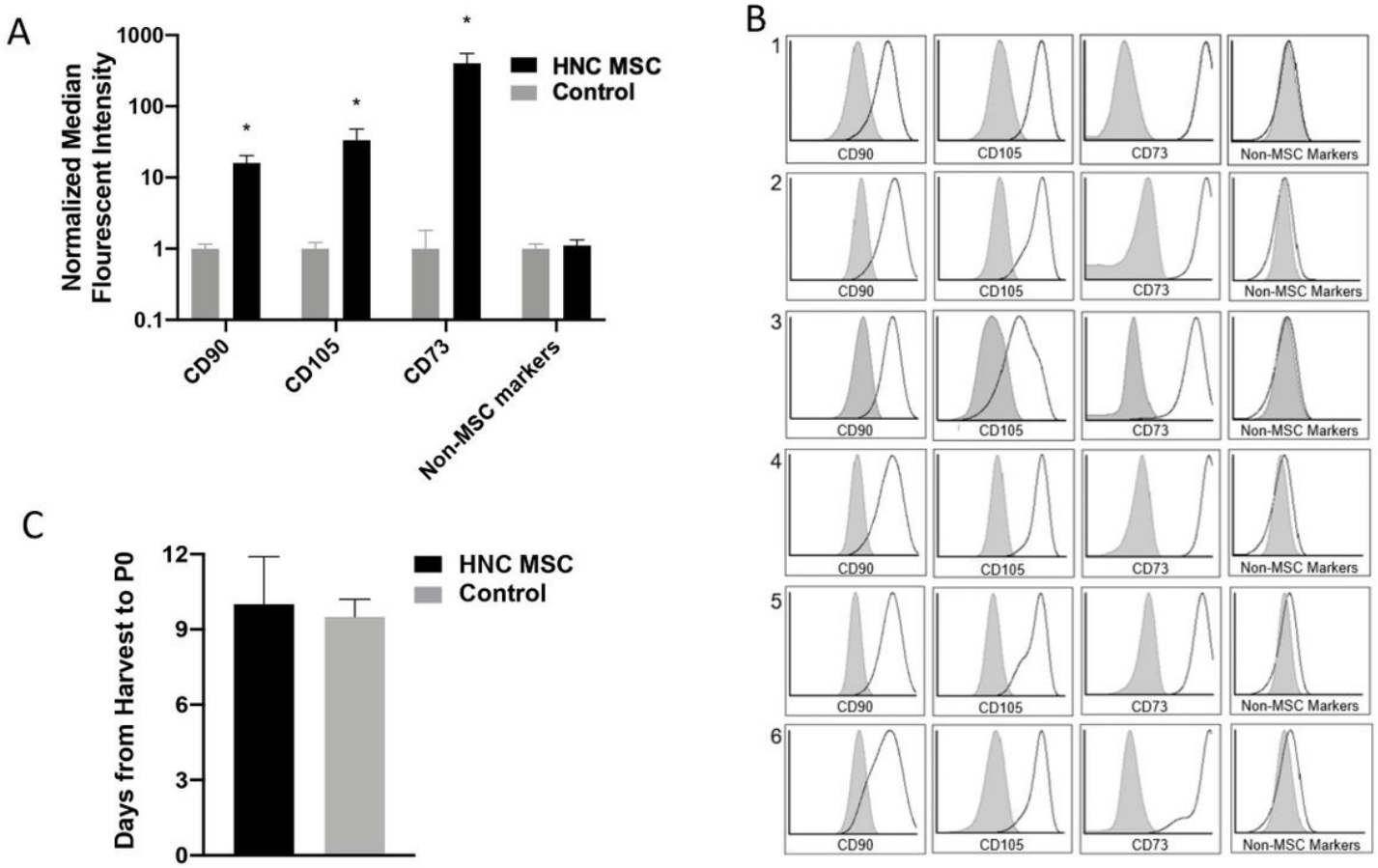

Figure 1

Characterization of bone marrow derived mesenchymal stromal cells (BM-MSCs) from patients with head and neck cancer (HNC). A) Graph of the normalized median fluorescent intensity of classical markers of HNC BM-MSCs. There was a significant increase of CD90, CD105, CD73 as compared to isotype control (*p=<0.01), with no significant difference in the median fluorescent intensity between MSCs and isotype control for the non-MSC markers (CD14, CD20, CD34, and CD45). B) Histograms of the 6 HNC BM-MSCs showing the expression of classical markers (CD90, CD105, and CD73) and no expression of non-MSC Markers (CD14, CD20, CD34, and CD45) (BM-MSCs shown as black outline, isotype control shown as

gray curve) C) Duplication time of BM-MSCs from HNC patients (mean of 10 days) and healthy controls (mean of 9.5 days, p=ns).

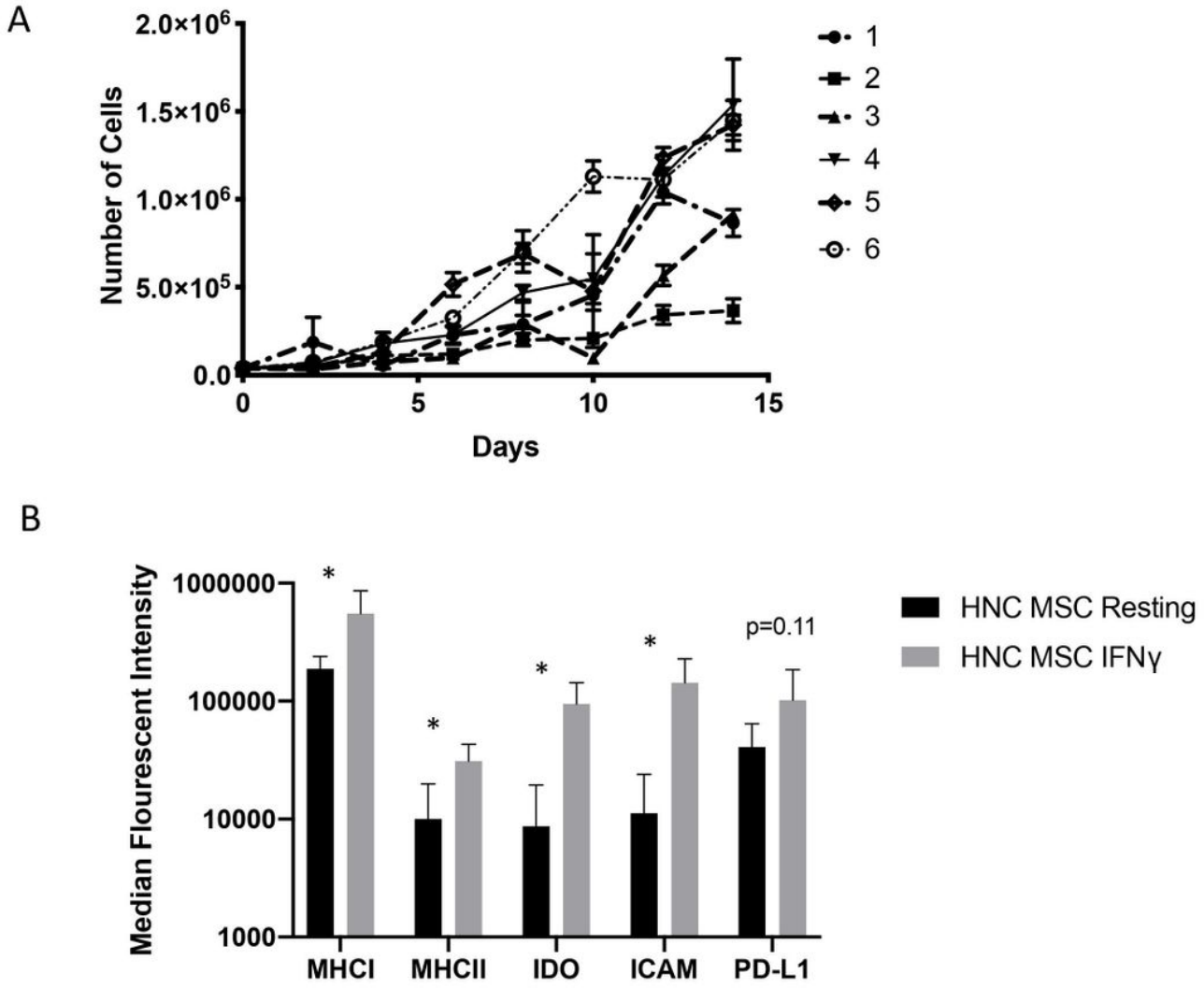

Figure 2

Interferon gamma (IFN□□□stimulated bone marrow derived mesenchymal stromal cells (BM-MSCs), growth and function. A) Growth curve of the 6 HNC MSCs after IFN□ stimulation, cryopreservation, and thawing showing variable speeds of continued growth over two weeks. B) Median fluorescent intensity (MFI) of immunosuppressive functional markers of head and neck cancer patients' MSCs with and without IFN□ stimulation. There is a significant increase in MHC I, MHC II, IDO, and ICAM-1, with a trend in increased PD-L1 expression without statistical significance, all MFIs are median with standard error bars, *p<0.05.

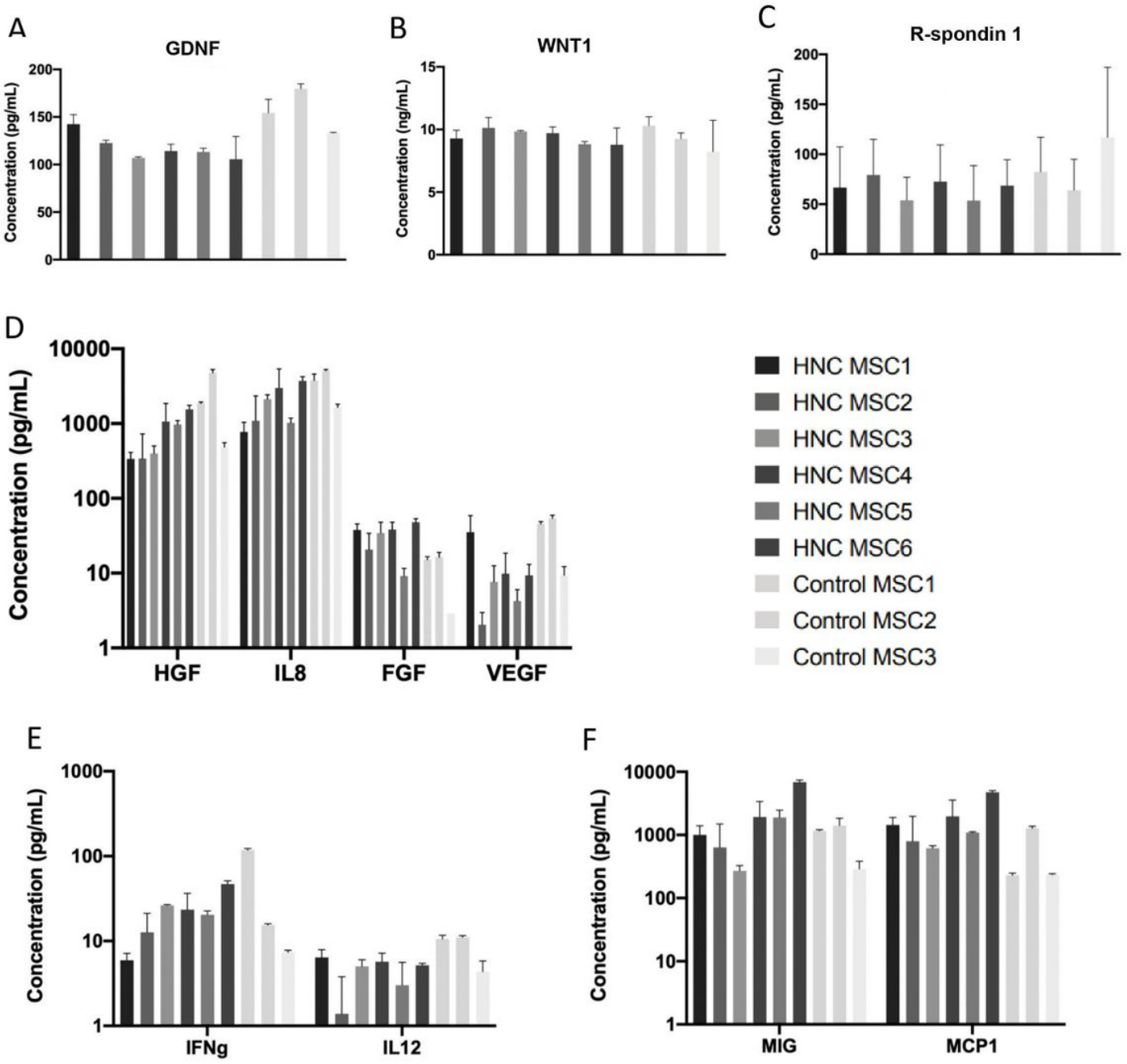

Figure 3

Cytokine expression of IFNγ stimulated bone marrow derived mesenchymal stromal cells (BM-MSCs) from patients with treated head and neck cancers and healthy donors after cryopreservation and culture rescue. A) GDNF expression, mean GDNF for HNC MSCs was 117.5 ± 15.9 pg/mL (standard deviation 15.9 pg/mL) mean GDNF for healthy donors was 155.7 ng/mL (standard deviation 21.6 ng/mL). B) WNT1 expression, mean WNT1 was 9.4 ng/mL (standard deviation 0.8 ng/mL, mean WNT1 for healthy donors was 9.3 ng/mL (standard deviation 1.6 ng/mL). C) R-spondin 1 expression, mean R-spondin 1 was 65.8 pg/mL (standard deviation 29.7 pg/mL), mean R-spondin 1 for healthy donors was 87.8 pg/mL (standard deviation 48.1 pg/mL). D) Pro-angiogenesis cytokines, means reported for all MSCs: HGF 1310.9 pg/mL, mean IL8 2464.0 pg/mL, mean FGF 24.8 pg/mL, and mean VEGF 19.7 pg/mL. E) General

immunomodulatory cytokines, means reported for all MSCs. Mean IFN 30.6 pg/mL, mean IL12 5.9 pg/mL. F) Immunomodulatory cytokines specifically affecting monocytes, means reported for all MSCs. Mean MIG 1715.5 pg/mL, mean MCP1 1386.6 pg/mL.

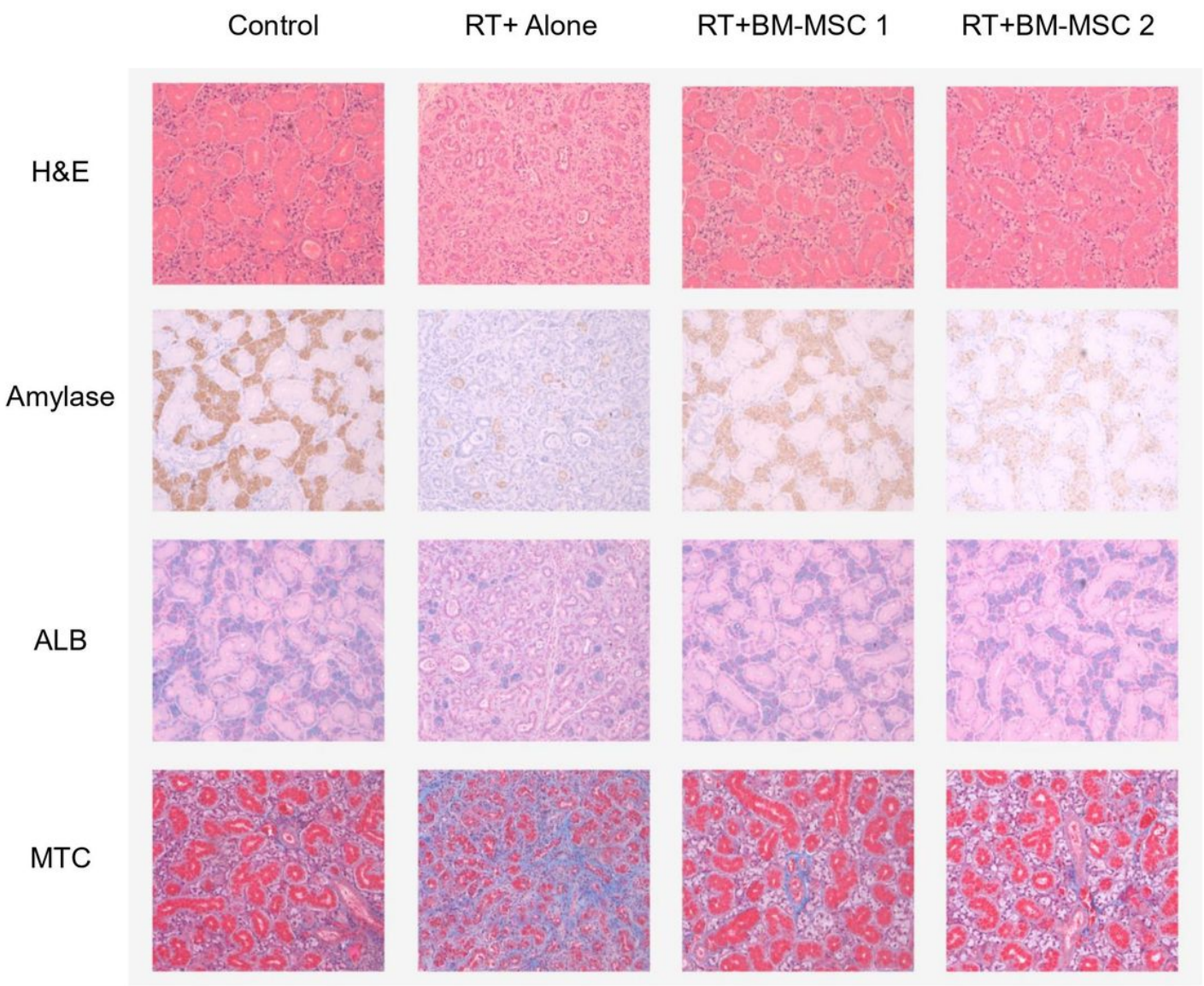

Figure 4

Murine submandibular glands at 20x magnification. Mice were irradiated with a single dose of 15 Gy radiation directly to the submandibular gland. One day after radiation the two treatment groups of mice received injections of 1x106 IFNγ stimulated bone marrow derived mesenchymal stromal cells (BM-MSCs) to the submandibular glands and the control and radiation (RT) only groups received PBS injections. The submandibular glands were harvested and prepared as detailed in methods 3 months after radiation. ALB – Alician blue stains mucin, MTC – Massons tri-chrome stains collage fibers.

Supplementary Files

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