Cell Extracts Mitigate Irradiation-injured Salivary Glands

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ABSTRACT

Salivary hypofunction, also known as xerostomia, is a major side effect of radiotherapy for head and neck cancer patients. Current treatments of irradiation (IR)-injured salivary glands (SGs) remain palliative. Our previous studies showed that mouse bone marrow cell extract (BMCE) could restore the secretory function of IR-injured SGs and that native proteins in BMCE were the main effective ingredients for the treatment. However, BMCE therapy requires multiple injections and protein denaturation is a concern during BMCE storage. The first aim of this thesis was to preserve, by lyophilization (freeze-drying), the bioactive factors in BMCE. We developed a method to freeze-dry BMCE and then to analyze its ingredients and functions in vivo. Results showed that the presence of angiogenesis-related factors and cytokines in freeze-dried (FD) BMCE remained comparable to those found in fresh BMCE. Both fresh and FD-BMCE restored comparably saliva production, protected salivary acinar cells, parasympathetic nerves, and blood vessels, increased cell proliferation, upregulated regenerative/repair genes in the irradiation-damaged salivary glands. Lyophilization of BMCE maintained its bioactivity and therapeutic effect on irradiation-injured salivary glands.

A mouse bone marrow cell extract could restore salivary secretory function to irradiation-injured mice. However, the components and therapeutic effects of human BMCE remain unknown. In addition, the whole bone marrow consists of heterogeneous cells, including red blood cells (RBCs), granulocytes (GCs), and mononuclear cells (MCs). This increases the complexity of the cell extract and consequently, increases the difficulties in identifying and analyzing the effective proteins in the cell extract. The second aim of this thesis was to test the therapeutic effect of the cell extract

from human bone marrow (BMCE) and to test whether the effect observed could be from a specific cell sub-population on IR-injured SGs. Results showed that BMCE and cell extract of RBCs, GCs and MCs (RBCE, GCE, MCE) contained various proteins/growth factors. MCE treatment provided the best therapeutic effect, and BMCE therapy could also restore the function of SGs, while RBCE treatment did not improve the secretory production when compared with the negative control group.

Studies suggest that tissue-specific cells may be more suitable for treating their original tissues/organs. Furthermore, the procedure of BM cell harvesting remains invasive and in severe cases, could even lead to life-threatening complications. Therefore, the third objective of this thesis was to test if human labial gland stem cells (LSCs) could be used as an alternate source of cell extract for the repair of IR-injured SGs. Results demonstrated that several angiogenesis-related factors were detected in LSCE. LSCE treatment restored 50%-60% of saliva secretion, protected acinar cells, blood vessels and parasympathetic nerves, promoted cell proliferation, and up-regulated the expression of tissue repair/regeneration proteins and genes.

In summary, lyophilization is a reliable approach to preserve the bioactive ingredients of BMCE in a powder form for long-term storage. Human BMCE, GCE and MCE successfully restored IR-induced salivary hypofunction and MCE provided the best therapeutic effect. LSCE was also an effective treatment in repairing irradiated-SG in the mouse model. These treatments showed the clinical potential to mitigate irradiated SGs.

RÉSUMÉ

L'hypofonctionsalivaire, également appelée xérostomie, est un effet secondaire majeur de la radiothérapie pour le cancer de la tête et du cou. Les traitements actuels des glandes salivaires (SG) blessées par irradiation (IR) restent palliatifs. Nos études précédentes ont montré que l'extrait de cellules de moelle osseuse de souris (BMCE) pouvait restaurer la fonction sécrétoire des SGs lésées par IR et les protéines natives dans BMCE sont les principaux ingrédients efficaces pour le traitement. Cependant, la thérapie BMCE nécessite plusieurs injections et la dénaturation des protéines est une préoccupation pendant le stockage BMCE. Le premier objectif de cette thèse était de préserver, les facteurs bioactifs du BMCE par lyophilisation. Nous avons développé une méthode pour lyophiliser le BMCE et analyser ses ingrédients et ses fonctions in vivo. Les résultats ont montré que la présence des facteurs liés à l'angiogenèse et de cytokines dans le BMCE lyophilisé (FD) restait comparable à celles trouvées dans le BMCE frais. Les produits frais et FD-BMCE ont restauré une production de salive comparable, des cellules acineuses salivaires protégées, des nerfs parasympathiques et des vaisseaux sanguins, une prolifération cellulaire accrue, des gènes de régénération / réparation régulés à la hausse dans les SGs endommagées par IR. La lyophilisation du BMCE a maintenu sa bioactivité et son effet thérapeutique sur les SGs lésées par IR.

Un extrait de cellules de moelle osseuse de souris pourrait restaurer la fonction de SG des souris lésées par IR. Cependant, les composants et l'effet thérapeutique du BMCE humain restent inconnus. En outre, la moelle osseuse entière est constituée de cellules hétérogènes, y compris les globules rouges (globules rouges), les granulocytes (GC) et les cellules mononucléaires(MC). Il

augmente la complexité de l'extrait cellulaire et entraîne des difficultés pour identifier et analyser les protéines efficaces dedans. Le deuxième objectif de cette thèse était de tester l'effet thérapeutique de BMCE et de tester si l'effet observé pouvait provenir d'une sous-population cellulaire spécifique sur des SG lésées par IR. Les résultats ont montré que le BMCE et l'extrait cellulaire des globules rouges, GC et MC (RBCE, GCE, MCE) contenaient diverses protéines / facteurs de croissance. Le traitement MCE a fourni le meilleur effet thérapeutique, les thérapies GCE et BMCE ont également pu restaurer la fonction des SG, tandis que le traitement RBCE n'a pas amélioré la production sécrétoire par rapport à celui du groupe témoin négatif.

Des études suggèrent que les cellules spécifiques aux tissus peuvent être plus adaptées au traitement de leurs tissus / organes d'origine. Et la procédure de récolte des cellules BM reste invasive et pourrait entraîner des complications potentiellement mortelles dans les cas graves. Par conséquent, le troisième objectif de cette thèse était de tester si les cellules souches de la glande labiale humaine(LSC) pouvaient être utilisées comme source alternative d'extrait cellulaire pour la réparation des SG lésées par IR. Les résultats ont démontré que plusieurs facteurs liés à l'angiogenèse ont été détectés dans LSCE. Le traitement LSCE a restauré 50% à 60% de la sécrétion de salive, protégé les cellules acineuses, les vaisseaux sanguins et les nerfs parasympathiques, favorisé la prolifération cellulaire et régulé à la hausse l'expression des protéines et des gènes de réparation / régénération tissulaire.

En résumé, la lyophilisation est une approche fiable pour conserver les ingrédients bioactifs du BMCE sous forme de poudre pour un stockage à long terme. Le BMCE, le GCE et le MCE humains ont réussi à restaurer l'hypofonction salivaire induite par l'IR et le MCE a fourni le

meilleur effet thérapeutique. LSCE était également un traitement efficace pour réparer le SG irradiée dans le modèle de souris. Ces traitements ont montré un potentiel clinique pour atténuer les SGs irradiées.

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CONTRIBUTIONS OF AUTHORS

This thesis includes two published papers and an unpublished manuscript of which the doctoral candidate is the first author. Contributions of all authors are provided as below:

Chapter 2

Su X, *Fang D, Liu Y, Ruan G, Seuntjens J, Kinsella JM, Tran SD. Lyophilized bone marrow cell extract functionally restores irradiation-injured salivary glands. Oral Dis. 2018; 24:202–206.*

The candidate designed and performed all experiments and data analysis, and wrote the manuscript.

Fang D assisted in experiment design and proof-read the manuscript.

Liu Y assisted in designing and performing experiments.

Ruan G assisted in designing experiments.

Seuntjens J conducted the animal irradiation and assisted in designing experiment and proof-read the manuscript.

Kinsella JM assisted in designing experiment.

Tran SD designed and supervised the whole study, and wrote and final approved the manuscript.

Chapter 3

Su X*, Liu Y, Elkashty OA, Seuntjens J, Tran SD. Cell Extract from Human Bone Marrow Mitigates Irradiation-injured Salivary Glands. -In preparation*.

The candidate designed and performed all experiments and data analysis, and wrote the manuscript.

Liu Y assisted in designing and performing experiments.

ElKashty OA assisted in designing experiments.

Seuntjens J conducted the animal irradiation and assisted in designing experiment and proof-read the manuscript.

Tran SD designed and supervised the whole study, and wrote and final approved the manuscript.

Chapter 4

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The candidate designed and performed all experiments and data analysis, and wrote the manuscript.

Y. Liu assisted in designing and performing experiments.

M. Bakkar assisted in performing experiments.

O. ElKashty assisted in designing experiments.

M. El-Hakim provided the human samples.

J. Seuntjens conducted the animal irradiation and assisted in designing experiment and proof-read the manuscript.

S.D. Tran designed and supervised the whole study, and wrote and final approved the manuscript.

ABBREVIATIONS

3D	Three-dimensions
Ach	Acetylcholine
Ad5	Adenovirus type 5
AdhAQP1	Adenoviral vector encoding human aquaporin-1
alpha-MEM	Minimum Essential Medium Eagle-Alpha Modification
ANOVA	Analysis of variance
AQP5	Aquaporin-5
BCA	Bicinchoninic acid
BM	Bone marrow
BMC	bone marrow cell
CD11b, 24, 29, 34, 44, 49f, 90, 117	Cluster of differentiation 11b, 24, 29, 34, 44, 49f, 90, 117
CK5	Keratin 5
CMC	Carboxymethyl cellulose
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DT	Doubling time
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FD	Freeze drying

FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Granulocyte
GDNF	Glial cell line-derived neurotrophic factor
GFRα2	GDNF family receptor alpha-2
GM-CSF	Granulocyte- macrophage colony-stimulating factor
Gy	Gray
HA	Hyaluronic acid
HEC	Hydroxyethyl cellulose
HGF	Hepatocyte growth factor
HPMC	Hydroxypropyl methylcellulose
HSG	Human ductal epithelial salivary gland
IGF-1	Insulin-like growth factor-1
IL-1ra, 6, 10	Interleukin- 1ra, 6, 10
IMRT	Intensity-modulated radiotherapy
IR	Irradiation
KGF	Keratinocyte growth factor
LG	Labial gland
LSC	Labial stem cell
LSCE	Labial stem cell extract
MC	Mononuclear cell
MMP	Matrix metallopeptidase
MSC	Mesenchymal stem cell

NaCl	Sodium chloride
NGF	Nerve growth factor
OPN	Osteopontin
PAG	Parotid gland
PAI-1	Plasminogen activator inhibitor-1
PAS	Periodic acid-Schiff
PBS	Phosphate buffered saline
PCNA	Proliferation cell nuclear antigen
PDL	Periodontal ligament
PF4	Platelet factor 4
PGM	Polyglyceryl methacrylate
PLGA	Poly lactic-co-glycolic acid
PTX3	Pentraxin 3
qRT-PCR	Quantitative real-time polymerase chain reaction
RBC	Red blood cell
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAA	Serum amyloid A
Sca-1	Stem cell antigen-1
SDF-1	Stromal cell-derived factor-1
SDF-1	Stromal cell-derived factor-1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFR	Salivary flow rate
SG	Salivary gland

sIgA	Secretory immunoglobulin A
SLG	Sublingual gland
SMG	Submandibular gland
TF	Transfer factor
TGF-β1	Transforming growth factor beta 1
TREM-1	Triggering receptor expressed on myeloid cells-1
VEGF	Vascular endothelial growth factor
α-SMA	α-Smooth muscle actin

Chapter 1 – Introduction

Salivary gland and saliva

Salivary glands

Human salivary glands (SGs) are the indispensable exocrine organs that produce saliva to maintain the homeostasis in the oral cavity. SGs consist of three pairs of major salivary glands (parotid, submandibular, and sublingual glands) and hundreds (600-1000) of minor salivary glands[1]. 90% of saliva is secreted from the major salivary glands and less than 10% is from minor salivary glands[2-5]. Major SGs play a vital role in most of the salivary secretion, while a large number of minor salivary glands contribute to lubricating the mucosa as they dispersedly distribute throughout the mucosa in palatal, buccal, lingual and labial tissues [6, 7]. SGs can also be classified as serous, mucous or seromucous(mixed) types according to the histochemical nature of the secreted saliva. Human parotid glands (PAGs) strictly contain pure serous acinar cells, submandibular glands (SMGs) are seromucous (compose both serous and mucous acinar cells), and sublingual glands (SLGs) mainly comprise of mucous acinar cells. Most minor SGs are mixed glands, except the palatine glands (strictly mucous) and lingual von Ebner's glands (strictly serous) [8]. During the rest, the majority of saliva composition is mainly contributed by serous glands (70% for SMGs, 26% for PAGs and 5% for SLGs)[9].

SGs each consist of acinar cells, myoepithelial cells, a duct network and complex neural and blood supply. Acinar cells are classified into serous and mucous acini according to their secretion[1]. Serous saliva contains a high level of amylase, ions and water. Mucous saliva produced by mucous SGs contains large amounts of mucins, immunoglobulins, lysozymes and salivary acid

phosphatase. Viscous saliva plays a role in protective mechanisms of oral mucosa and enamel surface. Specifically, it can prevent the physical surface barrier over the oral mucosa, and also forms the dental biofilm in the tooth surface to reduce surface-colonizing microorganisms[1].

All of the salivary fluid synthesized by acini is secreted into the duct complex. There are three different types of ducts, which are known as intercalated, striated and excretory ducts [1]. Intercalated ducts directly connect to the acinar secretory portions and receive the primary saliva. Several studies reported that the progenitor cells are located in the intercalated ducts and could differentiate into acinar, myoepithelial and ductal cells [1, 10]. Striated ducts, which connect an intercalated duct to an interlobular duct, contribute to modify the isotonic saliva to hypotonic saliva by mediating the ionic changes. The interlobular excretory ducts collect saliva and continue to reabsorb sodium and potassium, then finally transport the saliva into the oral cavity. The ducts in different types of salivary glands vary. For example, the ducts in major SGs are long and branched, whereas the minor salivary glands have shorter tracts and several small ducts directly spread to the oral mucosal surface [1].

Myoepithelial cells, as a kind of specialized smooth muscle cells, surround the secretory units between the acinar basement membrane and mesenchyme, and support the parenchymal cells in SGs[1, 11-13]. They are essential for the salivary secretion due to their contractile property. They mediate the secretory pressure, reduce the luminal volume in glandular endpieces, and consequently accelerate salivary flow [12, 14]. Besides the primary contractile role, myoepithelial cells have proliferative capacity, tumour suppression and basement membrane production abilities [1, 12, 14, 15].

SGs are innervated by the autonomic nervous system, especially controlled by the parasympathetic and sympathetic systems [5, 16, 17]. Neurotransmitter stimulation plays an essential role in salivary secretion. Autonomic nerves mediate both the volume and composition of the saliva [1, 17, 18]. Specifically, HCO_3^- secretion and the increase of saliva volume are under the control of the parasympathetic system. For example, salivary secretion is mediated by muscarinic receptors which are activated by acetylcholine (Ach) released from efferent nerves. In addition, parasympathetic nerves are involved in salivary gland development and are associated with the branching morphogenesis [1]. On the other hand, the sympathetic stimulation increases the protein secretion in the saliva and decreases the production of saliva by decreasing the blood flow to the SGs.

The systemic circulation is important for salivary secretion. The external carotid artery provides the arterial blood supply to the major salivary glands. In addition, there are many capillaries and arterioles surrounding the secretory units. Studies showed that blood flow provides the water for salivary secretion through the tight junctions and aquaporin channels [1, 5]. When maximum salivary secretion is required, the blood flow could increase up to 20 folds under the control of parasympathetic vasodilator fibres [1, 19]. Furthermore, blood-based molecules could transport to the SGs by the transcellular pathway. Therefore, saliva had been reported as a diagnostic body fluid for many diseases, such as cancer [20, 21], oral diseases [22], kidney [23] and heart diseases [24, 25]. However, further investigation is required to unveil the role of the vasculature during the branching morphogenesis[1].

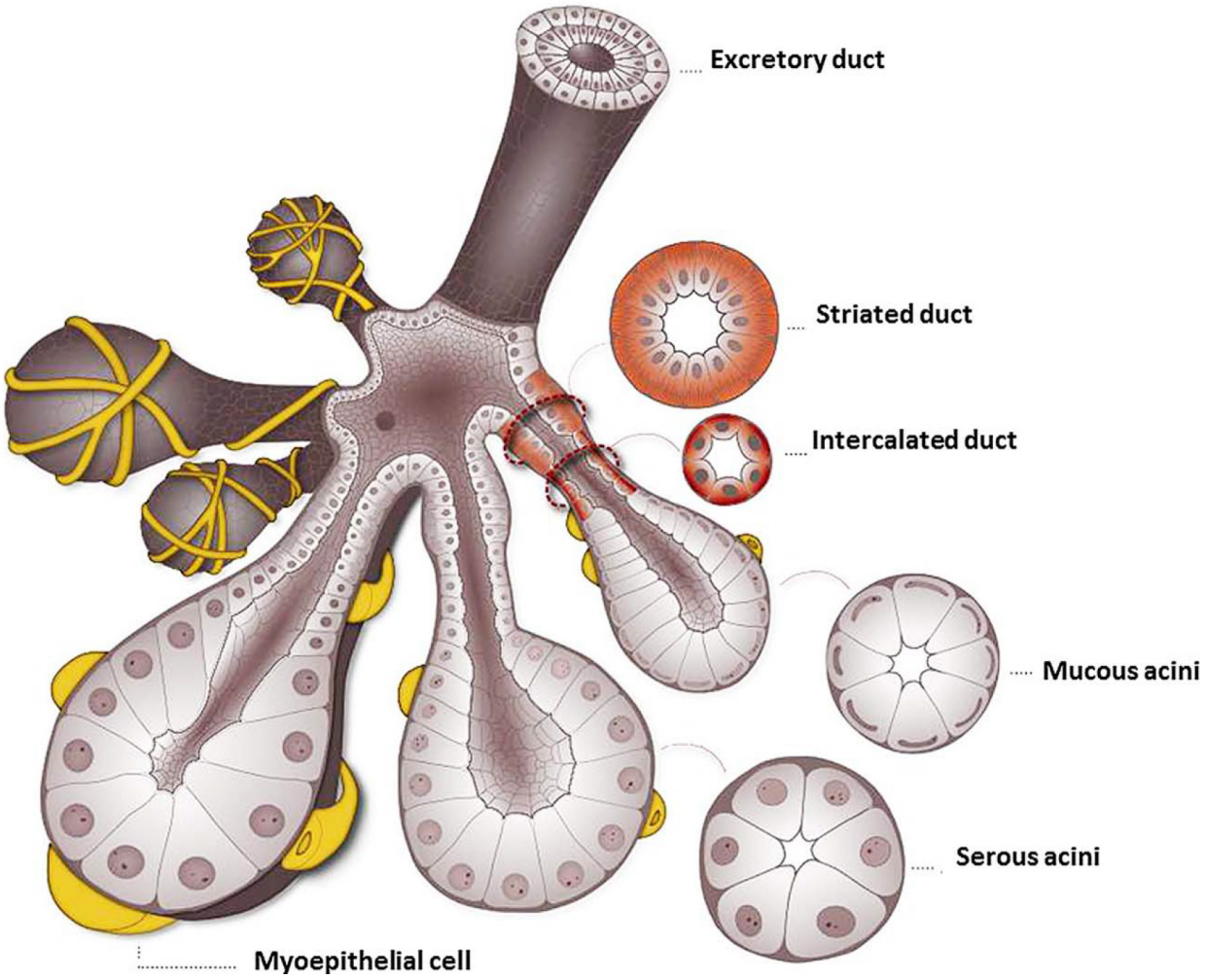

Figure 1. Schematic figure of the seromucous salivary gland composed of mucous and serous acini[1].

Saliva

Saliva is a complex body fluid composed of water (99.5%), organics (0.3%) and inorganic molecules (0.2%) [5, 19, 26]. The complex of organics in saliva includes mucoprotein, immunoglobulins, enzymes, urea, uric acid and maltase, while the inorganics components are Na^{+}, K^{+}, $Ca2^{+}$, Cl^{-}, HCO_3^{-} phosphate, minerals and gases (CO_2, O_2 and N_2) [5]. The pH of the normal

saliva is 6.0-7.0. The salivary flow rate is around 0.3-0.4ml/min at rest and up to 1.5ml/min during sensory, electrical or mechanical stimulations[19, 26]. Besides the saliva secreted from SGs, the whole saliva also contains desquamated epithelial cells, other cellular components, bacteria and their products, viruses, fungi, gingival crevicular fluid, mucosal transudate, nasal and expectorated bronchial secretions, as well as the blood derivatives of oral wound [26-31].

Salivary secretion is essential for overall health maintenance in humans. The multiple functions of saliva could be classified as follows: 1) digestive function 2) lubrication and moistening, 3) antimicrobial action and microbial homeostasis, 4) oral clearance, 5) buffering, 6) tooth protection, 7) tooth mineralization, 8) taste and smell, 9) wound healing and tissue repairs, 10) the facilitator of mastication, swallowing and speech [5, 8, 19, 32]. These functions served by salivary secretion are relative to the specific constituents and properties of saliva. For example, immunoglobulin (such as IgA) is involved in the inhibition of microbial adherence/ colonisation, promotion of phagocytosis, and aggregation of microorganisms [1, 5, 19, 33]. Another important component in saliva is mucins which are mainly secreted by minor SGs[1]. They play a role in lubricating the oral surface, protecting the oro-oesophageal mucosa from injury, dissolving and transporting the taste substances to taste buds, and facilitating the speech and swallowing[19]. As described above, saliva has been recognized as a kind of body fluid to monitor the health and diseases, such as Sjögren syndrome[34], periodontal disorder, cardiovascular, renal, and hereditary diseases, malignancy and infectious diseases[26]. These results indicated the indispensable role of saliva. Correspondingly, the decrease of saliva secretion inevitably causes complications, such as secondary rampant caries, chewing and swallowing disorders, loss of taste, and inflammation of oral mucosal tissues [35].

There are many factors that have an influence on salivary secretion. For example, the volume, flow and composition of unstimulated saliva vary at different times of the day due to circadian rhythms. The peak level of unstimulated saliva flow rates appears in the afternoon whereas the highest protein level happens in the early morning [5, 36-40]. These results indicate that the normal salivary secretion is affected by the circadian clock. Besides, the gland size, hydration levels of the body and age can also influence the function of salivary glands [5]. Smaller SGs, dehydration conditions and older age are associated with the decrease of saliva output. Meanwhile, the position of the body (standing or lying) could affect the salivary flow rate as well [5, 38, 41]. In addition, not only the volume varies, but the concentration of salivary components would also change after various stimulations, such as masticatory, taste and pharmacological stimulation. For example, both the structure and function of SGs are influenced by the utilization of drugs or alcohol[1]. Moreover, head and neck radiotherapy would also have an impact on the secretory function of SGs. All of these conditions should be considered when saliva is being measured for clinical and research purposes.

Irradiation (IR)-induced salivary gland hypofunction (Xerostomia)

Xerostomia (dry mouth) is a term clinically defined as the subjective symptom of oral dryness induced by a lack of saliva. The subjective sensation of dry mouth would appear when the unstimulated flow rate reduces to 50% [32, 38, 42]. Hyposalivation is designated as a pathologically low saliva secretion where the unstimulated saliva flow rate is below 0.1ml/min and/or chewing-stimulated saliva flow rate is below 0.5-0.7ml/min[5, 32, 43-45]. Severe oral dryness is commonly associated with the increased activity of tooth caries, oral fungal infections

and mucosal inflammation[19]. Several patients have to change their behavior and dietary intake because of dysphagia or due to trauma caused by salivary hypofunction. Such related symptoms would consequently affect the individual's nutritional intake, speech and sleeping, thus, reducing their quality of life. The prevalence of xerostomia is increasing with age. Up to 50% of elderly patients have xerostomia and the prevalence rate in the general population is around 20% (range from 0.9% to 64.8%) [5, 46]. However, the influence of gender remains controversial. Several studies pointed out that the incidence rate among women is higher than among men [5, 19, 47-49], while other studies showed that no difference had been found between genders[46, 50]. These differing results may be due to the complex pathogenesis of xerostomia. Indeed, dry mouth can occur for a variety of reasons. Dry mouth symptom can be reversed if it is induced by anxiety, dehydration or acute infection. However, radiotherapy, autoimmune inflammatory diseases, glandular pathologies or polypharmacy would cause permanent xerostomia[5].

Radiotherapy is a common treatment for the patient with head and neck cancer, especially for squamous cell carcinomas. For these patients, a total absorbed dose ranging from 50 to 70 Gray (Gy) is given for curative purposes. There are around 600,000 newly diagnosed patients worldwide with head/neck cancer annually [51, 52]. However, 80% of the patients who received radiotherapy consequently suffer from xerostomia because of the irreversible damage of acinar cells in SGs caused by irradiation[5, 32, 53]. Depending on the grade and location of the tumor and affected lymph nodes, irradiation portals and doses vary and inevitably may affect the SGs of those patients [54-56]. Various levels of damage of SGs would occur according to the volume of SGs involved in the radiation area and the cumulative dose of radiation for the SGs [19, 32]. Doses of 30-50 Gy may cause the reversible SG hypofunction, but doses higher than 60 Gy would likely cause

irreversible SG damage [19]. A rapid decrease of salivary secretion would occur in the first week post-irradiation therapy, and further major decrease is observed during the 3 months after radiotherapy[53]. In addition to volume, other factors such as salivary electrolyte levels, buffering property and antibacterial systems of saliva are additionally changed post-radiotherapy[5, 53]. The pH of the saliva reduces from 7.0 to 5.0 [53, 57, 58] and the concentration of inorganic salt (such as sodium, chloride and calcium) and organics (such as immunoproteins and lysozyme) are increased. Nevertheless, the total solid substance remains decreased due to the significant decrease in the salivary flow rate[53].

The process of irradiation(IR)-induced damage of SGs can be divided into four stages based on the time phases in the rat model[59-62]. In phase one (0-10 days), the secretion function decreases obviously due to the impairment of water channels. One reason is that the M3-muscarinic receptor in the plasma membrane is compromised by the irradiation. However, no severe apoptosis or cell loss is observed in this initial period [62-66]. The amylase secretion does not significantly decrease until phase two (10-60 days). In phase two, acinar cells in the SGs are disappearing. Phase three (60-120 days) is a comparable stable stage following phase two. Then, a lower salivary flow rate is observed in Phase four (120-240 days). This significant decrease in secretory function is caused by a lack of functional cells and the death of the reproductive cells in SGs. The mechanism behind the IR-induced SG injury is complex. To explain the acute damage of irradiation, Takagi et al. demonstrated that irradiation directly caused the severe dysfunction of water excretion and damaged the aquaporin 5 from the plasma membrane during and after the irradiation [67]. This result indicates that the plasma membrane is the early target of radiation and directly links to the reduction of salivary secretion. In addition to the plasma membrane, the other primary radiation

target of the early damage is the microvascular endothelial cell in SGs[68, 69]. Mizrachi et al. elucidated the acute damage of microvascular endothelial cells and further explored the mechanism behind it. They reported that the generation of ceramide and reactive oxygen species (ROS) resulted in the SG hypofunction via mediating endothelial apoptosis and microvascular dysfunction [68]. For the late damage, several studies demonstrated that irradiation damaged the cellular DNA and induced the death of the reproductive cell (acinar progenitor cells) [68, 70, 71]. It could be an interpretation of the later cell loss, cell apoptosis and the reduction of salivary secretion in the delayed phase[64]. To summarize, the irradiation-induced SG damage can be divided into two mechanisms, 1) acute phase: cellular dysfunction due to the membrane damage; 2) delayed phase: a classical cell killing of progenitor cells because of DNA damage and disorder of the cellular microenvironment[62].

Current treatments for the irradiation-injured salivary glands

There are four objectives of xerostomia treatments, including 1) replacing lost saliva or increasing the saliva flow, 2) maintain oral health, 3) control of dental caries, 4) control of possible infections [53, 72]. Most of the current treatment options for IR-induced SG hypofunction are palliative[73] or preventive and result in a limited therapeutic effect [74-76].

Palliative therapy

Palliative therapy is a widely used treatment for the irradiation-induced salivary gland hypofunction, such as the salivary substitutes and sialagogues. The main objective of palliative therapy is to relieve the symptoms and reduce the discomfort in patients with xerostomia.

Salivary substitutes

In the clinic, the utilization of salivary substitutes is the most common treatment to relieve the xerostomia through maintaining the lubrication in the oral cavity [77, 78]. There are various forms of salivary substitutes, including sprays, gels and solutions. In general, salivary substitutes contain agents to increase viscosity, including carboxymethyl cellulose (CMC), hydroxypropyl methylcellulose (HPMC), hydroxyethyl cellulose (HEC), and polyglyceryl methacrylate (PGM) [32, 77, 79]. Additionally, the function of anti-microorganisms is also provided in the salivary substitutes to mimic the natural saliva [80]. The other possible palliative substitutes are homeopathic remedies, including aloe vera gel, olive oil and rape oil spray[53, 81, 82]. These salivary substitutes lubricate the oral mucous membrane and relieve the symptoms, nevertheless, their effects are short-lived [50, 83]. Patients can also take sips of water, saline or bicarbonate solutions to keep oral tissue moist [80]. However, these solutions still cannot replace natural saliva. One of the reasons is that they are unable to provide satisfactory lubrication because these alternatives are not as viscous as saliva [83]. To sum up, the salivary substitute treatment is an effective symptomatic therapy, but it cannot stimulate the salivary gland to secret the nature saliva and the effect of the salivary substitutes is transient.

Sialagogues

Sialagogues, such as pilocarpine, are used to stimulate the secretory function of SGs. Taniguchi et al. reported that long-term administration of pilocarpine improved the salivary flow in irradiated mice and reduce the apoptosis in SGs post-irradiation[84]. However, the therapeutic effect of sialagogues (e.g. pilocarpine) in the clinical studies varied from a positive effect to no effect [84-90]. LeVeque et al. reported that the responses to pilocarpine treatment appeared only when a

higher dose (10mg three times a day) was used for some patients. This finding indicated that the negative results may due to under-dose for certain patients [90]. The other possible explanation is that the degree of salivary dysfunction varies from patient to patient. Sialagogues treatment would become ineffective if the SGs are completely destroyed. On the other hand, for the other patients who have positive outcomes during the pilocarpine treatment, they would require sialagogues for a lifetime as the function of salivary secretion would decrease after cessation of sialagogues treatment [91]. There are many cholinergic side effects related to generalized parasympathetic stimulation of sialagogues, including nausea, sweating, lacrimal and nasal secretion, as well as joint pain and change in frequency of urination [77, 92, 93]. In addition, there are some contraindications for the use of sialagogue, including narrow angle glaucoma, kidney stones, heart or liver diseases, and uncontrolled asthma[91]. Therefore, sialagogues must be given with caution, especially for patients with advanced age[91]. To date, sialagogues continue to be used in the clinic although it has severe side effects and inconsistent outcomes.

Other palliative therapies

There are several other palliative treatments available for xerostomia, including gustatory stimulation (such as the use of citric acid and malic acid)[77, 94, 95], mastication stimulation (such as chewing gum) [32, 96, 97], electrostimulation [98-103], hyperbaric oxygen therapy [104, 105], low-level laser therapy [106] and acupuncture[98, 104, 107]. However, they still have limitations. For example, the use of malic acid may increase the risk of dental erosion and dental caries[77]. To add, there is insufficient evidence to support these interventions in the management of xerostomia [77, 99, 104].

Preventive therapy

Preventive therapy is the most cost-effective treatment for many diseases, including irradiation-induced dry mouth [108]. In this section, preventive therapies were categorized into four groups: intensity-modulated radiotherapy (IMRT), radical scavengers, growth factors and salivary gland transfer.

Intensity-modulated radiotherapy

Intensity-modulated radiotherapy (IMRT) is an advanced organ-sparing radiotherapy technique that can reduce the damage of major SGs (especially the parotid gland) during radiotherapy [109]. According to the computer algorithm, IMRT can deliver an accurate irradiation dosage to the specific tumor mass based on the tumor location and grade, then IMRT can reduce the damage to the surrounding tissues, such as SGs[32]. Nutting et al. demonstrated that fewer patients suffered from severe xerostomia in the IMRT group when compared with that in the conventional radiotherapy group at all time point post-treatment [109, 110]. On the other hand, the regions of SG containing stem/progenitor cells are closely linked to the function of SGs post-irradiation [111]. Therefore, the protection of these regions would further improve the beneficial effect of IMRT because of the preservation of the stem cell niche. However, there are several clinical trials showed that no difference in physician-assessed oral dryness between conventional radiotherapy and the IMRT group [112, 113] although the symptom of sticky saliva was fewer in IMRT group[112]. The other challenge is to spare the minor SGs through IMRT. Moreover, the large midline cancers and bilateral metastases are the contraindications of IMTR[114].

Radical scavengers (Radioprotectors)

The generation of reactive oxygen species (ROS) is one of the major causes of the acute phase of irradiation-induced SG damage. The ionization of water in SGs produces highly reactive radicals that have an unpaired electron. These free radicals in the nucleus or membranes of cells are unstable, therefore, they would either donate or accept an electron from other molecules[115], then consequently attack and damage the relevant molecules, such as DNA, proteins, lipids and carbohydrates [116]. In SGs, these chain reactions of free radicals impair the water channels by affecting the plasma membrane and the microvasculature. Thereby, radioprotection can be achieved by using radical scavengers, such as Amifostine and Tempol. Amifostine is an oxygen radical scavenger and cytoprotective agent. It can protect the target molecules from irradiation-induced free radicals. The efficacy of Amifostine has been reported in a large number of clinical trials [109, 117-119]. However, several patients still suffered from the experience of xerostomia after Amifostine treatment [78, 117]. In addition, intravenous administration of Amifostine is accompanied by several side effects, such as nausea and hypotension[78, 109, 120]. Currently, the subcutaneous administration of Amifostine becomes an alternative treatment with better toleration [121-123]. However, there are some concerns associated with Amifostine treatment, such as the high costs and the probability of tumor protection. Tempol, a stable nitroxide, is another radical scavenger with antioxidant and radioprotective activities. Mizrachi et al. reported that microvessel density was significantly increased in the irradiation-injured SGs in the Tempol treatment group when compared with that of the control group[68]. Furthermore, Tempol provided SG protection but did not protect the tumor cells [78, 124]. Therefore, one could induce that there are fewer side effects accompanied by Tempol treatment in comparison to Amifostine therapy [125]. These studies provided a possibility of radical scavengers in treating the irradiation-induced SG damage.

Growth factors

Growth factors are the potential preventive agents for SG hypofunction, including insulin-like growth factor-1 (IGF-1), keratinocyte growth factor (KGF/ FGF-7), basic fibroblast growth factor (bFGF/ FGF-2) and other growth factors (such as glial cell-derived neurotrophic factor (GDNF) and colony-stimulating factor) [78, 126-128]. FGF-2 promotes fibroblasts, epithelial and endothelial cells proliferation, and facilitates regeneration of tissues and blood vessels [129, 130]. It also accelerates the growth of acinar cells [131], myoepithelial cells and ductal cells [129], thereby promoting the development and regeneration of salivary glands [129, 132]. Cohen-jonathan et al. demonstrated that FGF-2 extended the G2 arrest of SG cells post-irradiation and consequently enhanced the clonogenic survival of cells by allowing more time for cell recovering from the damage of irradiation [133]. Additional studies reported that FGF-2 protected SGs by protecting the acinar cells and inhibiting irradiation-induced apoptosis in-vitro and in-vivo [131, 134, 135]. Similarly, many studies suggested that IGF-1 could protect SGs from irradiation by reducing cell apoptosis, facilitating DNA repair and increasing cell proliferation rate in SGs [126, 136, 137]. However, IGF-1 not only enhances the growth of normal cells, but also the growth of tumor cells and protects them from radiotherapy in vivo and in vitro [138]. KGF, also known as FGF-7, have been administrated prior and after irradiation to protect SGs. Chois et al. reported the radioprotective effect of KGF in treating the irradiation-induced SGs hypofunction[139]. The radioprotection effect of KGF may be due to the mitigation of DNA damage as KGF possesses scavenge ROS ability [140, 141]. Other studies revealed that KGF could protect both acinar and fibrotic cells by reducing apoptosis and increasing stem cell survival rate in the SGs [142, 143]. Furthermore, Zheng et al. demonstrated that KGF gene transfer restored the function of SGs without promoting the proliferation of squamous cells[144]. Other growth factors, such as GDNF

was reported to enhance the regenerative potential of SG stem cells by promoting stem cell proliferation[145]. Taken together, growth factor therapy is a potential treatment for the SG hypofunction. Further study is required to unveil the efficacy and safety of the growth factor treatments in humans.

SG transfer

Salivary gland transfer is a surgical prophylaxis for the irradiation-induced SGs. This technique shows the potential to prevent SGs from radiotherapy damage by relocating the SGs to a new position that receives a minimal dose of irradiation. For example, the submandibular glands can be preserved by transferring to the submental space[120, 146]. Then, there is no need to modify the irradiation prescription according to the position of the SGs (such as the IMRT). Currently, many studies have reported that SG transfer increased the secretory function of SG post-IR [146-150]. Furthermore, the procedure of SGs transfer is considered to be oncologically safe [146, 150]. However, SG transfer is not appropriate for all patients. The eligible patients are those who have clinically negative cervical lymph nodes. Then, their SGs can be translocated to that position with no or a low dose of irradiation during radiotherapy[120, 146]. Meanwhile, there are several surgical complications associated with the SG transfer, such as ipsilateral facial edema, neck numbness, wound infection and bleeding formation [146, 150, 151].

Experimental restorative therapy and clinical trial

Although there are many preventive and palliative treatments in the clinical, many patients are still living with a life-long dry mouth as there is no available conventional therapy for xerostomia, especially for patients whose functional SG cells are completely lost. To solve this problem,

several new therapies are being developed with the aim of restoring SG secretory function. Current knowledge in restoring saliva secretion comes from experimental approaches, such as gene therapy[152-155], tissue engineering[156, 157], cell-based therapies [158-161], and cell-free therapies [162, 163].

Gene therapy

Gene therapy is a promising approach for irradiation-induced salivary hypofunction. The preclinical studies of gene therapies could be classified into four categories based on their mechanisms, including secretory gene therapy (Aquaporin 1 gene transfer), reparative gene therapy (FGF-2, VEGF, KGF gene transfer), compensatory growth gene therapy (Tousled-like kinase 1B gene therapy) and anti-apoptosis gene therapy (Protein Kinase C delta, Sonic hedgehog and Heat shock protein gene therapies)[164]. There is only one group, using a serotype 5, adenoviral (Ad5) vector encoding human aquaporin-1 (AdhAQP1), that has advanced to the phase I/II clinical trial in treating the SGs hypofunction [52, 165]. The human aquaporin-1 (hAQP1) gene encodes a water channel protein that plays an important role in regulating the secretory function in SGs. Baum group delivered AdhAQP1into irradiated submandibular gland and parotid gland in rat [166] and miniature pig model [167], respectively. These treatments resulted in an increase of secretory function when compared with that in the untreated group. Then, Baum and colleagues evaluated the efficacy and safety of the AdhAQP1-mediated gene therapy to the patients with SG hypofunction[165]. No dose-limiting toxicity or severe side effect was detected. Recently, the results of the clinical trial showed that all participants had a significant increase in the secretory function of SGs 3-4.7 years after treatment ended [125, 168]. Gene transfer performed via intraductal cannulation has advantages as the treatment procedure is less invasive and the

concentration of vectors would not be diluted by body fluids[169]. However, gene therapy has its limitations as the vector used in gene therapy may induce an immune reaction[170]. Moreover, gene transfer would be difficult to perform if the cannula injection is inaccessible due to jaw opening limitations or the lack of patency in the main excretory ducts [52]. Long-term observation of gene therapy is yet to be done. Thus, it is still unknown how long the effects would remain in humans.

Tissue engineering of SGs reengineering

In general, salivary gland tissue engineering requires three components which are cells, three-dimensional (3D) scaffold and extracellular matrix (ECM) proteins [127]. This novel 3D organotypic system aims to create a functionally artificial SG by integrating different cellular components with or without biomaterial. Selection and cultivation of SG-specific and/or non-SG specific cells are essential for SG reengineering. The human ductal epithelial salivary gland (HSG) cell line was used widely in SG tissue engineering [171]. However, as a cell line, HSG cell has limited potential of transformation [127] and had been recently reported as contaminated [172]. SG primary cell was reported as another cell candidate for tissue engineering. Joraku et al. subcutaneously transplanted the primary salivary cells with polyglutamic acid into the athymic mouse model and reported that the SG cells presented the salivary cell markers (AQP5, AE1/AE3 and cytokeratin) and produced α -amylase [173]. Ductal-like human submandibular cells with tight junctions were isolated and used for the artificial SGs [174]. However, the life spans of SG primary cells are limited and it is difficult to isolate and cultivate the functional acinar cells in vitro. One solution for this obstacle is to use stem cells with self-renewal capacity. SG-derived Kit^+ (c-Kit, CD117) cells were able to differentiate into organoids with ductal structures in 3D matrix mixture

with collagen and Matrigel in vitro [175], and SG spheroid-like progenitor cells could restore the function of SGs in vivo [161]. No tumor formation was found during the four-month observation[175], but a long-term study of tumorigenic safety is required. In addition to the mouse SG progenitor cells, human dental pulp-derived stem cells tagged with magnetic nanoparticles were studied in the novel 3D spheroid bio-printing system [176-179]. Bone marrow-derived stem cells expressing the α -amylase were reported after co-culturing with neonatal rat parotid acinar cells for 3 weeks[180]. These results indicated the possibility of using adult stem cells in SG tissue engineering. However, the stem/progenitor cells have to be used in a limited time window (such as passage 3-5) which is not convenient for clinical application.

3D scaffolds are utilized to structurally and functionally mimic cellular environment and native ECM during cell cultivation. Scaffolds facilitate the cell adhesion, migration or differentiation according to their various properties, such as porosity, stiffness, strength and biodegradability [157]. In addition to the benefits above, evidence has supported that cells cultured with 3D scaffold produced more native ECM components when compared with 2D culture[157]. Various kinds of scaffolds are developed and used in SG tissue reengineering, including collagen, Matrigel, hyaluronic acid (HA), and poly lactic-co-glycolic acid (PLGA). The formation of functional salivary spheroid was reported when SGs cells were cultured with Matrigel and collagen type I [161, 175, 181-183]. Nevertheless, these animal-derived biomaterials are not compatible with humans. To solve the xenogeneic problem, soft hyaluronic acid (HA) hydrogel was used as a human-compatible biomaterial. Pringle et al. reported that human SGs primary cells formed functional 3D spheroids with acini-like structures in vitro and in vivo [184]. However, it still has a limitation as the polarity of cells was reversed in the latter structure with HA-gels cultivation.

Recently, 3D bio-printing nanotechnology has been developing and therefore, has made achievements in SG engineering. Adine et al. showed that SG cells cultured with magnetic bio-printing system-generated organoids with innervation and secretory function, and maintained the epithelial polarity[185]. The other promising novel 3D scaffold is the natural ECM which can be isolated from the decelluar SGs tissue. It is a xeno-free biocompatible scaffold and can provide the native microenvironmental cues. Furthermore, it can release some ECM related proteins to support cell growth, migration and/or differentiation [127, 186]. ECM related proteins are also considered a vital element for SG engineering. Jang SI et al. reported that a high calcium concentration (0.05mM) is essential for the acinar cell growth and maintenance of polarization[187]. Meanwhile, it is worth noting that different ECM proteins would induce different impacts. For instance, Laminin-111 promoted the apico-basal polarization and epithelial tight junctions of SG epithelial cells, while chitosan induced a negative impact [188]. Therefore, it is essential to select beneficial ECM proteins and optimal concentrations for SG reengineering.

SG tissue engineering is a promising approach for SG hypofunction. Remarkable progression has been made by numerous studies in the past decades. However, it is still a challenge to generate ideal SG-like organoids with functional acinar, ductal, myoepithelial cells, as well as the networks of blood vessels, parasympathetic nerves and lumenized ducts. For the transplantation in vivo, it is also a challenge to connect the ideal organoids with cells in the transplanted area[111, 157]. So far, the isolation and cultivation of functional acinar cells are still the main obstacles in SG reengineering. In summary, further studies are required to create a functional and larger SG organoid for long-term SG transplantation [157]. To add, developing a surgical technique for in vivo transplantation with 3D organoids is necessary.

Cell-based therapy

Stem cell therapy

Stem cell types can be classified into three groups according to their potential use in stem cell therapy: embryonic stem cells, induced pluripotent stem cells and adult stem cells[189]. Currently, various mesenchymal stem cells (MSCs) have been reported as a possible regenerative therapy option to increase the salivary flow rate and relieve xerostomia caused by radiotherapy [190-193]. Ono et al. reported that induced pluripotent stem cells' cocultivation promoted the embryonic SG cells to develop a better epithelial structure and present fewer undifferentiated specific markers [194]. Their study indicated that induced pluripotent stem cells facilitated the differentiation of SG development and regeneration[194]. However, the tumorigenicity of induced pluripotent stem cells remained a major issue that has yet to be resolved, and a few studies have attempted to test the embryonic or induced pluripotent stem cells in treating the irradiation-induced SG hypofunction. Herein, we will focus on the adult stem cells which had been widely studied in the past decades.

Adult stromal/ mesenchymal stem cells are the fibroblast-like plastic-adherent cells derived from many organs with the abilities of self-renewal and multiple differentiations [195]. There are two advantages to the utilization of adult stem cells for disease treatment when compared to embryonic stem cells and induced pluripotent stem cells. First, adult stem cells provide a possibility for autologous cell utilization in the clinic. For instance, pre-radiotherapy isolation of adult stem cells followed by post-radiotherapy transplant into the patient could be a practical strategy for the treatment of salivary hypofunction following radiotherapy. Moreover, adult stem cells present

stable phenotypes and lack ethical problems when compared with embryonic stem cells. Bone-marrow-derived stem cells are the most studied adult stem cells for regenerative medicine. Specifically, it was proposed as potential candidates for the treatment of salivary hypofunction induced by the irradiation. Woodward et al. reported that bone marrow (BM) MSC secreted growth factors, regulated the immune response, reduced the inflammation and promoted the remaining MSCs proliferation and differentiation in SGs [196]. Another study demonstrated that BMMSC restored the secretory function of SGs by differentiating into acinar cells in a mouse model [191]. However, challenges that still remain are the lack of easy accessibility and the lack of sufficient human stem cells derived from bone marrow.

In addition to bone marrow-derived stem cells, adult tissue-specific stem cells have been isolated from many other tissues, including liver[197], intestine[198], lung [199], adipose tissue[193], dental pulp[200], hair follicles[201], skin[202], muscles[203] and salivary glands[161]. Several of these tissues showed potential in treating salivary hypofunction[190, 191, 193, 204]. Adipose-derived MSC is a relatively non-invasive stem cell source that could be harvested by fat aspiration. Moreover, adipose tissues have a higher yield of the MSCs when compared with bone marrow and it is unaffected by the donor's age[190]. Furthermore, it had been reported with non-toxic and non-tumorigenic in animals and human studies [205]. Three studies have revealed that adipose-derived stem cells alleviated the hypofunction of salivary gland post-irradiation in mouse [190, 206] and rat model[207]. To add, a research group cocultured human hypoxia adipose MSCs with IR-injured SG epithelial spheroids in vitro. Results showed that hypoxic conditions increased the therapeutic effect of adipose MSCs by promoting cell secretion of growth factors (e.g. FGF10) and activating FGFR-PI3K signaling. Adipose MSC treatment also reduced apoptosis and protected the structure

and function of acinar cells [193]. Recently, adipose stem cells had been tested in phase I–II clinical trials[208].

The other candidate cell source for the treatment of irradiation-induced hyposalivation is the stem/progenitor cells in salivary glands. There are various cells derived from different origins in SGs, such as stromal cells, parenchymal cells, neural and blood vessel cells [189]. The SG stem cells could be selected by their specific surface markers. In 2004 and 2007, the Endo group firstly isolated the progenitor cells from the murine and swine injured SG by fluorescence-activated cell sorting with Sca-1(+)/c-Kit(+) markers [209, 210], and these cells could differentiate into a hepatic lineage. Afterwards, the Coppes group isolated and cultured the murine SG cells into the spheres and reported that spheres contained the cells positive to stem cell markers, such as a CD117, CD24, CD29, CD49f, CD44, CD90, CD34, Sca-1, Mushashi-1 and c-Kit [161]. Then, the human SG-derived spheres were successfully formed with the cells positive to c-Kit [125, 183, 211]. These c-Kit positive stem cells could self-renew for more than 48 weeks in vitro and in vivo [125, 160, 161, 212]. Furthermore, the c-Kit$^+$ cells rescued hyposalivation and maintained the homeostasis of SGs post-irradiation [160, 161]. More recently, the studies about SG c-Kit$^+$ cells were tested in phase I–II clinical trials [125, 208, 211, 213]. However, the minimal number of stem cells is still unknown and depends on the individual patient (e.g. age, dose and location of irradiation)[183, 184]. Indeed, most patients with head and neck cancer were elders. Preclinical results showed that less sphere formation was observed when cells were isolated from aged mice [183]. However, another study reported that aged SG stem cells still could potentially be used to alleviate SG dysfunction[214]. In addition to the c-Kit$^+$ cells, there is another tissue specific-stromal/ mesenchymal stem cell isolated from human major SGs [204, 215], that was capable of multiple

differentiation, including osteogenesis, adipogenesis, chondrogenesis and could generate the epithelial cell type (epithelial and hepatic cells). Furthermore, these stem cells showed capabilities in treating SG hypofunction induced by irradiation.

Stem cells derived from SGs provide advantages in comparison to other stem cells. Mimeault and Batra suggested that tissue-resident adult stem cells could release growth factors to repair damaged tissues or differentiate into tissue-specific cells [216]. Indeed, MSCs from different tissues show some differences [217-219]. For example, bone marrow-derived MSCs exhibited better osteogenic and chondrogenic differentiation abilities, while MSCs derived from adipose tissue exhibited greater potential of immunomodulatory effects [220]. Moreover, transplantation of periodontal ligament (PDL) stem cells resulted in more cementum-PDL formation than that of the bone marrow MSCs group [221]. These examples suggest that origin-specific MSCs may be more suitable to treat diseases in their original tissues/organs. However, the procedure to harvest the MSC from major SGs is invasive which is impractical for clinical application. Recently, MSC was successfully isolated from human minor SGs. These cells showed mesenchymal stem cell type and exhibited epithelial characteristics, which suggested that they were epithelia-mesenchymal cells [204]. These pieces of evidence indicated that MSCs derived from SGs are more promising for remodeling salivary glands than MSCs from other tissues. But it is worth noting that minor SG-MSCs have yet to be tested in the irradiation-damaged salivary gland model. Further studies are required to test the efficacy of minor SG MSCs and compare SG MSCs to other MSCs.

Other cell therapies

In addition to MSCs, there are several other cell sources that have been studied in SG regeneration. We will introduce the utilization of the four kinds of cell candidates (SG cells, bone marrow cells, dental pulp cells and peripheral blood mononuclear cells) and two potential cell sources (bone marrow-derived mononuclear cells and spleen cells) in this section. Generally, most of these cell sources contain a portion of stem cells or cells positive to several stem cell markers.

Whole bone marrow contains heterogeneous cells, including stromal cells (fibroblasts, macrophages, adipocytes, osteoblasts, endothelial cells) and hematopoietic cells (myelopoietic cells, erythropoietic cells, megakaryocytes, plasma cells, reticular cells, lymphocytes and monocytes). There are two populations of stem cells in whole bone marrow: hematopoietic stem cells and mesenchymal stem cells. Studies reported that human bone marrow cells had differential abilities [222] and showed the therapeutic effect to non-hematologic diseases, such as cardiovascular and autoimmune diseases [223, 224]. Sumita et al. transplanted bone marrow-derived cells into irradiated mice by intravenous administration. Results showed that salivary output was increased and the weight of SGs was heavier after treatment when compared with that in the non-treated group. Furthermore, bone marrow-derived cell treatment resulted in fewer apoptosis cells, a higher percentage of cell proliferation rate and higher formation of blood vessels in SGs. The donor-derived cells (bone marrow cells) were also observed in the region of SGs with a higher ratio of acinar cells [225]. Overall, bone marrow-derived cell is a promising cell candidate in treating the damage of irradiated SGs.

Peripheral blood mononuclear cell is the other cell candidate for the treatment of SG hypofunction. One recent study reported that mouse peripheral blood mononuclear cells could be induced to

effectively conditioned cells through a simple and effective culture method[226]. Additionally, the effective peripheral blood mononuclear cells contained enriched cells positive to CD11b/CD206 (M2 macrophage-like) cells and presented more anti-inflammatory and vasculogenic phonotypes. Specifically, the effective cell contained 66% lymphocytes, 13% monocytes/macrophages, and 2.5% endothelial stem/progenitor cells. These cells were then transplanted into a mouse model of irradiation-injured atrophic submandibular glands via intro-glandular administration. Results showed that the transplantation treatment increased 3.8 folds of SG secretory function, reduced the expression of inflammatory genes in SGs, and promoted cell proliferation, blood vessel formation and tissue regeneration. Furthermore, the transplanted cells were observed in the vascular endothelium and perivascular gland tissues at 2 weeks post-irradiation. This study indicated that the effective peripheral blood mononuclear cells ameliorated the hypofunction of SGs through vascular differentiation and/or paracrine manner.

According to the effect of bone marrow-derived cells and mononuclear cells from peripheral blood discussed above, we would propose a promising cell type that has not yet been used in treating SGs dysfunction: the bone marrow-derived mononuclear cells. Autologous bone marrow-derived mononuclear cell transplant was proved as a safe and effective approach for patients of traumatic brain injury [227], spinal cord injury[228], myocardial infarction[229], and cardiomyopathy[230] in clinical trials. Research reported that instead of the small number of stem cells in bone marrow, the beneficial effects of mononuclear cell treatment might be attributed to the combined effects of all mononuclear cells[229, 230]. For example, the lymphocytes in mononuclear cells play a role in cardiovascular disease treatment by secreting numerous factors of angiogenesis[231]. Therefore,

it is reasonable to believe that bone marrow-derived mononuclear cells would be a cell candidate for the SGs treatment. However, more experiments are required to support this hypothesis.

The other two cell candidates are dental pulp endothelial cells and spleen cells. Yamamura et al. demonstrated that dental pulp cells containing the stem cell source were able to differentiate into dental pulp endothelial cells. The dental pulp endothelial cells could reorganize the tube-like structure in the Matrigel and could mitigate the hypofunction of SGs following by the irradiation in vivo [192]. Therefore, dental pulp cells are a potential cell source for the salivary hypofunction. The spleen contains a special stem cell population ($Hox11^{+}$) that had been used as the regenerative medicine[232-234] for restoring the function of cranial nerves [235], hearts [236], inner ear [235], pancreatic islets [237] and salivary gland with Sjögren's-like disease [238]. Fang and colleagues showed that spleen cell extract could rescue the function of SGs hypofunction following irradiation[234]. Accordingly, although it is not direct evidence, spleen cells might be a viable cell candidate in treating irradiation-injured SG.

Cell transplantation is an effective approach for functional restoration of SGs, however, several limitations for cell-based therapies remain [226, 239-242]: 1) there is a lack of method and source to easily obtain a sufficient population of human adult stem cells for transplantation; 2) the expansion process of stem cells in vitro might result in the loss of their plasticity; 3) the capacities of the stem cells are particular depending on the patient's age and morbidity; 4) cell-therapy carries the risks of thromboembolic, tumorigenesis and the possible cell rejection; 5)few transplanted cells were alive and were able to successfully engraft and differentiate into the target SG cells [225]; 6) cells have to be used in the limited passages.

Mechanism for the cell therapy

Increasing studies have reported that cell-based therapy is able to restore SG hypofunction following irradiation. However, therapeutic mechanisms behind the cell-based therapies remain unknown. Currently, many studies have proposed the hypotheses of the therapeutic mechanisms of cell-based therapy, including the cell-cell contact (transdifferentiation and fusion) and paracrine effect[162, 193, 243].

Transdifferentiation was originally proposed as the mechanism underlying the cell-based therapy[244]. The engrafted cells undergoing the epigenetic modifications would differentiate into various organ lineages in vivo[245, 246], such as bone, cartilage, adipose, and epithelial cells. Meanwhile, the transdifferentiation capacity of engrafted cells has been demonstrated as one of the therapeutic mechanisms in diverse disease models, such as brain strokes[247], infarcted myocardium [248], wood healing [249-251] and salivary hypofunction[225]. Sumita et al. transplanted bone marrow-derived cells into the 18Gy-irradiated mice via intravenous administration. After 24 weeks, 9% of donor-derived cells were detected in the SGs and positive to the epithelial cell markers [225]. These results indicated that bone marrow-derived cells could restore the SG function in head and neck irradiated mice by differentiating into the salivary epithelial cells. Recently, another study reported that the adipose-derived stem cells could transdifferentiate into SG acinar-like cells in vitro, which is in agreement with our previous study in vivo[252]. However, the transdifferentiation pathway is still controversial. For example, several investigators demonstrated the transplanted MSCs were differentiated into the epidermal

keratinocytes in the wound repair model [249-251] while other investigators failed to detect the differentiated cells in the animal model [253].

In addition to direct differentiation, cell fusion is considered as another possible mechanism of cell-based therapy. Noiseux et al. injected bone marrow-derived MSCs into the infarcted myocardium mouse and observed MSCs fusion in three days with a significant increase of cardiac function [248]. This study implied that cell fusion of bone marrow-derived MSCs plays a role in cardiac repair. Bone marrow-derived MSCs could also be fused with the epithelial cells in the liver post-irradiation[245, 254]. However, a low frequency of cell fusion was detected in the study [245]. It indicated that cell fusion might not be the principal pathway for cell therapy.

Lack of replacement of stem/progenitor cells in the SGs would result in the handicapping of the natural tissue regeneration and induce the damage of SGs post-irradiation[62, 125, 143, 255]. Therefore, a number of investigators believe that cell-cell contact (differentiation and fusion) is the principal pathway of cell-based therapy because it could directly ameliorate the lack of stem/progenitor cells and recover the damage of SGs. As discussed above, although MSCs or other cells could transdifferentiate into or fuse with other cells, few donor-derived cells (range from 0% to 10%, approximately) were found successfully engrafted and differentiated/fused into the target cells [225, 245, 248, 256, 257]. Furthermore, a low survival rate of engrafted MSCs or other engrafted cells had been reported and long-term engraftment survival cell number was negligible [258, 259]. These pieces of evidence refute the original hypothesis that cell-cell contact is the principal mechanism of cell-based therapy and indicate other effects involved in the beneficial effects.

The paracrine effect is the alternative mechanism of cell-based therapy proposed by the investigators. The paracrine cytoprotective effects of cell-based therapy were reported in various diseases, including contractile cardiomyocytes [260-262], renal ischemia/reperfusion injury[263], brain injury (gnocchi 61 62), and irradiation-induced SG injury[162, 193, 225]. Our previous study showed that cell extract from bone marrow was as effective as bone marrow cells in restoring the secretory function of SGs damaged by the irradiation[222]. It indicated the important role of the paracrine effect and the intact cells/ MSCs might not be necessary for the treatment. Shin and colleagues explored that paracrine factors secreted from human adipose MSCs (such as FGF10) could protect the SGs from irradiation-induced apoptosis [193], which is in agreement with our previous study. Indeed, there are numerous growth factors and cytokines (such as FGF-1, -2, -7, VEGF, HGF) released from engrafted cells and worked as the natural nutrition to facilitate the native cells repair and regeneration. Many studies unveiled that paracrine factors released from cells provided the possibility of tissue regeneration by modulating the immune reaction, mitigating inflammation and fibrotic effects, promoting the angiogenesis and neurogenesis as well as preventing the apoptosis [163, 193, 264, 265]. For example, the adipose-derived stem cell treatment recovered the salivary production of irradiation injured SGs by protecting differentiated cells (mucin-producing acini, myoepithelial cells) and progenitor cells (c-Kit cells), increasing microvessel density, promoting anti-apoptotic and anti-oxidative effects in SGs[193, 266, 267]. This indicated that the comprehensive benefits from the cell-based therapy might result from the diversity of the paracrine factors.

Cell-free therapy

There are several obstacles for the stem/progenitor cell therapies, such as the limited time window of cell lifespan (e.g. passage 3-5) for utilization[268] and the potential risks of immunoreaction and tumorigenesis. Furthermore, the paracrine effect had been proposed as a potential pathway for cell-based therapy. Based on this theory, cell-free therapies were developed in the past decade, including cell extract therapy, conditioned medium therapy and exosome therapy.

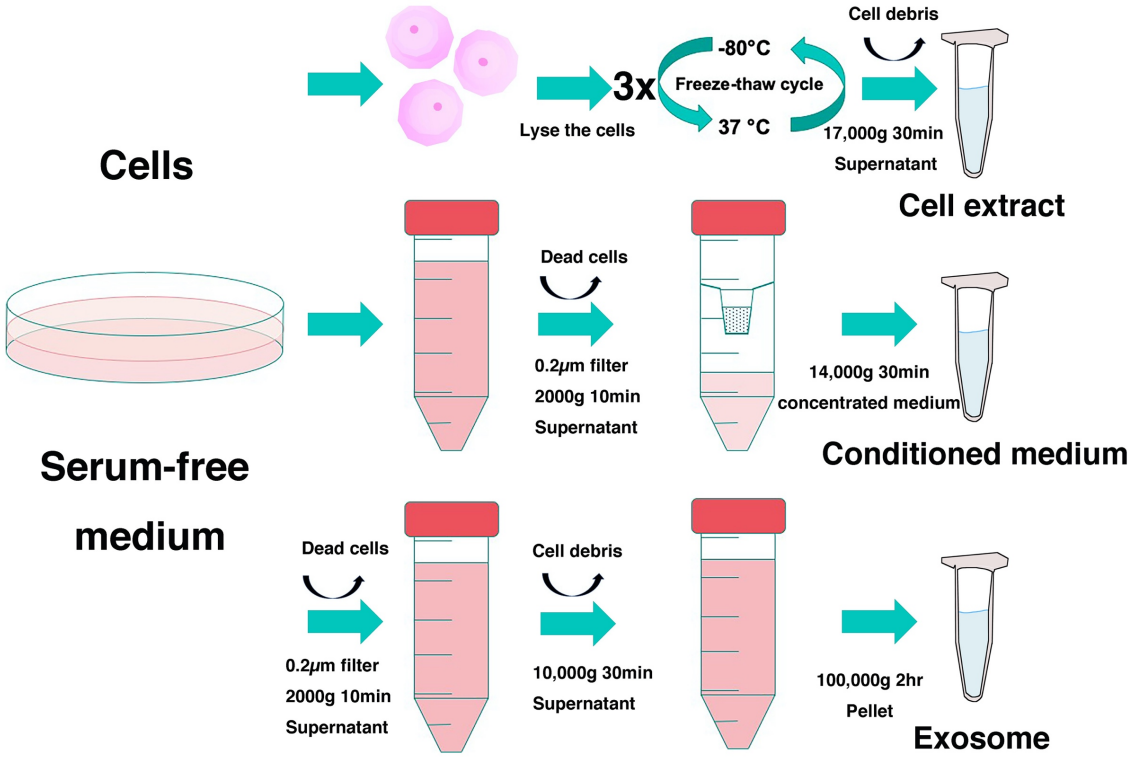

Figure 2. Illustration of the cell-free product isolation, including cell extract, conditioned medium and exosome.

Cell extract therapy

Cell extract is the heterogeneous mixture isolated from the soluble components of cell lysates by three freeze-thaw cycles. It contains proteins, nucleic acids, lipids and carbohydrates[264]. Cell extract harvested from different cell sources have been analyzed, including bone marrow cells[162, 269], bone marrow stem cells[270], bone marrow mononuclear cells[271], adipose stem cells[234, 272], spleen tissues[234] and white blood cells[273].

Currently, cell extract had been used as the cell-free agent for various diseases, such as wood healing[272], myocardial infarction[271], osteoradionecrosis [269], Sjogren's syndrome[270] and irradiation-induced salivary injury[162]. Yeghiazarian's group first isolated the bone marrow cell extract and compared its efficacy with the intact bone marrow cells on the myocardial infarction model[224]. Results showed that cell extract was as effective as alive cells in reducing infarct size and cell apoptosis, enhancing vascularity and improving the cardiac function. The results indicated that paracrine is the major mechanism of cell-based therapy and cell extract might be the potential agent to alternate the intact cell treatment. In the following years, the efficacy of the cell extracts was reported by other research groups. Na et al. demonstrated that adipose-derived stem cell extract promoted wound healing by increasing the dermal fibroblast proliferation, migration and extracellular matrix production[272]. Michel and colleagues reported that bone marrow cell extract significantly enhanced the new bone formation in the irradiated bone in a rat model[269]. Another study suggested that the white blood cells extract induced cancer cell line apoptosis, but no sign of cytotoxicity for the noncancerous Vero and HaCaT cells was found[273].

In agreement with the Yeghiazarian's studies, our previous study reported that bone marrow cell extract was as effective as the bone marrow cells in repairing irradiation-damaged SGs[162]. However, the procedure to harvest the bone marrow cells remains invasive for the donor. Therefore, Fang and colleagues tested two other cell extracts from adipose stem cells and spleen tissues as alternate cell sources [234]. Results suggested that both spleen and adipose stem cell extracts could mitigate irradiation-injured SGs. Nevertheless, the active factors in cell extract remain unknown. To investigate the active components, proteinase K followed by heating was utilized to deactivate the proteins in the bone marrow cell extract [264]. The results revealed that the "deactivated bone marrow cell extract" was no better than the negative control, while bone marrow cell extract treatment restored the secretory function of SGs. It implied that rather than the other components in bone marrow cell extract, native proteins were the effective ingredients for the treatment of salivary hypofunction.

Based on this finding, our group preliminarily identified the protein components in the bone marrow cell extract. Numerous growth factors and cytokines had been identified by protein array assays, such as CD26, HGF, FGF, MMP-8, -9, OPN, PF4, SDF-1, IL-1ra and IL16. These proteins in the cell extracts could be divided into several groups according to the cellular activities they involved, such as tissue remodeling proteases (MMP-8,-9) and its inhibitor (TREM-1), stem cell homing chemokines (SDF-1), anti-inflammatory cytokines (IL-1ra and IL16), pro-angiogenic growth factors (HGF and FGF) and an enzyme associated with immune regulation (CD26). Additionally, we found that the components in cell extracts derived from various cell populations were different. For example, 2 folds of identified angiogenesis growth factors were detected in adipose stem cell extract when compared with that in cell extract derived from bone marrow cells

or spleen tissues[234]. Furthermore, although these cell extracts could all mitigate the hypofunction of SGs, their therapeutic effects were not all exactly the same. Fang et al. demonstrated that spleen and bone marrow cell extracts had comparable efficacies while the adipose stem cell extract had a relatively shorter therapeutic effect on the restoration of SG hypofunction. These results indicated that the components and benefits of cell extracts isolated from various cell sources were different. Therefore, further studies are required to investigate cell extracts from more cell sources and unveil the differently characteristic properties of them.

Although cell extract treatment has been found to be efficacious for irradiated SGs, there are several challenges associated with the clinical infusion of cell extract. First, it is necessary to develop a long-term and convenient storage approach for the large-scale production of cell extract in the future. Second, it should be noted that cell extract is made of complicated components and there is a high possibility that not all of the ingredients in cell extract are the effective factors. Furthermore, the active proteins in cell extract are still unknown. Therefore, future studies should narrow down the complex of the cell extract by purifying the active factors specific to the desired targets, as this might reduce side effects and promote therapeutic outcomes. Third, no human source cell extract was tested for the irradiation-injured SGs model. Last, the treatment of cell extract needs to be assessed extensively, especially for its safety and efficacy, to bring this cell-free treatment a step closer to clinical reality.

Conditioned medium therapy

Over the past decade, increasing numbers of research groups have analysed the conditioned medium isolated from various cells, such as progenitor/mesenchymal stem cells[163, 274-281],

fibroblasts[282, 283] and amniotic cells[284]. The conditioned medium contains a group of proteins released from the cultured cells. The concentration and type of the secretome from different cells varied. For example, a high level of IL-6, TGF-β1 and IGF-1 was detected in adipose stem cell-conditioned medium [163, 275], while almost no IGF-1 was detected in the conditioned medium from periodontal ligament stem cells by protein array assay [278]. Furthermore, the secretome is modified by environmental conditions, such as the kinds of culture medium[285] and the hypoxia condition[163]. An et al. reported that the hypoxia condition promoted the GM-CSF, IGF-1 and VEGF secretion from the MSCs and resulted in a better therapeutic effect for the SG hypofunction [163]. Maarof et al. compared the total number of the identified proteins in the conditioned medium of fibroblasts when using the EpiLife™ or Defined Keratinocyte Serum-free culture medium. Results showed that the fibroblasts cultured with the former medium released more identified proteins[285]. In addition to the secretory proteins, studies revealed that several intracellular/non-secreted proteins were identified in the conditioned medium from the cell lysis or the dead cells in the medium[283, 286], which increased the complexity of the conditioned medium. The computational analysis preliminarily classified the function of identified factors in the conditioned medium into several sections, including metabolism, tissue differentiation, defense response, vascularization, hematopoiesis and the development of skeletal[244]. Indeed, the proteomic technique is a promising approach to characterize the protein complex. Nonetheless, the definitive list of each conditioned medium has yet to be completed. Further experiments are needed to characterize and quantify the dynamic expression profile of conditioned medium and its functional factors.

To date, the conditioned medium has been reported as a cell-free treatment for many diseases, such as amyotrophic lateral sclerosis[274], Huntington's disease[284], radioactive dermatitis/ radiation-induced skin wound [276], spinal cord injury[277], periodontal disease[278] and irradiation-induced SGs hypofunction[163]. Chou et al. reported that SG-derived fibroblasts or the conditional media from the fibroblasts could enhance the expression of alpha-amylase in human SG-derived acinar cells. This study indicated that paracrine factors (such as FGF-2) play a role in regulating the alpha-amylase expression in the acinar cells in vitro[282]. Another study administrated the conditioned medium of the adipose stem cells to the irradiation-induced SG hypofunction mice. The conditioned medium treatment protected the epithelial cells, endothelial cells, myoepithelial cells, and progenitor cells, and thereby remodelled the damaged SGs[163]. Therefore, conditioned medium treatment is a potential therapy for salivary hypofunction. However, the mechanisms behind the protection and regeneration effects remain unclear.

Exosome therapy

There is a growing interest in exosome research in the past decade. Exosomes are the membrane-bound extracellular vesicles (30-100nm in diameter) containing lipids, proteins, DNA and RNA released from an original cell to the extracellular environment[287-289]. Exosomes could deliver biological information over long distances to their target cells and could result in information exchange and host cell reprogramming [290, 291]. Thereby, exosomes play an important role in intercellular communication in vivo. The target cells could uptake the exosome by several hypothesized pathways, including incorporation, endocytosis, micropinocytosis, incorporation through the plasma membrane and phagocytosis [287, 292-296].

Exosomes will be introduced in this section as a possible cell-free therapy according to its promising potential, although no study has tested the efficacy of exosome treatment for the salivary hypofunction. Currently, exosome had been harvested in vitro for treatment purposes. It has been reported as the treatment for various diseases, such as liver disease[288], acute and chronic skin wound[297], acute lung injury[298] and irradiation-induced lung injury[299], osteoarthritis[300], kidney diseases[301], ocular diseases[302], spinal cord injury[303], acute myocardial ischemia[304] and neurological disorders[287]. Many investigators reported that exosome treatment could improve cell proliferation and reduce apoptosis, modulate the immune system, reduce the fibrosis and may induce the death of tumor cell[246, 288, 305]. Furthermore, exosomes showed the potential to reduce oxidative stress, which is one of the main rationales for the irradiation-induced SG hypofunction. Yan et al. reported that MSC-derived exosome treatment protected the oxidative injury of the lung by protecting the mitochondrial membrane and preventing the reactive oxygen species-induced apoptosis through the extracellular signaling receptor kinases 1/2 pathway [288, 306]. Interestingly, the exosomes derived from the fibroblasts did not protect the liver cells from reactive oxygen species. This study indicated that the therapeutic capacity of exosomes relies on the type of cells and the therapeutic benefit of exosomes may be an MSC-specific effect[288]. It is worth noting that the impact of exosomes on tumor progression is controversial[307]. Several studies suggested that MSC-derived exosomes promoted the tumor growth[308, 309], while others demonstrated the suppressing impact of exosomes by inhibiting the tumor cell proliferation and promoting apoptosis[310-312].

One limitation of exosome treatment is the low yield of exosomes which induces the difficulty of the large-scale production for clinical application. Currently, several investigators contributed to

improving the exosome yield by developing cell culture methods. The technology, such as microcarriers and hollow-fibre bioreactors could be used for the large-scale expansion of cells and consequently, increase the product yield of the exosomes [313, 314] as well as the other cell-free agents (cell extract and conditioned medium). However, there are other challenges that remain. Currently, the purification and isolation of exosomes are not uniform, and the active factors in the exosome complex have yet to be identified. The safety assessments, such as cytotoxicity and side effects, require clarification as well.

Advantages of cell-free therapy

Nowadays, cell-free therapy is widely used to treat various diseases. Indeed, accumulating evidence shows the advantages of cell-free therapy when compared with cell-based therapy. Angeli et al. reported that cell extract treatment improved the function of infarcted hearts without a severe immune reaction in an immune-competent mouse model [271]. It is in agreement with our previous study, which reported that bone marrow cell extract contained fewer histocompatibility antigens than the intact cells[264]. These results indicated that cell-free agents would elicit a weaker immune response. Also, we had reported that cell extract treatment is not patient-specific [162]. Therefore, cell-free therapy provides the possibility of allogeneic treatment in the future. Second, cell-free treatment is theoretically less risks of tumorigenesis. Even though none in-vivo studies reported the increase of cancer cells associated with MSC transplantation, cell-based treatment still has the potential risk to stimulate cancer cells grow or differentiate into cancer cells, while cell-free treatment is theoretically considered safer in this sense [162, 264]. In addition to safety, the cell-free application is more convenient and practical. Our group recently reported that cell extract could be cryopreservation in a -80 °C freezer for 12 months without

protein degradation[234]. Furthermore,1-year cryopreservation did not affect the therapeutic effect of cell extract. Although experiments are needed to develop a more practical strategy for long-term storage, such as liquid nitrogen and lyophilization, the normal cryopreservation results indicated that cell-free agents could be simply stored for up to one year and ready to be used at any time. In contrast, it is difficult to perform treatment with alive cells at any given time. As cryopreservation is inevitable when cells at the appropriate passages are expected to be transplanted at the right time points. As a result, it is time-consuming to thaw and re-expand cells before each treatment. Taken together, we believe that cell-free therapy is a promising approach for the patient suffered from IR-injured SG hypofunction.

Rationale, Hypothesis, and Aims

The following three hypotheses are investigated in this thesis:

Hypothesis 1 -Lyophilization is a useful process to maintain the bioactivity and therapeutic effects of bone marrow cell extract for irradiation-injured salivary glands.

Rationale:

Salivary hypofunction, also known as xerostomia, is one of the prominent sequelae of radiotherapy for head and neck cancer patients. Current treatments of irradiation-injured SG remain palliative. Cell-free therapy had been recently reported to restore the secretory function of irradiation-induced SGs[162]. Our previous studies showed that mouse bone marrow cell extract restored comparably salivary production, increased cell proliferation, protected the acinar cells, parasympathetic nerves and blood vessels, and upregulated repair/ regenerative genes in the SGs post-irradiation[162].

Additionally, native proteins in the bone marrow cell extract are the main effective ingredients for the treatment[264]. However, proteins are not stable for long-term storage and for that reason, they would be degraded quickly once extracted from their native cellular environment. Therefore, cell extract has to be stored in a strict condition or used in a limited period of time. To solve this challenge, we expect to develop a technique for long-term storage of bone marrow cell extract. Lyophilization, a dehydration technique, is widely used to prolong the storage time of the biological products, such as platelet-rich-plasma [315]. The aim is to develop an approach to lyophilize the effective ingredients (proteins) in bone marrow cell extract and to compare the therapeutic effects of the lyophilized and freshly prepared cell extracts in treating the irradiation-induced SG hypofunction.

Hypothesis 2 -Cell extract from cell subpopulations in human bone marrow could mitigate irradiation-injured salivary glands.

Rationale:

Our previous study showed that mouse bone marrow cell extract could restore the secretory function of irradiation-induced SGs[162]. However, the efficacy of the human whole bone marrow cell extract remains unknown. Besides, the whole bone marrow consists of heterogeneous cells, which could be categorized into three major subpopulations, including red blood cells (RBCs), granulocytes (GCs), and mononuclear cells (MCs). The heterogeneity of human bone marrow cells increases the complexity of the cell extract and result in the difficulties to identify and analyze the effective proteins in the cell extract. Therefore, in this study, we aim to 1) test the therapeutic effect of human bone marrow cell extract, 2) preliminarily purify the cell extract via separating the three

major cell subpopulations in human whole bone marrow, 3) unveil the beneficial effect of cell extracts from human RBCs, GCs and MCs (RBCE, GCE and MCE). Narrowing down the complex of bone marrow cell extract would result in a better therapeutic effect and could bring cell extract therapy one step closer to the clinical reality.

Hypothesis 3- Cell extract from human minor salivary glands (labial glands) can rescue the hypofunction of salivary glands.

Rationale:

Adult stem cells play an important role in tissue repair and regeneration. Various adult stem cells have been used to treat the irradiation-induced salivary glands [192, 193, 215]. A study reported that tissue-resident stem cells might be better than the other adult stem cells because they can release growth factors to repair the damaged tissues or differentiate into tissue-specific cells [216]. Indeed, multipotent-specific stem cells isolated from human major SGs could restore SG hypofunction induced by irradiation [204, 215]. However, the procedures to harvest the major SGs are invasive and unpractical for the patients with head/neck cancer. Currently, one research group had isolated the mesenchymal stem cells from human minor SGs and used them as the treatment for liver diseases[316, 317]. Therefore, according to the accessibility and origination, minor SG stem cells would be considered as the candidate cell source for the treatment of SG hypofunction. On the other hand, cell-based therapy provides potential risks, such as tumorigenesis and immunoreaction when compared with cell-free therapy. Moreover, our recent study reported the efficacy of adipose stem cell extract in treating the salivary hypofunction [234]. Our study indicated the efficacy of cell extract from the mesenchymal stem cells. Taken together, the

administration of cell extract from minor SG stem cells might be an optimal treatment in treating the SG hypofunction. In this study, we aim to prepare the cell extract from human minor SG stem cells and to test the therapeutic effect of this novel cell-free therapy for the irradiation-injured SGs.

In this thesis, the C3H or C57BL/6 mouse model of irradiation-injured SG was used to test these three hypotheses. Each chapter is a paper manuscript published in peer-review journals or under preparation for publication.

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Chapter 2 - Lyophilized bone marrow cell extract functionally restores irradiation-injured salivary glands

Preface

Our previous studies showed that mouse bone marrow cell extract could restore comparably salivary production, increased cell proliferation, protected the acinar cells, parasympathetic nerves and blood vessels, and upregulated repair/ regenerative genes in the SGs post-irradiation. We also reported that the native proteins in the bone marrow cell extract are the main effective ingredients for the treatment. However, protein is not stable for long-term storage and easy to degrade once extracted from their native cellular environment.

In this chapter, we developed a lyophilization method to protect the proteins in bone marrow cell extract from degradation. In addition, the components and therapeutic effect of lyophilized and freshly prepared cell extract were compared in vivo. Results showed that the components in lyophilized and freshly prepared cell extract were comparable. Moreover, the functionality of the freeze-dried bone marrow cell extract is also comparable to that of the freshly prepared cell extract in treating the salivary hypofunction in the mouse model.

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Lyophilized bone marrow cell extract functionally restores irradiation-injured salivary glands

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Abstract

OBJECTIVE: Bone marrow cell extract (BMCE) was previously reported to restore salivary gland hypofunction caused by irradiation injury. Proteins were shown to be the main active factors in BMCE. However, BMCE therapy requires multiple injections and protein denaturation is a concern during BMCE storage. This study aims to preserve, by lyophilization (freeze-drying), the bioactive factors in BMCE.

METHODS: We developed a method to freeze-dry BMCE and then to analyze its ingredients and functions in vivo. Freeze-dried (FD) BMCE, freshly-prepared BMCE (positive control) or saline (vehicle control) was injected into the tail vein of mice that had received irradiation to damage their salivary glands.

RESULTS: Results demonstrated that the presence of angiogenesis-related factors and cytokines in FD-BMCE remained comparable to those found in fresh BMCE. Both fresh and FD-BMCE restored comparably saliva secretion, increased cell proliferation, upregulated regenerative/ repair genes, protected salivary acinar cells, parasympathetic nerves, and blood vessels from irradiation-damaged salivary glands.

CONCLUSION: Lyophilization of BMCE maintained its bioactivity and therapeutic effect on irradiation-injured salivary glands. The advantages of freeze-drying BMCE are its storage and transport at ambient temperature.

Keywords: bone marrow cell extract, salivary glands, freeze drying, irradiation, head and neck cancer, lyophilization

Introduction

Recently, a bone marrow cell extract (BMCE) was reported to repair irradiated-injured salivary glands and osteoradionecrotic bone [1-3]. The process to obtain this cell extract consists of three cycles of freeze-thawing at -80°C /+37°C to lyse the bone marrow cells. Then the cell lysate is centrifuged at 17,000 g and the supernatant, named as BMCE or also BM Soup, is aliquoted and injected into the diseased animal [1, 2, 4, 5]. The native proteins were demonstrated to be the main effective ingredients in BMCE[2]. However, proteins can be degraded once extracted from their native cellular environment, and thus current uses of BMCE required freshly-prepared or strict storage conditions to preserve the biological activities of BMCE, such as at -80°C or in liquid nitrogen [2].

To overcome the instability of biological ingredients, a widely used process is lyophilization (also termed as freeze-drying) which is a dehydration technique used for long-term storage of biological products, purification and manufacturing of protein biomolecules [6, 7]. Several studies have indicated that a lyophilized format provides a longer shelf life and functional preservation for cytokines [8], growth factors [9], vaccines [10], and other biological products such as platelet-rich plasma [11]. In addition to protein preservation, lyophilization was reported effective in the long-term storage of DNA samples [12] and of high-quality RNA from tissues [13]. The aim of this study is to test a method to lyophilize (freeze-dry) the protein ingredients in BMCE (such as the growth factors) and to test their functions post-lyophilization in vivo. We hypothesize that the functionality of freeze-dried BMCE (FD-BMCE) is comparable to that of freshly-prepared BMCE in restoring salivary secretory function to IR-injured mice.

Methods

Animals. 6 to 8 weeks old C3H mice were used in this study and approved by McGill University animal care committee.

Preparation of Freeze-dried (FD) BMCE. The preparation of BMCE was similar to our previous reports [1, 2]. For FD-BMCE, aliquots of BMCE (300 µl) were placed in Eppendorf tubes, sealed with parafilm, and frozen overnight in a -80°C freezer. The parafilm was then pierced using a needle and the tubes placed in a VirTis glass jar and set up to the VirTis Benchtop Freeze Dryer (SP Scientific, Warminster, PA, USA) for 12 hours until all the liquid was sublimated and only powder remained in the tube. For the experiments reported here, FD-BMCE powder was stored at -20°C for 2 months, while the fresh BMCE was stored in a -80°C freezer. Protein concentrations in BMCE and FD-BMCE were measured by bicinchoninic acid assay (BCA) following the manufacturer's instructions.

Characterization of fresh and FD-BMCE. Proteome Profile Mouse Arrays (ARY015 and ARY006, R&D Systems, Minneapolis, MN, USA) were used to detect angiogenesis-related factors and cytokines in FD-BMCE and in BMCE. A 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was run at 120V for 60 min, and Coomassie blue staining to visualize the protein fractions in FD-BMCE and BMCE. All the procedures followed the manufacturer's instructions and were described previously in Fang *et al*. (2015)[2].

Irradiation (IR). Female C3H mice were irradiated with a single dose of 13 Gy using the methodology described in Fang *et al*. (2015)[2]. Twenty-four mice were divided into 4 groups (n=6 per group): (1) Sham irradiation group (no irradiation, normal saline injection; Control Group); (2) Irradiation with normal saline injection (NS Group); (3) Irradiation with BM cell extract injection (BMCE Group); (4) Irradiation with FD-BMCE injection (FD-BMCE Group). 100 µl of normal saline, BMCE, or FD-BMCE was injected through the tail vein at 5-7 days

post-IR, twice a week for two consecutive weeks, as described in Fang *et al*. (2015)[2]. Mice were sacrificed after 8 and 16 weeks post-IR.

Measurements. Salivary flow rate (SFR) and lag time measurements were done at week 4, 8, 12 and 16 post IR, as described in Fang *et al*. (2015)[2]. Histological analyses, such as hematoxylin/eosin and PCNA, immunofluorescent staining using aquaporin 5 (to detect acinar cells), smooth muscle actin (myoepithelial cells), cytokeratin 5 (basal ductal cells and a subpopulation of myoepithelial cells), GFRα-2 (parasympathetic nerves), CD31 (endothelial cells), and quantitative real- time PCR (qRT-PCR) for EGF, VEGF, IGF-1R, HGF, FGF2, GFRα2, AQP5, CK5 and GAPDH were done as previously described in Fang *et al*. (2015).

Results

1. Proteins in FD-BMCE were well preserved when compared to fresh BMCE.

Following lyophilization, FD-BMCE appeared as a loose and porous powder (Fig. 1a) that could easily be reconstituted to its liquid form by adding distilled water. The protein concentration of FD-BMCE (2.3±0.38 mg/ml) was statistically not significantly different from BMCE (2.1±0.34 mg/ml) (Fig. 1b). This suggested that no proteins were degraded or insolubilized during the freeze-drying and reconstitution processes. To visualize proteins after lyophilization, Coomassie blue staining was performed (Fig. 1c). The pattern of protein bands was comparable between the fresh and FD-BMCE. This indicated that the proteins, at different molecular weights, remained during the freeze-drying process. Angiogenesis-related growth factors and cytokines in fresh and FD-BMCE were detected by protein arrays (Fig. 1d,1e). The angiogenesis factors (FGF-1, HGF, MMP-8, -9, OPN, SDF-1, PF4, CD26) and cytokines (IL-1ra, IL-16 and TREM-1) detected in fresh versus FD-BMCE were at comparable levels. (Fig. 1d,1e). Normal saline was used as the

negative control. Thus, our protein array analysis demonstrated that growth factors and cytokines from fresh BMCE could be preserved by freeze drying without any loss in quantity.

2. Both fresh and FD-BMCE restored function to IR-injured salivary glands in vivo

Salivary flow rates (SFR) of both fresh and FD-BMCE therapies were comparable in restoring salivary function. At 8, 12 and 16 weeks post-IR, both BMCE and FD-BMCE treatment groups had higher SFR levels (~50%-60%) when compared to the normal saline (NS) vehicle control group (Fig. 2a, $p<0.05$). The lag time for saliva secretion in mice injected with the vehicle control (saline) was longer than that of mice injected with FD-BMCE (Fig. 2b). The histology, gene and protein expression of submandibular gland (SMG) and parotid gland (PAG) were assessed. At week 8 post-IR, PCNA staining indicated that the cell proliferation rate in treated groups were higher than that of the saline group (Fig. 2c, $p<0.05$). The proportion of acinar cells was comparable between the fresh and FD-BMCE groups (64.7%±2.7% and 64.9%±1.3% in SMG, Fig. 2d; 78.5%±4.7% with 81.2%±0.9% in PAG, data not shown, respectively), which were significantly higher than the vehicle control (saline) group (59.9%±2% in SMG, Fig. 2d, $p<0.05$; 70%±1% in PAG, data not shown).

Salivary glands of FD-BMCE treated mice had a higher density of blood vessels (CD31, Fig. 2e) and of parasympathetic nerves (GFRα2, Fig. 2f) than that in the normal saline group ($p<0.05$). Similarly, more cells in the BMCE and FD-BMCE groups were positive for Aquaporin 5 (AQP5, marker for acinar cells), Keratin 5 (CK5, marker for basal ductal cells and some myoepithelial cells) and alpha-smooth muscle actin (α-SMA, marker for myoepithelial cells) when compared to the normal saline group (Fig. 2g-i, $p<0.05$). qRT-PCR results revealed that the expression levels of genes related to tissue repair/regeneration were up-regulated after the BMCE and FD-BMCE

treatment, especially for the expression of EGF and GFRα2 gene (parasympathetic nerves) (Fig. 2j, $p<0.05$).

Discussion

The findings of this study showed that: 1) freeze drying is a reliable approach to preserve the bioactive ingredients of BMCE in a powder form for long-term storage, and this approach will promote the distribution, standardization and transportation of BMCE; 2) FD-BMCE functionally restored IR-injured SGs at a comparable level as fresh BMCE.

Proteins within a solution are usually stored at -80 °C in a freezer or at -196 °C in liquid nitrogen to protect their structures from change [6, 14]. Another issue is the transport of these frozen protein samples requires chemicals such as dry ice or liquid nitrogen to maintain a low temperature. Protein structure and stability can be preserved by removing water molecules; because water accelerates degradative processes [7]. Freeze-drying maintains protein structure and stability during long-term storage and shipping. Storing FD-BMCE is advantageous when compared to storing fresh BMCE. First, freeze-drying is a cost-effective technique for transporting BMCE when compared to current expensive and hazardous chemicals. Second, the protein sample in its solid form can be stored under ambient conditions after freeze-drying [7]. Third, the freeze-dried powder is reduced in volume, which further facilitates BMCE storage and transportation. Fourth, the protein concentration of FD-BMCE can easily be titrated or standardized when reconstituted in its liquid form. For example, we extract BMCE from bone marrow cells of several mouse donors (up to 10 mice totaling 8 ml of BMCE) and at different times; we were able to reconstitute FD-BMCE at a normalized protein concentration for our experiments. Standardization of protein concentrations from a large pool of BMCE donors can thus be done for future human BMCE studies, as is the proposed strategy for platelet-rich plasma [15].

Cryoprotectant/ lyoprotectant is a substance used to minimize freezing damage to a biological sample. Adding lyoprotectants during freeze-drying can provide additional protection to proteins [6]. We decided not to include any additional cryoprotectants/ lyoprotectants to BMCE because it is being injected in vivo to mice, and in the future to patients. Also, the in vivo bioactivity of FD-BMCE was similar to the fresh BMCE, when tested on IR-injured salivary glands. Therefore, there was no need for additional cryoprotectants such as sugars, polyols, polymers and salts added to FD-BMCE. The only salt added to BMCE was the 0.9% sodium chloride (NaCl) from normal saline used to resuspend BM cells during the BMCE processing step.

One limitation of this study was the storage time of only two months for the fresh and FD-BMCE. However, our previous study had reported that BMCE can be stored at -80°C for over 1 year without losing its bioactivity. Thus theoretically, we assume FD-BMCE can be stored for a longer period than its liquid form at -80°C. We have plans to test this batch of FD-BMCE again at 1 and 3 years of storage at -20°C and +4°C.

Conflict of interest

The authors declare no competing financial interests.

Author Contributions

This study was designed by X.S., Y.L., D.F., and S.D.T.; X.S., D.F., Y.L., J.S., G.R., and J.M.K conducted experiments. X.S., D.F., Y.L., and S.D.T. wrote the manuscript. S.D.T. supervised this study and directed the final version of all contents. All authors reviewed and approved the manuscript.

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Figures

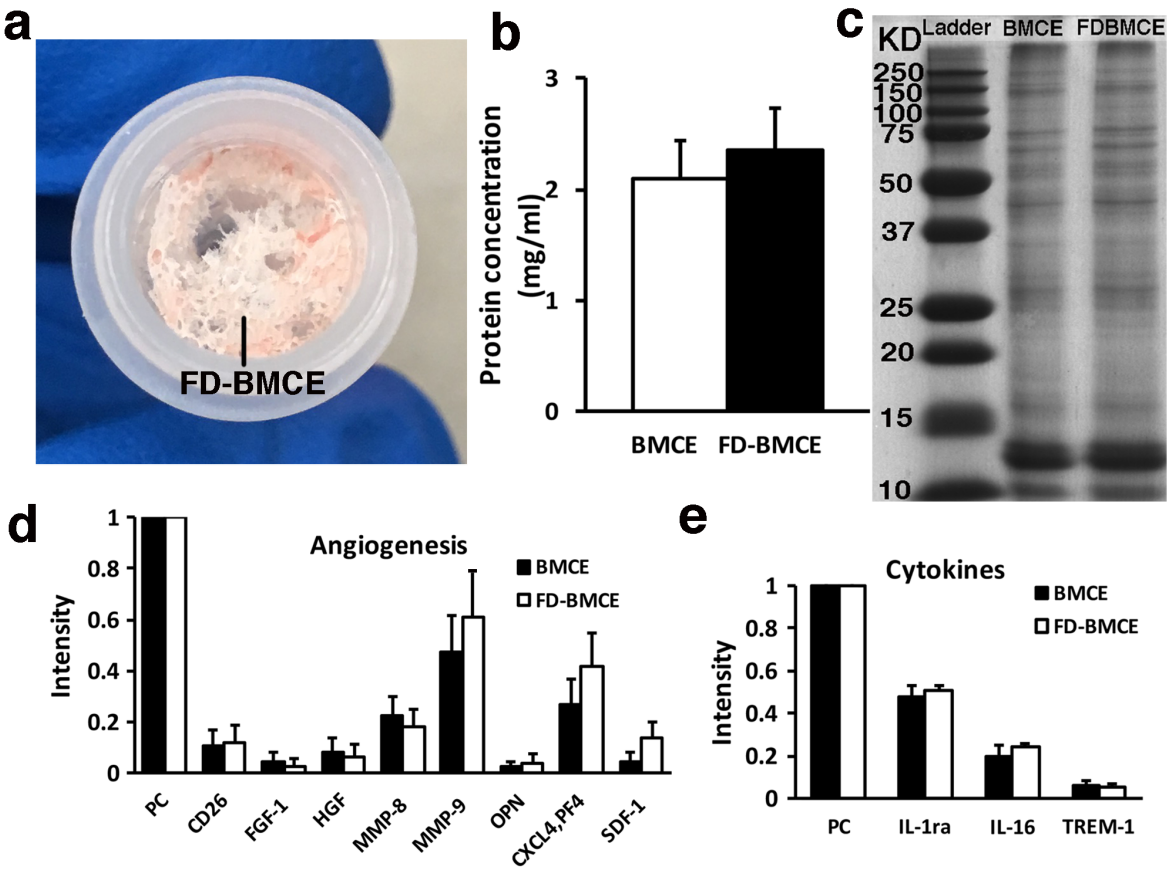

Figure 1. Characterization of FD-BMCE. Proteins in FD-BMCE were well preserved when compared to fresh BMCE. (a) FD-BMCE powder was loose, porous, and easy to dissolve in distilled water. (b) Bicinchoninic acid assay (BCA) was used to compare the total protein concentrations between fresh BMCE and FD-BMCE. (c) SDS-PAGE and Coomassie blue staining were used to visualize the protein patterns and molecular weights between BMCE and FD-BMCE. (d, e) Two protein arrays were used to characterize the angiogenesis-related growth factors and cytokines in BMCE. Relative quantification of angiogenesis-related growth factors and cytokines were analyzed by NIH Image J software. All data were normalized to the positive control (PC). Three independent experiments were performed for each assay. All the data are reported as mean± S.D.

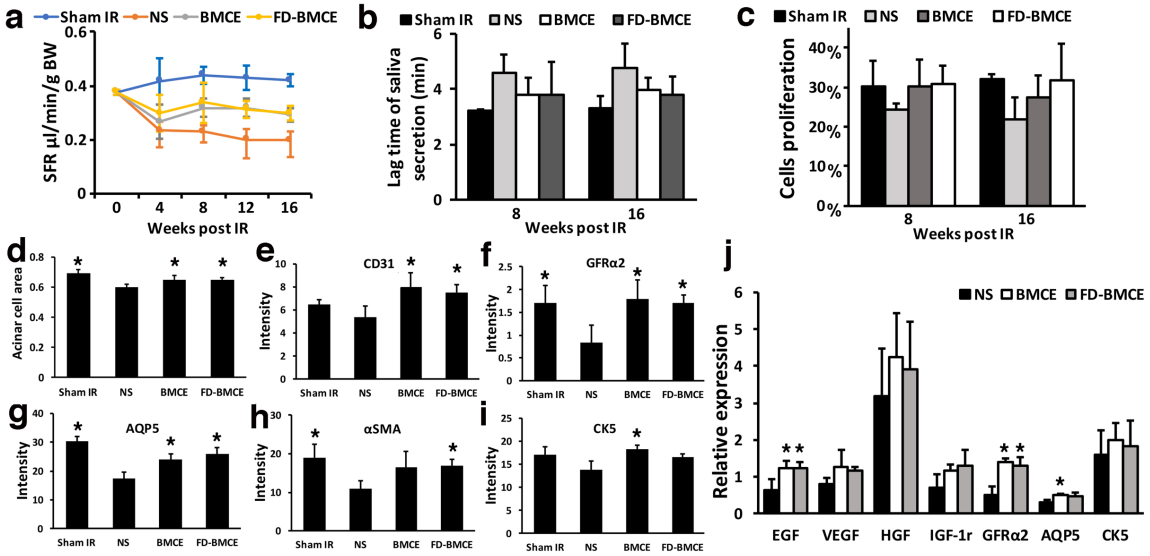

Figure 2. **FD-BMCE functionally restores IR-injured salivary glands.** (a) Salivary flow rate (SFR) was measured by volume of saliva/10min/g of body weight at pre-irradiation, 4, 8, 12 and 16-week post-IR. (b) Lag time is the measure for the first drop of saliva observed in mice after pilocarpine stimulation. Saliva lag time was measured at 8 and 16 weeks post-IR. (c) PCNA assay was used to calculate the salivary cell proliferation rate at 8 and 16-weeks post IR. Five to eight photographs at 400X magnification were counted for each sample with Image J software (NIH). (d-i) Specimens were harvested at 8 weeks-post IR and analyzed with Image J software (NIH) (n=6 mice per group). (d) H&E staining was used to identify the percentage of surface area occupied by acinar cells at 200X magnification, 6 fields/ gland. (e-i) Cells positive for (e) CD31, (f) GFRα2, (g) AQP5, (h) α-SMA and (i) CK5 were detected on frozen sections. Semi-quantification of these proteins through immunofluorescent staining of 6 random fields/ glands at 200X magnification. (j) Relative gene expression levels by quantitative RT-PCR. The expression of GAPDH was used as the endogenous reference. All these gene were normalized to mice from the sham-IR group. All data were presented as mean± S.D, *p<0.05.

Chapter 3- Cell Extract from Human Bone Marrow Mitigates Irradiation-injured Salivary Glands

Preface

Our previous study showed that mouse bone marrow cell extract restored the secretory function of irradiation-induced SGs. However, the components and effect of the human whole bone marrow cell extract remain unknown. Besides, whole bone marrow consists of heterogeneous cells, including red blood cells (RBCs), granulocytes (GCs), and mononuclear cells (MCs). The heterogeneous cells in human bone marrow increase the complicatedness of cell extract and result in the difficulties to identify and analyze the effective proteins in the cell extract.

In this chapter, we preliminarily identified the proteins in human bone marrow cell extract (BMCE) and tested the therapeutic effect of cell extract in restoring the salivary hypofunction. In addition, we separated three major cell subpopulations in the human whole bone marrow into RBCs, GCs and MCs, and prepared them into cell extracts (RBCE, GCE and MCE). Various proteins/growth factors were detected in BMCE, RBCE, GCE and MCE. MCE contained more kinds of angiogenesis-related growth factors when compared with other cell extracts. Four kinds of cell extracts were injected into mice with irradiation-injured SGs. Results showed that MCE treatment provided the best therapeutic effect, GCE and BMCE therapies could also restore the function of SGs, while RBCE treatment did not improve the secretory production when compared with that in the negative control group.

The study presented in this chapter is in preparation for publication.

Cell Extract from Human Bone Marrow Mitigates Irradiation-injured Salivary Glands.

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Abstract

A mouse bone marrow cell extract (msBMCE) has the property to restore saliva secretion to irradiation (IR)-injured salivary glands (SGs). However, to bring this potential therapy to the clinic, a cell extract originating from human bone marrow(BM) needed to be tested, which was the first objective of this study. In addition, the heterogeneity of BM cells would impact on the effect of BMCE treatment. Therefore, to improve the efficacy and unveil the active ingredients in BMCE, the effect of cell extracts from three BM cell-subpopulations was investigated as the second aim. The minor aim was to test the effect of human cell extract from different genders.

Human bone marrow cell extract (BMCE) was obtained from three-freeze-thaw cycles. Subpopulations, such as red blood cells (RBCs), granulocytes (GCs) and mononuclear cells (MCs) of BM were separated with density centrifugation (Ficoll-Paque) and the extracts from each of these cell subpopulations were also obtained (RBCE, GCE, and MCE). Protein arrays were used to identify the factors from BMCE, RBCE, GCE, and MCE. These cell extracts were injected into an IR-injured SG mouse model.

Human BMCE was as effective as msBMCE in treating the salivary hypofunction although their protein compositions were identified as different. MCE contained most angiogenesis-related growth factors than that in BMCE, GCE and RBCE. Human BMCE, GCE and MCE significantly restored 50%, 60% and 73% of saliva secretion when compared with that in the control group. Furthermore, BMCE, GCE and MCE protected acinar cells, blood vessels, parasympathetic nerves, and increased cell proliferation. However, significant higher acute inflammation factors were detected in GCE-treated mice. Lastly, the gender of bone marrow donors did not influence the outcomes.

In conclusion, human BMCE could restore the function of IR-injured SGs. MCE provided the best therapeutic effect with less immune reaction and no gender limitation and showed the clinical potential to IR-injured SGs.

Introduction

Radiotherapy is a common treatment for millions of head and neck cancer patients worldwide. However, irradiation (IR) would cause symptoms such as xerostomia. New minimally invasive techniques such as intensity modulated radiation therapy (IMRT) [1, 2] are being developed to protect the salivary glands (SGs) from co-irradiation damage. Still, SGs are being injured due to factors such as tumor location and grade [3-5], especially acinar cells [6], which are the main site of salivary secretion. Current treatment options for the IR-induced SG hypofunction, such as pharmacological drugs (Sialagogues) and salivary substitutes are palliative[7], and result in a limited and ineffective therapeutic effect[8-10]. Current knowledge in restoring saliva secretion comes from experimental approaches, such as gene therapy[11-14], tissue engineering[15, 16], reimplantation of autologous SG cells[17, 18], and stem cell-based therapy [19, 20]. However, each approach has its limitations. For example, the vector used in gene therapy may create an immune reaction[21], and cell transplant carries the risk of thromboembolic complications or possible cell rejection [22]. Recently, a newly developed strategy is cell-free therapy/cell extract therapy. When compared to cell-based therapies, cell extract treatment is theoretically less tumorigenic and immunogenic since it only contains soluble contents lysed from the whole cells [23, 24]. Our previous study reported that the administration of cell extract from mouse bone marrow (msBMCE) was as effective as the treatment of bone marrow (BM) whole cells in restoring secretory function post-IR [25]. In addition to the therapeutic benefits, cell extract infusion solves the problem of low cell survival during cell transplantation[26]. Additionally, cell extract has other advantages that its application is more convenient and practical. Fang et al. revealed that cell extract can be stored for more than one year and can be used directly after thawing [27]. Furthermore, lyophilized cell extract provides long-term storage without protein

degradation and it is more convenient for transportation and ultimately reduced the cost of cell-free treatment [28]. Cell extract provides an "off-the-shelf" availability while the cryopreservation and constant care are inevitable for classical cell treatment.

In order to bring this potential therapy to the clinic, a cell extract originating from “human” bone marrow needed to be tested. The first objective of this study was to test the therapeutic effect of the cell extract from human bone marrow (BMCE) on IR-injured SGs in a mouse model. However, human bone marrow cells are heterogeneous which would increase the complexity of the compositions in cell extract and result in the difficulty to unveil the mechanism behind the cell extract treatment. Therefore, it is necessary to separate the cell subpopulations of human whole bone marrow and to test whether the therapeutic effect observed could be from a specific cell sub-population. To study this, we preliminarily separated human whole bone marrow into three majority types of cells fractions: 1) red blood cells (RBCs), 2) granulocytes (GCs), and 3) mononuclear cells (MCs). The extracts from each cell subpopulation (RBCE, GCE, and MCE) were tested for their therapeutic efficiency in restoring salivary function. Finally, as a minor objective, we compared the effect of the cell extracts from male versus female donors to study if gender would be a factor influenced the outcomes. In summary, our objectives were to test the effect of human BMCE and investigate whether a specific cell fraction in bone marrow contributed the most to repair IR-injured SGs, and with a minor aim to compare the gender of bone marrow donors.

Herein, we revealed that human BMCE treatment provided a beneficial effect in restoring the function of IR-injured SGs. Moreover, we preliminarily identified the growth factors in human

BMCE, RBCE, GCE and MCE and unveiled that the cell extract from mononuclear cells acted as the major effective constituents in human BMCE, while RBCE did not significantly repair the damage in SGs. Although GCE treatment repaired the IR-injured SGs, it resulted in an acute inflammatory response. Last, the genders of donor did not affect the effect of cell extract from human bone marrow. These findings indicated the promise of MCE therapy as the potential therapeutic clinical application with many advantages described here, 1) a best therapeutic efficacy in restoring the function of IR-damaged SGs, 2) less immunologic rejection, 3) an accessible cell resource, 4) no gender limitation, 5) easy for storage and transport.

Materials and Methods

Subpopulation cells isolated from human BM

Human bone marrow samples (n=4, 2 male and 2 female, 20-34 years old) were purchased from the company (Lonza, Walkersville, MD, USA). Ficoll-Paque PLUS density gradient media (GE Healthcare Life Sciences, Marlborough, MA, USA) and RBC lysis buffer for human (J62990, Alfa Aesar, Tewksbury, MA, USA) were used to separate human bone marrow samples into three fractions: red blood cells (RBCs), granulocytes (GCs) and mononuclear cells (MCs). The procedures were performed following the manufacturer's instruction. Briefly, human bone marrow was diluted with an equal volume of balanced salt solution and carefully layered over the Ficoll gradient media. After centrifugation at 400g for 40min with Ficoll-Paque PLUS at room temperature, BM sample was separated into 4 layers (from top to bottom): plasma layer, mononuclear cell layer, Ficoll-Paque media layer and granulocytes and erythrocytes layers. The upper layer containing plasma and platelet was drawn off and the mononuclear cell layer (white second layer) was left at the interface and transferred to a new tube. After carefully drawing off

the third layer with Ficoll-Paque media, the undisturbed fourth layer included GC enriched layer and RBC fraction could be obtained. The GCs were collected from a thin white cell layer above the RBC layer and transferred to a new tube, then RBC lysis buffer was added to lyse the RBCs mixed with GCs. Meanwhile, RBCs were harvested from the cells remained in the original tube. All of the isolated cells were ready to use after three times washing with normal saline.

Preparation of mouse bone marrow cells

Mouse bone marrow cell extract was prepared as described previously[23]. Briefly, BM cells were flushed from tibias and femurs from C57BL/6 mice with PBS. After centrifugation, the cell suspension was filtered through a 70μm cell strainer, flowed by 400g centrifugation for 5min.

Preparation of the cell extracts

Cell extract was prepared as our previous study described[23, 25]. In brief, cells were resuspended with 0.9% saline to the concentration of 10^7 cells /100 μl. Then three cycles of freeze (-80°C) and thaw (37°C) were performed to lyse the cells. After centrifugation at 17,000g for 30min at 4°C, supernatant cell extract was stored at -80°C.

Characterization of human cell extract from BM, RBCs, GCs and MCs

The total protein concentration was measured by the bicinchoninic acid assay (23225, BCA: Thermo Scientific, Pierce, IL, USA). All procedures were according to the manufacturer's instruction. The concentration of each cell extract was adjusted to 2mg/ml with normal saline.

To profile the angiogenesis-related factors in BMCE, RBCE, GCE and MCE, the Proteome Profile Human Array (ARY007, R&D Systems, Minneapolis, USA) was conducted. According to the manufacturer's protocol, 200µl of cell extract/ saline was incubated with biotinylated detection antibodies and the chemiluminescence was detected using the ChemiDoc™ Touch Imaging System (Bio-Rad). The semi-quantification of the relative intensity in the membrane was calculated by Image J software (NIH). Saline solution was used as the background (negative) control.

Animals and irradiation

According to the protocol imposed by the Canadian Council on Animal Care, all the experiments with animals were approved by the University Animal Care Committee (UACC) at McGill University (Approved protocol #5330, www.animalcare.mcgill.ca). Eight weeks old C57BL/6 mice were purchased from Charles River (Montreal, QC, Canada).

According to our previous study, the irradiated mice model was developed[23]. C57BL/6 female mice were anesthetized with 0.3 µl/g body weight of 60 mg/ml Ketamine and 8 mg/ml Xylazine (02239093, Novopharm, Toronto, Canada) diluted in saline. 13 Gy irradiation was slit-collimated to the salivary glands (head and neck area) performed by a clinical 6MV accelerator on typically 5 mice simultaneously at 5.5Gy/min, as determined using small-field detectors. No more than 3% out-of-field dose affected on the surrounding tissues area.

Ninety-six mice were divided into 7 groups (n=12-15): (1) Sham IR group (no irradiation, no injection); (2) Saline group (IR + normal saline intravenous injection); (3) BMCE group (IR +

human BMCE intravenous injection); (4) RBCE group (IR+ human RBCE intravenous injection), (5) GCE group (IR + human GCE intravenous injection), (6) MCE group (IR + human MCE intravenous injection), (7) msBMCE group (IR+ mouse BMCE intravenous injection). According to the grouping, mice were injected with 100μl normal saline/ cell extract through tail vein at 5~7 days post-irradiation, once a week for two consecutive weeks. Mice were sacrificed at 3/ 24 hours after treatments or 8/16 weeks post-IR in each group. Human cell extracts (male, female or mixture with two genders) were injected randomly into mice, respectively.

Salivary flow rate (SFR) and lag time measurement

0.3 μl/g body weight of 60 mg/ml Ketamine and 8 mg/ml Xylazine were used as the anesthetic to measure the salivary flow rate (SFR) of mice at week 4, 8, 12and 16 post-IR. As previously described[25], saliva was collected in the following 10 minutes after the stimulation by 0.5mg/kg body weight of pilocarpine (P6503, Sigma-Aldrich, ST. Louis, USA). We assumed the density of saliva is 1g/ml, which means the weight of saliva can represent the volume of it. In addition, the lag time of the first saliva secretion was measured to analyze the function of the salivary gland.

H&E staining

Specimens were fixed in 4% paraformaldehyde (P6148, Sigma-Aldrich) and embedded in paraffin. Each sample was sliced into 8 μm thickness followed by the staining with Hematoxylin and Eosin (H&E). The magnification was 200x, and 5-8 fields were random choose for counting. NIH Image J software was used to calculate the percentage of the surface area of acinar cells/ the whole tissue area.

PCNA staining

Cell proliferation in SG tissues was detected by PCNA staining. Samples in paraffine were sliced into eight-micrometer sections. After deparaffinization and rehydration, heat-induced antigen retrieval was performed for 30 min at 95 °C with 10 mM Citrate Buffer solution (pH 6.1) and cooled down for 30 min at room temperature. Then, Immunohistochemistry (IHC) process was performed as the manufacturer 's instruction. Briefly, slices were blocked with 10% goat serum for 1 hour at room temperature. Primary antibody, rabbit anti-PCNA (1:500, ab92552, Abcam, Cambridge, MA, USA) or PBS (negative control) was incubated overnight with SG tissues at 4°C. Then slices were incubated with secondary antibody (1:200) for 1 hour at room temperature. The secondary antibody was Goat anti-rabbit-IgG (HRP) (ab 97051, Abcam). DAB (DAB Substrate Kit, ab64238, Abcam) was incubated with SG tissues for 3 min, followed by rinsing in water. Then hematoxylin staining was performed followed by dehydration in an ethanol series. The number of positive cells was counted under 400 x magnification of 8 fields/gland by Image J software.

Immunofluorescent (IF) staining

Submandibular gland and parotid gland were embedded into the optimal cutting temperature (OCT), and cut into 6-8μm thickness sections. After fixing with 4% paraformaldehyde (P6148, Sigma-Aldrich, ST. Louis, USA) for 15 min, slides were blocked with 10% donkey serum for 1 hour at room temperature. The primary antibodies were as follows: rabbit anti-aquaporin 5 (1:200, AQP5, ab92320, Abcam, Cambridge, MA, USA); mouse anti-alpha smooth muscle actin (1:200, α -SMA, ab7817, Abcam); rabbit anti-cytokeratin 5 (1:400, CK5, Sigma-Aldrich, Oakville, ON, Canada); goat anti-GFRα -2 (1:200, AF429, R&D Systems, Minneapolis, USA); goat anti-CD31

antibody (1:200, AF3628, R&D Systems, Minneapolis, USA); PBS was used as negative control. SG tissues were incubated overnight with primary antibodies or PBS at 4°C. After 3 times washing with PBS, slides were incubated with secondary antibody (1:200) for 1 hour at room temperature. Secondary antibodies were donkey anti-rabbi-Rhodamine Red™-X- conjugated, anti-mouse-Alexa Fluor® 594-conjugated, anti-goat-Alexa Fluor 488-conjugated. 4, 6-diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen, Ottawa, ON, Canada) was used to label the nucleus of the cell. Leica DM4000 fluorescent microscope was used to take 6 pictures for each sample, and the intensity of the fluorescence signal was analyzed by ImageJ software (NIH).

Quantitative real- time PCR (qRT-PCR)

Total RNA was extracted from the SGs with TRIZOL reagent (15596018, Invitrogen, Carlsbad, CA). First strand cDNA was synthesized from 50ng RNA per sample with the High-Capacity cDNA Reverse Transcription Kit (4368814, ThermoFisher Scientific, MA02451, USA). Triplicate qRT-PCR assays were performed by Step One Plus (Life Technologies) in TaqMan Universal Master Mix II (4440040, Applied Biosystem, Foster City, USA). The probes used in this study were EGF (Assay ID: Mm00438696), VEGF (Assay ID: Mm01281449), IGF-1R (Assay ID: Mm00802841), HGF (Assay ID: Mm01135193), FGF2 (Assay ID: Mm01285715), BMP7 (assay ID: Mm00432102), NGF (assay ID: Mm00443039), MMP2 (assay ID: Mm00439498), and AQP5 (assay ID: Mm99999915). Glyceraldehyde- 3-phosphate dehydrogenase (GAPDH, Assay ID: Mm99999915) was used as the endogenous reference. The cycles were programmed as follows: 50°C for 2 min, 95°C for 20 s, then 40 cycles at 95°C for 1 s and 60°C for 20 s. Results were expressed as fold changes in relative gene expression.

Enzyme-linked immunosorbent assay (ELISA)

Mouse ELISA Kits (Abcam, Canada) were used according to the manufacturer instructions to quantify pro-inflammation cytokines (interleukin (IL)-6 and serum amyloid A (SAA)) and anti-inflammation cytokines (interleukins-10 (IL-10) and interleukins-1ra (IL-1ra)) in mouse serum. All assays were performed in duplicates, the optical density was determined by Bio-Tek EL800 Universal Microplate Reader at 450 nm and averaged concentrations were calculated for each duplicate.

Statistical analysis

SPSS version 19 software (IBM, USA) was used to perform the statistical analysis. All data are presented with mean ± SD. Student's t-test or one-way ANOVA with Turkey's Post-Hoc was used to determine statistical differences ($p<0.05$) among each group.

Results

Characterization of human BMCE and cell extracts isolated from BM sub-populations

In order to preliminarily profiled the proteins in human BMCE and cell extracts of BM sub-populations, a protein array of angiogenesis-related growth factor was performed (Fig. 1). The results demonstrated that different cell extracts contained different protein components. Several constituents in human BMCE were not detected in mouse BMCE, such as TSP-1, pentraxin3, PAI-1, ET-1, Angiopoietin-2, Persephin and VEGF (Fig. 1a). On the other hand, MCE contained the most growth factors, including pro-angiogenetic factors (MMP-8, -9, VEGF, Angiopoietin-2, uPA, IL-8, Artemin and ET-1), anti-angiogenetic factors (TSP-1, PF4, TIMP-1, PEDF and PAI-1), and biphasic angiogenetic factors (TGF-β1 and PTX3). Whereas uPA, TGF- β1, IL-8, TSP-2 or FGF-

1 were not detected in RBCE, and VEGF, PEDF, CD26 or EG-VEGF were not detected in GCE. Although many of the detected growth factors were all presented in BMCE, RBCE, GCE and MCE, the concentrations of them were different. For example, a higher level of TSP-1 and a lower level of Artemin and Persephin were detected in RBCE than those of in GCE. These results suggested that different pro- and anti-angiogenesis-related factors co-exist in BMCE, RBCE, GCE and MCE and resulted in a different therapeutic effect when injected in IR-injured SG animal model.

Human BMCE functionally restore IR-injured SGs in vivo

Our study firstly evaluated the efficacy of human BMCE in repairing the SG damaged by IR. One of the main findings in this study was that human BMCE was effective in repairing IR-injured SGs. Human BMCE-treated mice had a 50% and 43% increase in SFR at week 8 and 16 post-IR when compared to IR-mice injected with saline (negative control group) (Fig. 2a, $p<0.05$). The proportion of acinar cell area was measured by H&E staining at week 8 post-IR (Fig. 2c). The percentage of surface occupied by acinar cells was increased (68.1%± 5.1% in PAG and 59.0± 4.5% in SMG) in BMCE group versus saline group (59.4%± 9.1% in PAG and 50.1%± 5.7% in SMG). Cell proliferation ability was analyzed by PCNA staining (Fig. 2d). The proliferation cell rate in BMCE group (22.7%± 4.4%) was significantly higher than that in the saline group (13.4%± 4.9%, $p<0.05$). Blood vessel endothelial cells (CD31) and parasympathetic nerves (GFRα2) were localized and quantified by immunofluorescent staining. In addition, the other three functional cells in SGs, the acinar cells (AQP5), ductal cells (CK5) and myoepithelial cells(α-SMA) were measured in the study. Results showed that BMCE treatment resulted in a ~2.5 folds higher blood vessel density and 2 folds higher innervations when compare to the group without treatment.

Similarly, human BMCE-treated mice had a higher number of cells positive to AQP5, CK5 and α-SMA markers, especially the cells in the PAG when compared to the untreated mice (Fig.4b-f, $p<0.05$). Meanwhile, all the genes detected in this study were up-regulated after the BMCE treatment (Fig. 5a). The gene expression of growth factors involved in SG repair/development (EGF, IGF1r, and BMP7), blood vessels and nerves repair/regeneration (MMP2 and NGF) were significant up-regulated (Fig.5a, $p<0.05$). The ELISA results showed that the concentration of pro-inflammatory cytokines in human BMCE, mouse BMCE and saline-treated mice were comparable (Fig. 2e, f). Human BMCE restored the function of IR-damaged SGs with no additional acute inflammatory reaction.

The effects of cell extracts isolated from Sub-population of human bone marrow were different

The second part of this study compared the effect of cell extracts lysed from three kinds of human bone marrow subpopulations, including RBCE, GCE and MCE. At week 8 post-IR, MCE treated-mice had a 73% improvement of SFR when compared to saline group (3.12±0.72 μl/g versus 1.8±0.20 μl/g, $p<0.05$), while GCE-treated group had a 60% increase of SFR (2.88±0.49 μl/g, $p<0.05$). The RBCE-treated mice did not have a statistically significant increase of SFR than that of saline-treated mice (31% more SFR, 2.36±0.55μl/g, $p>0.05$). At week16 post-IR, MCE-treated mice still had their SFR 50% increase ($p<0.05$), while the improvement in GCE group reduced rapidly to16% and SFR maintained at 28% increase in the RBCE group (Fig. 3a). Additionally, the lag time of saliva secretion was significantly reduced in the MCE group when compared to the untreated group at week 8 post-IR (Fig. 3b). These data indicated that MCE

treatment restored the secretory function of SGs in vivo lasted for 16 weeks and GCE had a relatively short efficacy, while RBCE was not superior in restoring the function of IR-SGs.

MCE and GCE protected diverse cell populations in IR-SMG and -PAG. Firstly, PCNA staining results showed that the percentage of proliferation cells in SMG was higher in the MCE group (25.2%±1.0%) versus that in the saline group (13.4%±4.9%, Fig. 3c, $p<0.01$). H&E staining revealed a higher occupied area of acinar cells in both PAG and SMG in GCE and MCE groups when compared with that in the saline group (Fig 3d, $p<0.05$). Furthermore, MCE-treated mice had a significantly higher acinar cell area (75.5%±0.9%) than the mice treated with RBCE (64.1%±4.5%) in PAG ($p<0.05$). MCE and GCE-treated groups were found to protect both parenchymal (acinar, ductal) and stromal cells (endothelial, myoepithelial, nerves) in irradiated-injured SGs (Fig 4). Whereas only the expression of α-SMA was increased in RBCE-treated group (Fig 4d, $p<0.05$). Lastly, gene expressions were up-regulated in the treated groups (Fig 5a). Specifically, gene expression of IGF1r, BMP7 and MMP2 were significantly up-regulated in both MCE, GCE and RBCE groups ($p<0.05$). Besides, HGF, AQP5 and FGF2 genes were up-regulated in the MCE group ($p<0.01$), and higher expression of EGF gene was detected in the GCE group.

Both pro-inflammatory (IL-6 and SAA) and anti-inflammatory (IL-10 and IL-1ra) plasma cytokine concentrations were measured by ELISA. The IL-6 concentration was slightly increased ($p>0.05$) in cell extract-treated groups at 3hr post-injection when compared to that in the saline group, and it reduced and became comparable to that in the Sham IR group at 24hr (Fig. 5b). Another inflammatory cytokine, SAA was significantly higher in GCE group ($p<0.001$) at 24hr post-injection (Fig. 5c). On the other hand, GCE-treated mice had a higher concentration of IL-10

($p>0.05$) and a significantly higher concentration of IL-1ra ($p<0.05$) at 3hr post-stimulation of injection. While the other treatment groups (human BMCE, MCE, RBCE) showed comparable results when compared with the saline group (Fig. 5d, e). These results indicated that the immunogenicity of BMCE would concentrate into GCE, while MCE, RBCE and BMCE were comparable safer, although MCE induced a mild acute inflammation when utilized in the mouse model. Taken together, cell extract from mononuclear cells acted as the major effective constituent in human BMCE, while RBCE did not restore the function of IR-injured SGs. GCE repaired the secretory function in SGs, but it resulted in an acute inflammatory response.

BMCE from both male and female donors was equally effective in treating salivary hypofunction

The third part of the study evaluated the efficacy of cell extracts from male and female donors. The data demonstrated that cell extracts harvested from female and male were both effective in repairing IR-injured SGs (Fig. 6). This finding suggested that the whole bone marrow could be harvested from both male and female. Both of them would induce a comparable efficacy in repairing irradiated SGs.

Discussion

The findings of this study were 1) human BMCE was as an effective treatment as mouse BMCE in repairing irradiated-SG in mouse model; 2) the protein constituents in the three subpopulations of human BMCE (i.e. MCE, GCE and RBCE) were variable, with the MCE having higher concentrations of angiogenic factors; 3) MCE had the best therapeutic effects when compared to GCE and RBCE; 4) gender of the donors did not influence the therapeutic outcomes.

Wide varieties of proteins were co-existed in BM sub-population cell extracts and resulted in different beneficial effects in restoring the function of IR-injured SGs. VEGF, an angiogenic factor with neurotrophic and neuroprotective effects[29], was detected in MCE with a relatively higher concentration when compared to the other cell extracts. Studies reported that other angiogenic factors were required as the complement of VEGF to promote vessel maturation, because the neovessels were unstable when the sole use of VEGF [30, 31]. It implies that the synergistic effect of diverse proteins played a role during the treatment and the therapeutic effect was attributed to the interactions of multiple growth factors in cell extract rather than one or two vital factors. In addition to angiogenesis growth factors, several neurogenesis factors were found in BMCE, GCE and MCE, such as the PTX3[32], Persephin (PSP) and Artemin (ARTN). The neovascularization and innervation induced by the growth factors had been suggested to be involved in functional salivary gland regeneration in the wound SMG mouse model[33]. Similarly, our results demonstrated that the expression of CD31 and GFRα2 were up-regulated in the BMCE, GCE and MCE groups (Fig. 4). Interestingly, a higher concentration of anti-angiogenesis factors (PEDF, TIMPs, TSP-1and PAI-1) was detected in MCE. In general, these factors inhibit cell proliferation, migration, and angiogenesis [34-39]. However, they showed diverse benefits in treating the IR-induced SG hypofunction. As an example, most of these anti-angiogenic factors are the natural inhibitor of tumorigenesis [35-37], which is a main advantage for head and neck cancer patients. Fang et al. reported that cell extract from bone marrow, which contained pro- and anti-angiogenesis factors did not promote the tumor cell proliferation [23, 27]. One reason may be the modulated interactions of anti-angiogenic growth factors. Additionally, the effect of such growth factors is complex that they have pleiotropic biological activities. For example, PEDF possesses the

neurotrophic activity[36], while TSP-1 is an inhibitor for inflammation [34, 35], and they play a role in repairing injured-tissue. Together, these results suggest that the therapeutic effects of different cell extracts are modulated by multiple factors as well as their interactions (cooperation and antagonism), and these factors are part of the complicated SG regeneration process.

In general, the immunocompromised or immunodeficient animal was commonly used for human cell transplantation. However, immunocompetent mouse was utilized as our animal model in the present study. The first reason is that human cell extract therapy had been reported as a relatively safe treatment for the xenograft. A study reported the immunocompetent mouse as a myocardial infarction model to test the therapeutic effect of human BMCE and MCE transplantation[24]. The results turned out to be positive with the improvement of cardiac function and without severe immune responses. It implied that the cell lysate treatment is safer with less immunogenic risk than the intact cell infusion. It also provided a possibility for the clinical allotransplantation of cell extract. The other reason for our animal model is that the immunocompetent mouse with the completed immune system is more clinically translatable to the healthy human when compared with the immunocompromised animals. Therefore, the immunogenicity of cell extract can be analyzed in this model. Meanwhile, considering the comparability of our previous results with mouse BMCE, we decided to keep the same animal model with our previous studies.

Our previous studies reported that mouse BMCE treatment preserved the salivary gland function and up-regulated the expression of repair/regeneration related genes and proteins in SG-injured model [23, 25]. In order to bring this potential therapy to the clinic, a cell extract originating from “human” bone marrow is essential to be tested. In the present study, the mouse BMCE was

considered as positive control. According to our results, human BMCE significantly increased the SG function, but the improvement of SFR and the cell proliferation rate were slightly lower than that in the mouse BMCE group. One reason could be the kinds of proteins and the protein constituents were different in human and mouse BMCE (Fig 1a). These differences might due to the diversity of different species and different cell harvest methods. In our study, around 40-50% of the marrow karyocytes were counted in the mouse BM while approximate 0.3-0.5% karyocytes were counted in the human BM samples (data not shown). These results indicated that the frequency of cell fractions in the mouse and human BM were different, and the concentration of the effective proteins in human BMCE might be lower. On the other hand, the acute inflammatory of human BMCE-treated mice was comparable to that in mouse BMCE-treated mice. This result suggested that human BMCE did not induce the severe immune responses in the mouse model and cell extract strategy would reduce the immunogenicity when compared with the cell-based therapy.

Human whole bone marrow contains heterogeneous cells, including red blood cells (RBCs), granulocytes (GCs) and mononuclear cells (MCs). These cells increase the complexity of BMCE and the difficulty for further studies, such as proteomics and RNA sequencing analyses. Therefore, the separation of whole BM cell subpopulations would be the first step to investigate the mechanism behind the cell extract treatment. Besides, Assmus et al. reported that the efficacy of MC therapy was impaired by the contamination of RBCs [40]. It implied that the ineffective cell extract fraction in BMCE may reduce the benefits of treatment and "purified" BMCE would induce a better efficacy. Meanwhile, a study reported that different fractions of bone marrow cells brought different outcomes in treating the infarcted heart [41] and promoting the myocardial regeneration[42]. Hence, there appears to be a cell extract treatment with a better efficacy from a

specific cell fraction in BM, such as GCE and MCE. The separation approach would also allow choosing the best cell extract product according to their efficacy. Currently, unselected mononuclear bone marrow cells (MCs) are commonly used in cell transplantation therapy [24, 43-45]. Meanwhile, the granulocyte colony stimulating factor (GCSF), which could be secreted by GCs had been reported to improve the cardiac function post-myocardial infarction [46]. These results suggested that different fractions in whole bone marrow would show various benefits. Taken together, it is therefore necessary to separate different fractions in human BM and test the potential of these cell extracts derived from GCs, MCs and RBCs.

The therapeutic effects in different BM subpopulation cell extract groups varied in the present study. For example, 73% improvement of secretory function at week 8 post-IR was observed in MCE group, while 60% and 50% were observed in GCE and BMCE groups, and 31% was found in RBCE group (Fig. 3a). Furthermore, gene expressions of FGF2 and MMP2 in the MCE group were significantly up-regulated when compared with that in BMCE and RBCE groups (Fig. 5a). These results indicated that MCE was the specific cell extract in terms of the active ingredients in BMCE. On the other hand, although BMCE contained a low percentage of MCE, less therapeutic effect was observed in BMCE group than that in MCE group. These results suggested that the concentration of the MCE directly influences the effect of treatment and a relatively low concentration of MCE may be still effective in treating the IR-injured SGs.

MC population is a heterogeneous fraction comprised of diverse cell types, including the differentiated cells (lymphocyte and monocyte) and progenitor cell populations. Autologous bone marrow-derived MC transplantation was proved as a safe and effective approach for the patients

of traumatic brain injury [43], spinal cord injury[47], myocardial infarction[48], and cardiomyopathy[49] in the clinical trials. Research reported that instead of the small number of stem cells in MCs, the beneficial effects of MC treatment might be due to the combined effects of all mononuclear cells[48, 49]. For example, the lymphocytes from MCs played a role in the process of vessel formation as numerous factors secreted by lymphocytes were involved in the angiogenesis in vivo [50]. Meanwhile, MC treatment has benefits when compared with the other cell source treatments. First, diverse cell types with multipotent capacities are relatively concentrated in adult BM-derived MCs, such as hematopoietic progenitor cells, endothelial progenitor cells, mesenchymal stromal cells and small embryonic-like stem cells[44, 51-54]. Second, it can be easily harvested and used with minimal processing when compared with cultured cells [42, 55, 56]. Moreover, when incorporated with the cell-free strategy, MCE therapy would become a more feasible and convenient treatment for the clinical application as it would induce less immune reaction and provide a possibility for long-term storage. With this knowledge in mind, our study proposed an effective, safe and the most readily available treatment for the salivary hypofunction: MCE therapy.

To test the immunogenicity of cell extracts, the pro- and anti-inflammatory factors were assayed. Mice in the GCE group had an acute inflammation factor (SAA) increase at 24hr post-injection ($p<0.05$), while a mild increase was detected in the MCE group when compared with the saline group (Fig. 5c, $p>0.05$). On the other hand, our results revealed a higher anti-inflammatory factor expression (IL-10 and IL-1ra) at 3hr post-GCE injection (Fig. 5d, e). It indicated that GCE was involved in immunomodulation via upregulating the IL-10 and IL-1ra. Studies reported that the lumbar puncture delivery was superior to intravenous delivery because of a less immune response

and a better tissue sparing for spinal cord injury[57]. Thus, the intra-glandular injection delivery route could be a possible approach to reduce the immunogenicity and increase the effects of the treatment[25]. On the other hand, a clinical trial with MC autogenous transplant for spinal cord injuries reported a mild adverse reaction (e.g. fever and headache) manifested in the first week after the therapy and become normal later with mediations[47]. Meanwhile, Cox et al. reported no serious complications in organs after the autologous transplant of human MCs for the patients with traumatic brain injury, but there was a dose-dependent pulmonary toxicity with a low-level lung injury[43]. These findings imply that the mildly acute reactions would appear even for the autologous MC infusion in a short period (a week) and a lower dose of MCs treatment was safer. Similarly, our results revealed that BMCE, consisted of a lower concentration of GCE and MCE induced a comparable level of inflammation factors when compared with that in the negative control (saline) group. This result further confirmed that the acute inflammatory could be possibly avoidable or minimized by dose optimizing. Furthermore, the human cell extracts from BM were utilized in the mouse model in the present study. Theoretically, the immunoreaction might reduce when human-derived cell extracts were autologous or allogeneic transplanted to the patients. The maximal effect with minimal immunoreaction can be achieved in the future by optimizing the concentration of the cell extract therapy. Hence, our cell extract treatment (e.g. MCE) is a promising alternative to the cell-based therapy for the patients in the future with a lower level of acute inflammatory and better efficacy.

To our knowledge, there is no study using the human unfractionated and fractionated BMCE as the treatment for the IR-injured SGs. However, there are several limitations to the present study. First, although multiple proteins had been detected in each cell extract, it remains unclear which

fraction of proteins act as the active factors during the treatment. Second, there is a need to verify if the age of the BM donor would influence the results, considering this study is limited to the bone marrow from the young people. And most patients with head and neck cancer are of the older age, thus, it is important to test the BM from the donor with older age to verify the possibility of autologous applications of cell extracts. Last, BMCs or MCs were not included in the comparison. Because the whole cells from the human sample may induce severe immunological rejection in immunocompetent animals. In the future, human cells such as BMs or MCs would be tested in other animal models.

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Figures

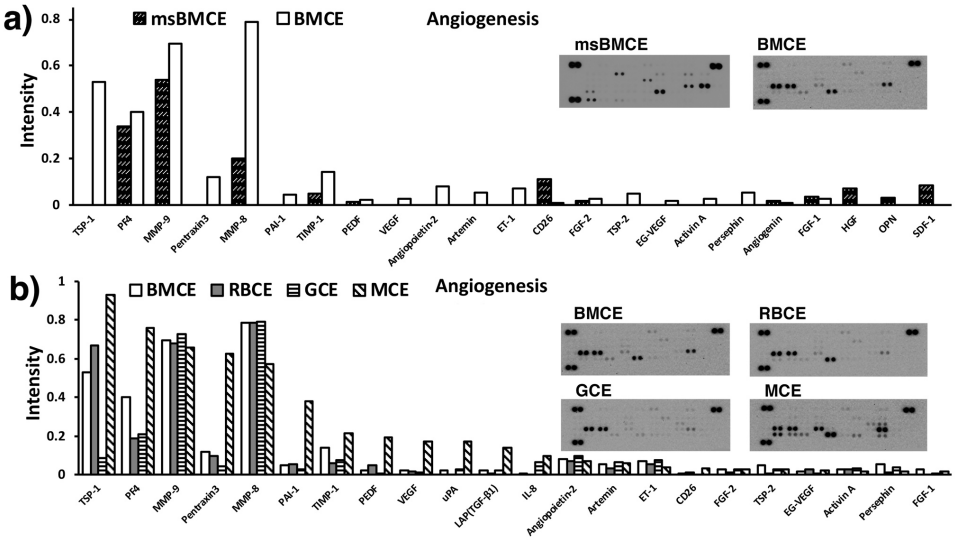

Figure 1. Screening of angiogenesis-related factors in cell extracts originating from bone marrow and three sub-populations. A protein array was used to identify angiogenesis-related growth factors presented in mouse BMCE and human BMCE, RBCE, GCE and MCE. Semi-quantification of angiogenesis-related factors (intensity) were presented in each cell extract.

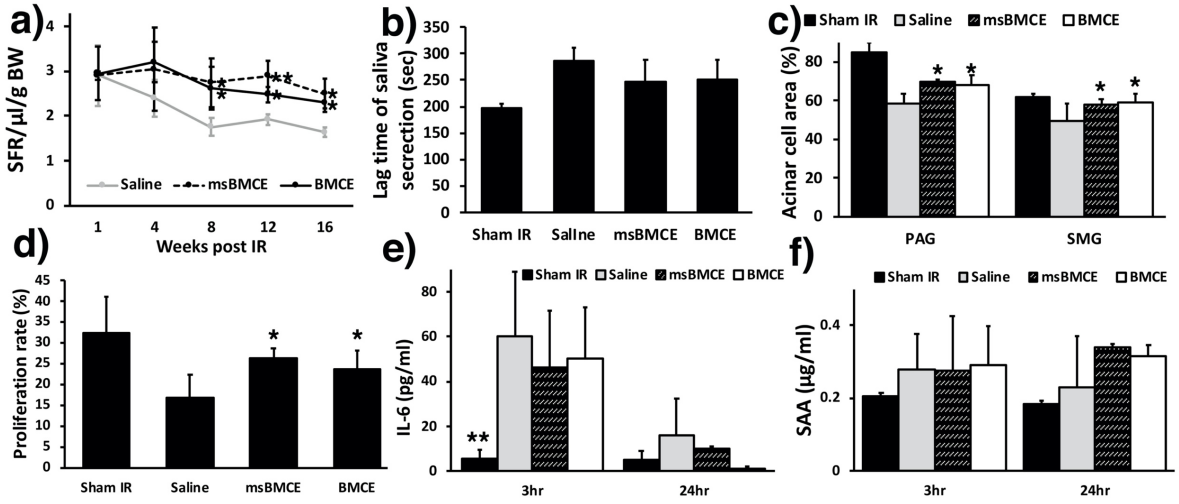

Figure 2. Cell extract from human bone marrow (BMCE) functionally restore IR-injured salivary glands as well as mouse BMCE (msBMCE). a) Salivary flow rate (SFR, µl/g body weight) was measured at week 1, 4, 8, 12 and 16 post-IR. **b)** Time to salivation (lag time) was measured at week 8 post-IR**. c)** Quantification of acinar cells area according to H&E staining in mouse parotid glands (PAG) and submandibular glands (SMG) at 8 weeks post-IR. **d)** PCNA assay was used to calculate the salivary cell proliferation rate in SMG at week 8 post-IR**. e- f)** Interleukin-6 (IL-6) and Serum Amyloid A (SAA) ELASA assays were tested at 3 and 24 hours post treatment, respectively. All data were presented with mean ± S.D; * $p < 0.05$, ** $p < 0.01$ when compared to the Saline group.

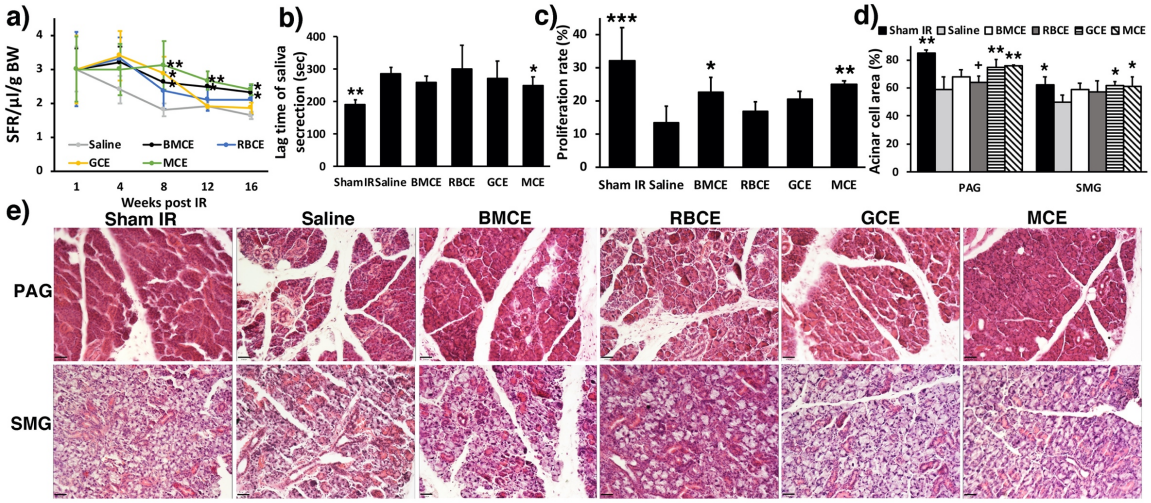

Figure 3. Cell extract from granulocytes (GCE) and mononuclear cells (MCE) functionally restore IR-injured salivary glands. a) Salivary flow rate (SFR, µl/g body weight) was measured at week 0 (before IR), 4, 8, 12 and 16 post-IR. **b)** Time to salivation (lag time) was measured at week 8 post-IR. **c)** PCNA assay was used to calculate the salivary cell proliferation rate in SMG at week 8 post-IR. Five to eight photographs at 400x magnification were counted for each sample with Image J software (NIH). **d)** Quantification of acinar cells area in submandibular glands (SMG) and parotid glands (PAG) in H&E staining. The percentage of acinar cells surface area at 200x magnification were analyzed with ImageJ software (NIH). e) H&E staining of SMG and PAG. Specimens were harvested at 8 weeks post-IR. Scale bar is 37µm. All data were presented with mean ± S.D; * $p < 0.05$, ** $p < 0.01$ when compared to the Saline group; + $p < 0.05$ when compared to MCE group.

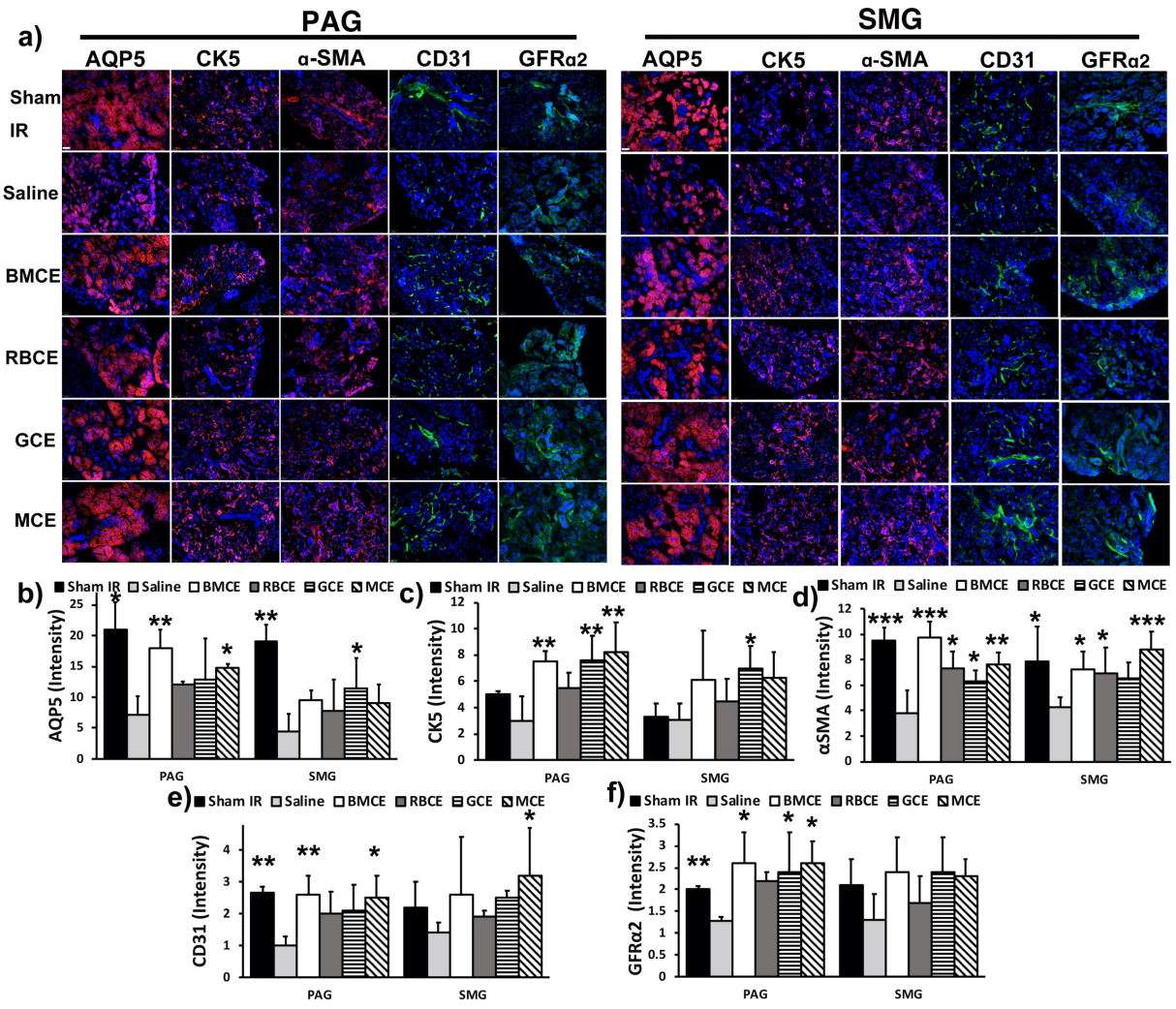

Figure 4. Cell extract from granulocytes (GCE) and mononuclear cells (MCE) protected the cells in IR-injured salivary glands a) Immunofluorescent staining for mouse submandibular glands (SMG) and parotid glands (PAG). Positive cells of AQP5, α–SMA, CK5, GFRα2 (marker for parasympathetic nerve) and CD31 (marker for blood vessel endothelial cell) were detected on frozen sections of salivary glands. Scale bar is 40μm. **b-f)** Semi-quantification of immunofluorescent expression for all the markers were analyzed by ImageJ software. six fields/gland. All data were presented with mean ± S.D; * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ when

compared to the Saline group.

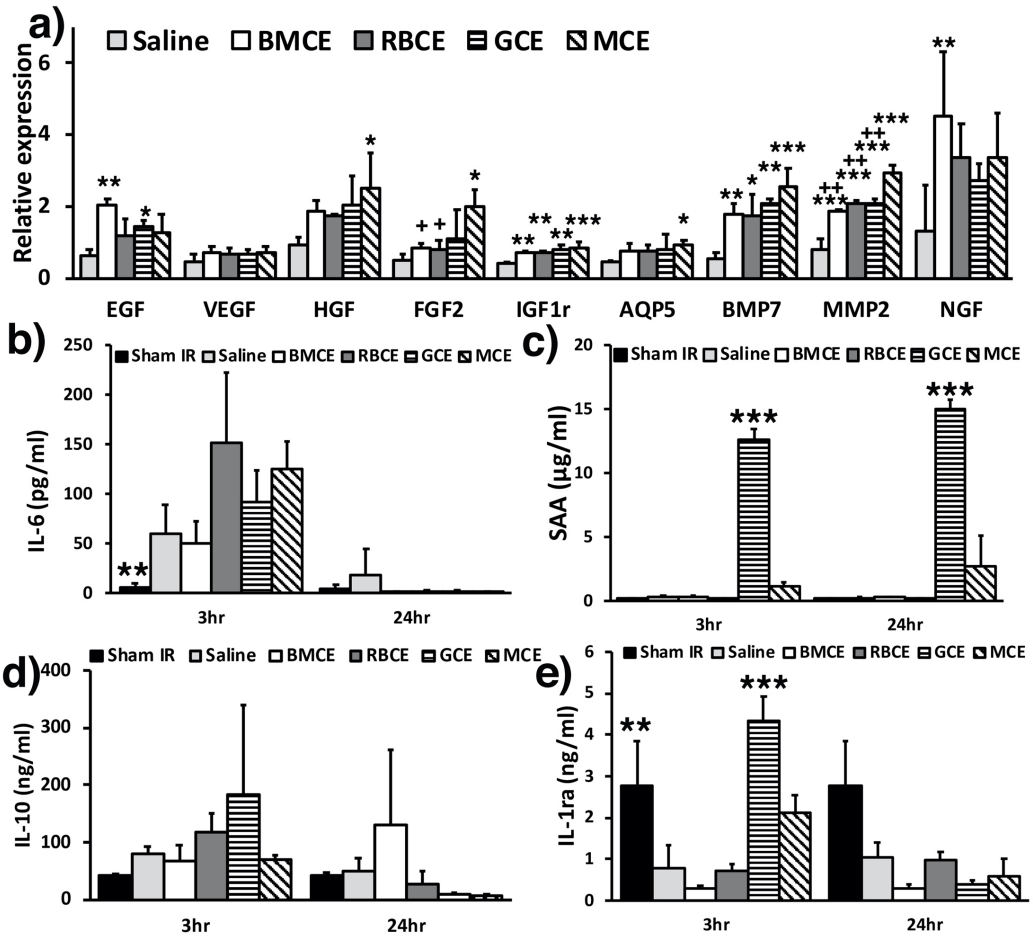

Figure. 5 The immunoreaction and gene expression post each cell extract treatment. a) Relative expression of genes related to tissue repair/regeneration was determined by quantitative real-time PCR. GAPDH was used as the endogenous reference. **b-e)** The immunogenicity of human cell extracts was measured by ELASA kits. Pro-inflammation factors (Interleukin-6 (IL-6), and Serum Amyloid A(SAA)) and anti-inflammation factors (Interleukin-10 (IL-10) and Interleukin-1ra (IL-1ra)) were detected from serum samples at 3hours and 24 hours post cell extract treatment. Three experimental replicates were performed for each sample. All data were presented

with mean ± S.D; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to the Saline group; + $p<0.05$, ++ $p<0.01$ when compared to mononuclear cell extract (MCE) group.

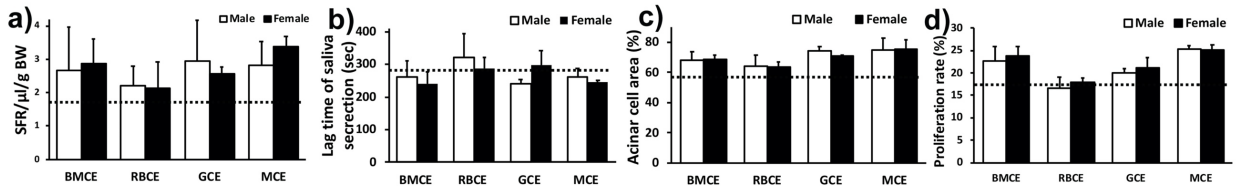

Figure 6. Comparison of the effect of cell extracts from male and female samples. a) Salivary flow rate (SFR, µl/g body weight) was measured at week 8 post-IR. **b)** Time to salivation (lag time) was measured at week 8 post-IR**. c)** Quantification of acinar cells area according to H&E staining in mouse submandibular glands at 8 weeks post-IR. **d)** PCNA assay was used to calculate the salivary cell proliferation rate in submandibular glands at week 8 post-IR**.** All data were presented with mean ± S.D; horizontal dashed line represents the data measured in saline group (negative control).

Chapter 4- Labial Stem Cell Extract Mitigates Injury to Irradiated Salivary Glands

Preface

Our previous studies showed that cell extract from human bone marrow and mononuclear cell could rescue the irradiation-induced salivary gland hypofunction. However, the procedures to harvest the human bone marrow cells are invasive and could lead to life-threatening complications in some severe cases. Therefore, it is necessary to find out an alternative cell source for the treatment of salivary hypofunction. On the other hand, multipotent-specific stem cell isolated from human major SGs was reported to restore SG hypofunction induced by irradiation. However, cell-based therapy provides potential risks, such as tumorigenesis and immunoreaction when compared with cell-free therapy. Furthermore, the procedures to harvest the major SGs are also invasive and unpractical for patients with head/neck cancer. Taken together, according to the accessibility, origination and safety, human minor SG stem cell extract was considered as the candidate source for the treatment of SG hypofunction in the present study.

In this chapter, we harvested a large number of human labial gland stem cells (LSCs) from small pieces of human labial gland tissues by explant culture method. The LSCs were lysed into cell extract (LSCE) by three freeze-thaw cycles. LSCE was injected into mice with irradiation-injured SGs. Results showed that LSCE contained numerous growth factors and restored saliva secretion, protected acinar cells, blood vessels and parasympathetic nerves, promoted cell proliferation, and up-regulated the expression of tissue repair/regeneration proteins and genes.

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Labial Stem Cell Extract Mitigates Injury to Irradiated Salivary Glands

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Abstract

Stem cell-based therapies could provide a permanent treatment for salivary gland (SG) hypofunction caused by ionizing radiation (IR)-injury. However, current challenges for SG stem cells to reach the clinic include surgical invasiveness, amount of tissue needed, cell delivery and storage methods. The objective of this study was to develop a clinically less invasive method to isolate and expand human SG stem cells, and then to obtain a cell-free extract to be used as a therapy for IR-injured SGs.

Methods: Human labial glands (LGs) were biopsied and labial gland mesenchymal stem cells (LSCs) were expanded by explant culture. The LSC extract (LSCE) was obtained by releasing the cellular components after three freeze-thaw cycles and 17,000g force centrifugation. LSCE was injected intravenously into mice that had their SGs injured with 13-Gy IR. Positive (non-IR) and negative (IR) control mice received injections of saline (vehicle control).

Results: Three pieces of LGs (0.1g weight) could expand 1 to 2 million cells. LSCs had a doubling time of 18.8hrs, could differentiate into osteocytes, adipocytes, chondrocytes, and were positive for mesenchymal stem cell MSC markers. Both angiogenic (FGF-1, FGF-2, KGF, angiopoietin, uPA, VEGF) and antiangiogenic factors (PAI-1, TIMP-1, TSP-1, CD26) were detected in LSCE. In addition, some angiogenic factors (PEDF, PTX3, VEGF) also possessed neurotrophic functions. Mice treated with LSCE had 50%-60% higher salivary flow rate than saline-treated mice at 8- and 12-weeks post-IR. Saliva lag time measurements also confirmed LSCE restored SG function. Histological analyses of parotids and submandibular glands reported a comparable number of acinar cells, blood vessels, parasympathetic nerves, and cell proliferation rates in sham-IR and LSCE-treated mice, while significantly lower in saline-treated mice.

Conclusions: An explant culture method can harvest a large number of LSCs from small pieces of LGs. LSCE showed clinical potential to mitigate IR-injured SGs.

Keywords:

salivary diagnostics, regeneration, saliva, growth factors, regenerative medicine, angiogenesis

Introduction

Salivary gland (SG) hypofunction with its symptom xerostomia (dry mouth) is a prominent sequela for millions of patients with head and neck cancer worldwide following radiotherapy treatment. In many patients, their salivary parenchymal tissue is lost, and side effects such as oral mucositis, infections, dental caries, speaking difficulty and dysphagia lead to a significant deterioration of their quality of life [1, 2]. Unfortunately, current treatments (e.g. pharmacologic drugs) for ionizing radiation (IR)-induced injury to SGs remain palliative as it depends on residual acinar cells. Stem cell-based therapies are being tested with the objective of providing a permanent treatment for IR-injured SGs [2, 3]. Stem cells originating from the bone marrow (BM) [4], adipose tissue [5, 6], and dental pulp [7] were reported to repair IR-injured SGs in animals. Also, it was suggested that tissue-resident stem cells, if preserved, could release growth factors to repair injured tissues or could differentiate into tissue-specific cells [8]. Tissue-specific stem cells were isolated from human major SGs (parotid and submandibular glands), and when transplanted in rodents, were capable of restoring function to IR-injured SGs [9, 10]. Although these advances are promising, there remain major challenges in the development pipeline to allow SG stem cells to be used in the clinic. The first challenge is the invasiveness of the surgical method to obtain SG stem cells. Second is the amount of tissue needed to expand SG stem cells. Third is the delivery method of the SG stem cells to the site of injury; for example, systemic intravascular injections of cells can result in thromboembolic complications. Fourth is the possible rejection of the transplanted cells, if allogeneic. The fifth challenge is the storage and transportation logistics of the SG stem cells.

The objective of this study was to develop a clinically less invasive method to obtain, isolate and expand human SG stem cells, and then to create a cell-free product to be used as a therapy to mitigate IR-induced injury to SGs. Here, we demonstrated that human minor SGs (i.e. labial glands)

could be obtained with minimal invasiveness, and that these small pieces of tissue (1-2mm in size) can provide a sufficient number of labial mesenchymal stem cells (LSCs) through the explant culture method. Moreover, we reported for the first time that cell extract from these LSCs (LSCE) could repair IR-injured SGs and restore secretory function in a mouse model. The advantages of our proposed method were, in accordance to the challenges described here: 1) a less invasive method to obtain the tissue, 2) small size of tissue allowing ample cell expansion, 3) a cell-free therapy, 4) minimal immunologic rejection, 5) and the ability to store (freeze or lyophilize) the LSCE.

Materials and Methods

Explant culture and expansion of LSCs

Human labial minor salivary glands (LGs) were dissected from discarded tissues of orthognathic surgery performed at the Maxillofacial Surgery Department, McGill University, and in compliance with guidelines from McGill Research Ethics Board. LGs from 3 patients (2 males and 1female; 30 to 55 year-old) were used in this study. The size of each labial gland was 5 to 7mm. These patients had received no radiotherapy or chemotherapy. LGs were rinsed twice in PBS, minced into 1-2mm pieces, and then deposited into a 60 mm dish with 1ml alpha-MEM (12571-063, Gibco, Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (10,000U/ml).

Cell proliferation

Cells at passage 3 were seeded at a density of $5x10^3$ per well in 96-well plate for MTT assay (M5655, Sigma). Triplicate wells were made for each sample and the absorbance was measured at

a wavelength of 570nm for 5 continuous days. Doubling time (DT) was calculated by the formula $DT=t[\log 2/(\log N-\log N_0)]$, t was the culture period, N and N_0 indicated cell number at the end and at the beginning of culture, respectively.

Multilineage differentiation of LSCs

Multilineage differentiation assays were done to confirm the ability of LSCs to differentiate into osteoblasts, adipocytes, or chondroblasts. Recipe for the differentiation media and staining methods were performed based on our laboratory protocol [11]. In brief, osteogenesis, adipogenesis and chondrogenesis differentiation were detected by Alizarin Red S (A5533, Sigma), Oil Red O (O1391, Sigma), and collagen type II staining, respectively (see Appendix).

Flow cytometry

Flow cytometry was performed as previously described [11]. LSCs were harvested by Accutase (423201, Biolegend, San Diego, CA, USA) and 10^6 cells were used for each test. All procedures followed the manufacturer's protocol for human mesenchymal stem cell (MSC) analysis (562245, BD Biosciences, San Jose, CA, USA). Post-acquisition analysis was performed using FlowJo (version 10, Tree Star Inc., OR). Isotype-matched control antibodies were used in the antibody analysis (see Appendix).

Preparation of LSCE

In this study, only LSCs at passages 3-5 were used for the preparation of LSCE. Trypsin was used to detach cells, which were then resuspended in 0.9% saline at a concentration of 10^7 LSCs /ml. The cell extract was obtained after three cycles of freeze-thaw (-80°C to +37°C) to lyse the cells,

as previously described [12]. The cell lysate was centrifuged at 17,000g for 30minutes at 4°C and the supernatant, defined as the labial gland stem cell extract (LSCE) was transferred into a new tube and stored at -80°C until use.

Characterization of human LSCE

The total protein concentration was measured by the bicinchoninic acid assay (23225, BCA; Thermo Scientific, Pierce, IL, USA). 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 120V, 60min) and Coomassie blue staining were used to visualize the protein fractions in LSCE.

The angiogenesis-related factors and cytokines within LSCE were detected with Proteome Profiler Human Angiogenesis Arrays (ARY007, R&D Systems, Minneapolis, USA). All procedures were done according to the manufacturer's instructions. Saline solution was used as the background (negative) control prior to analyzing the protein signals by ImageJ software (NIH).

Animals and Ionizing radiation (IR) injury to salivary glands

All procedures were performed under protocols approved by McGill University Animal Care Committee (Protocol #5330). Animal experiments were conformed to ARRIVE guidelines. All the mice were kept in clean conditions with food and water in the animal resource center at McGill University. Briefly, C3H female mice (8 weeks old; 18-20g; Charles River, Montreal, Canada) were anesthetized and the radiation field was slit-collimated to the head and neck area comprising the SGs. The dose delivered was 13Gy and irradiations were performed using a clinical 6MV accelerator on typically 5 mice simultaneously at 5.5Gy/min, as determined using small-field detectors. This study used 18 mice that were randomly divided into 3 groups (n=6 mice per group):

(1) “Sham IR” positive control group (these healthy mice received no IR and were injected with saline); (2) “Saline group” (these mice received 13Gy IR and were injected with saline); (3) “LSCE” group (IR-injured mice injected with the experimental treatment). Five to seven days post IR, mice were injected through their tail vein, according to their assigned groups, with either 100µl normal saline (vehicle control) or 100µl LSCE (1.95±0.2mg/ml; the rationale for this dose of 0.195mg proteins is explained in the discussion section), twice a week for two consecutive weeks. Mice were sacrificed after 8 and 16weeks post-IR, and their parotids and submandibular glands were harvested.

Measurements

Salivary flow rate (SFR) and lag time measurements were done at week 4, 8, 12 and 16 post-IR as described previously [13]. Histological analyses, such as hematoxylin/eosin and PCNA, immunofluorescent staining using aquaporin-5 (AQP5, to detect acinar cells), smooth muscle actin (α-SMA, myoepithelial cells), cytokeratin-5 (CK5, basal ductal cells and a subpopulation of myoepithelial cells), GFRα2 (parasympathetic nerves), CD31 (endothelial cells), and quantitative real-time PCR (qRT-PCR) for EGF, VEGF, IGF-1R, HGF, FGF-2, GFRα2, AQP5, CK5 and GAPDH were done as previously described [13].

Statistical analysis

SPSS (version19) was used to perform the statistical analysis. All data were reported with mean±S.D, Student’s t-test or one-way ANOVA, with Tukey’s Post-Hoc test. Statistical difference was defined as $p<0.05$.

Results

Isolation and characterization of human LSCs

After 3-5 days in culture, many viable cells migrated and rapidly expanded from the small pieces (1-2mm in diameter) of labial glands (Fig 1d-e). These labial-derived cells were attached and had a spindle-shaped morphology typical to mesenchymal cells. Cells were passaged at a ratio of 1:4 after 10-14 days from the initial explant culture, and subsequently every 5-7 days. LSCs had a doubling time of 18.8±0.8hrs (Fig 1c). LSCs were able to differentiate into various cell lineages, such as osteocytes, adipocytes, and chondrocytes (Fig 2a). LSCs at passages 3, 7 and 13 were positive for MSC markers (99.4%-99.7% CD44, 99.5%-99.9% CD73, 97.6%-99.2% CD90, 58.6%-77.2% CD105) and negative for hematopoietic cell markers (0.05%-0.4% for CD34, CD45, CD19, CD11b, HLA-DR) (Fig 2b).

LSCE contained numerous growth factors

LSCE contained a mixture of proteins of various molecular weights, with a preponderance of proteins with >30kDa (Fig 3a). Angiogenesis-related growth factors detected in LSCE were FGF-1, FGF-2, KGF, angiopoietin, uPA and VEGF (Fig, 3b-c). Anti-angiogenesis factors such as PAI-1, TIMP-1, TSP-1and CD26 were also detected. These findings suggested that both angiogenesis and anti-angiogenesis factors could co-exist in LSCE and resulted in a therapeutic effect when injected in IR-injured SGs.

LSCE Restored the Function of Salivary Glands injured by IR in-vivo

The body weights of LSCE-treated mice did not differ from Sham-IR mice (26.7±2.9g versus 29.7±4g), while the body weight of saline-treated mice was less at 8-week post IR (24.9±2.1g)

(Fig 4c). No adverse effect was observed in LSCE-treated mice. The salivary flow rate (SFR) was measured at 4, 8, 12, and 16-week post-IR to evaluate salivary function in vivo. At 4-week post-IR, saline-treated IR mice had their SFR level decreased to 55% when compared to that of sham IR (no IR) mice. This reduced SFR remained low, at ~45% for the saline-treated mice during the 16-week follow-up period. On the other hand, mice treated with LSCE had 50-60% higher SFR level when compared to saline-treated mice at 8-week (3.13±0.1μl/10min/g versus 2.1±0.4μl/10min/g; $p<0.05$), and 12-week (3.2±0.33μl/10min/g versus 2.0±0.1μl/10min/g; $p<0.05$) post IR (Fig 4a). At 16-week post-IR, the SFR difference between LSCE-treated and saline-treated mice was reduced to 32% (no statistical difference, $p>0.05$). The "saliva lag time" is the time lag between the injection of pilocarpine (to stimulate saliva secretion) and the onset of saliva secretion. The saliva lag time in saline-treated group (4.6±0.6min) was higher than that of the LSCE-treated group at 8-week (3.7±0.7min) and 16-week (4.8±0.6min versus 3.8±0.2min) (Fig 4b). Both SFR and lag time measurements confirmed that LSCE treatment restored SG function (Fig 4a-b).

We also examined the histology, gene and protein expression in IR-injured submandibular gland (SMG) and parotid gland (PAG) after LSCE treatment (Fig 4d-g and 5). The percentage of acinar cells was comparable between the sham-IR and LSCE-treated groups (69%±3%, 67%±5% in SMG and 82%±8%, 80%±2% in PAG), respectively; which were significantly higher than the saline group (60%±3% in SMG and 69%±1% in PAG) ($p<0.05$, Fig 4d-e). PCNA staining indicated that mice in the LSCE group had a higher percentage of cell proliferation rate than the saline group (Fig 4f-g). SGs of LSCE-treated mice showed a higher density of parasympathetic nerves (GFRα2) and of blood vessels (CD31) than saline-treated mice (Fig 5; $p<0.05$). Cells in LSCE-treated mice highly expressed AQP5, α-SMA and CK5 (Fig 5, $p<0.05$). qRT-PCR results revealed that the expression levels of genes related to tissue repair and regeneration were upregulated in LSCE-

treated mice (Fig 5g). Specifically, EGF, GFRα2 and IGF-1R were significantly higher ($p<0.05$). Taken together, these data demonstrated that LSCE mitigated salivary cells and blood vessels from IR-induced injury, up-regulated gene and protein expression, and restored SG function in vivo.

Discussion

The main findings of this study were that: 1) small pieces of human SGs, such as LGs of 5mm in size, could provide an ample number of expanded MSCs; and 2) the cell extract of these expanded human cells (LSCE) was effective in mitigating IR-induced injury to SGs by restoring salivary secretory function in a mouse model.

The first main finding of this study was that small and accessible salivary tissue could be used to provide sufficient MSCs for a therapeutic treatment. Although stem cells have been isolated from human parotid and submandibular glands [9, 10], the methods used to harvest tissues from these major SGs are invasive. In this study, we opted to harvest human "minor SGs" which required a less invasive surgery. To date, one research team has isolated and characterized stem cells derived from human minor SGs (labial glands), but their use has been limited to study liver regeneration [14, 15]. Our novelty was the use of LSCs to restore function to IR-injured SGs.

For expansion of stem cells from small pieces of tissues, the explant culture method was reported more advantageous when compared with classical enzymatic tissue digestion methods [16]. In our preliminary studies, the classical enzymatic digestion procedure used required 1 gram of tissue [17, 18], which was equivalent to the weight of 30-40 pieces of LGs to obtain 1-2 millions of cultured primary cells; an equal number of cells could be obtained with the explant culture method with only 0.1g of LGs (i.e. 1/10 less tissue). We observed that the explant method initially released fewer cells in the culture dish, as compared to the enzymatic method. However, after 10-14 days,

cells migrated from the tissue explant and the cell number was comparable to the enzymatic method.

The second major finding of this study was the positive therapeutic effect of LSCE in treating IR-induced hyposalivation. These results suggested that the duration of the therapeutic effect of LSCE could last for 9 weeks, but not be sustained to 16 weeks (Fig 4a). We observed the same trend for therapeutic duration from our previous work with mouse bone marrow cell extracts, and administering LSCE injections every 3 months would maintain a long-term therapeutic effect [19]. The rationale for starting injections of LSCE between 5-7 days post-IR was because both the IR and tail vein injection procedures were stressful to the mice, and this resting period allowed them to recuperate. We injected 100µl LSCE of a 1.95mg/ml solution (i.e. a dose of 0.195mg of proteins) because this was equivalent to the cell extract obtained from 10^6 LSCs. This (protein) dose is equivalent to $1x10^6$ MSCs; a cell number shown to be effective when injected for transplantation studies in mice with IR-injured salivary hypofunction [5, 20, 21]. Our study confirmed that injections of human LSCE were well tolerated in a mouse model, and that LSCE could potentially be used as either an autogenous or an allogeneic transplantation. Our study delivered LSCE with four intra-venous injections. An alternate route of delivery could have been by intra-glandular injection, as we have previously tested with a bone marrow cell extract [12]. The use of I.V. delivery in this study allowed us to inject a higher dose of human LSCE into immune-competent mice and to assess adverse reactions. Mice receiving LSCE injections did not have any significantly different body weights when compared to sham-IR normal controls (Fig 4c). Also, vital organs (liver, kidney, spleen and lung) from LSCE-treated mice showed no inflammatory or pathological changes (data not shown). An added advantage of cell extracts was its ability be stored

frozen or lyophilized for over one year and still maintaining its bioactivity when tested in vivo [22].

Many research groups, including ours, have previously focused solely on examining the submandibular glands to assess the effectiveness of a tested experimental therapy against IR-induced injury [9, 23]. However, examining parotids provided a better assessment of the therapy because: 1) parotid glands have a major function during stimulated saliva secretion [24, 25], 2) parotids are more sensitive to IR than submandibular glands [26], and 3) salivary hypofunction and cell apoptosis in mouse parotids are correlated to IR doses [26, 27]. Thus, this study examined the LSCE effect in both parotid and submandibular glands. In general, results of the histological and protein expression studies in parotids and submandibular glands were comparable. One advantage of using parotids was that the distribution and expression of AQP5 in healthy glands was two-fold higher than that in submandibular glands (AQP5 fluorescence relative intensity 43.6 versus 22.5; Fig 5b). Following IR-induced injury, both glands had AQP5 relative intensity between 15-16 and this implied parotid acinar cells were more radiosensitive. Then after LSCE treatment, the AQP5 relative intensity in parotids increased to 27. These results suggested that including parotids facilitated the detection of a sizable difference in AQP5 protein expression, which was a histologic indication that acinar cells were functional for saliva secretion.

Our previous study reported that proteins were the main effective factors in the cell extract from bone marrow cells[13]. In that work, we preliminarily screened proteins from three categories: angiogenesis, cytokines, and chemokines. Several angiogenic factors were detected while little cytokines and chemokines were identified [13]; this was our reason in this study to screen LSCE for angiogenic factors first. We believe LSCE was modulating angiogenesis as one of its therapeutic effects, but we do not know what the relative strength of this angiogenesis effect is,

when compared with other therapeutic effects from LSCE proteins yet to be identified . FGF-2, a well-known mitogen with important roles in angiogenesis and wound healing, was the most highly expressed protein in LSCE. Cotrim and colleagues demonstrated that transfection of FGF-2 to endothelial cells was crucial in treating IR-injured SGs [28, 29]. Additional studies have also reported that FGF-2 protected SGs by inhibiting radiation-induced apoptosis in-vitro and in-vivo [29-31]. In addition to FGF-2, LSCE was detected with several additional pro-angiogenic factors such as VEGF, FGF-1, KGF, HGF and PDGF as well as anti-angiogenic factors such as PEDF, TIMPs, TSP-1and PAI-1. Furthermore, several factors detected in LSCE were multifunctional. For examples, the angiogenic factors PEDF, PTX3, and VEGF also possess neurotrophic functions [32-34], and this may be a reason for a higher GFRα2 protein expression after LSCE treatment (Fig 5e-g). Taken these findings together, we propose that the therapeutic effect of LSCE was modulated by multiple factors and their interactions with each other, both antagonistic and synergistic, to provide restoration of SG function. More studies will need to be done to decipher these interactions.

The first limitation in this study was that the therapeutic effect of the cell extract (i.e. LSCE) was not compared directly to that of the intact (alive) LSCs. It is because we previously reported that intact cells, such as bone marrow cells versus their cell extracts resulted in a comparable therapeutic effect for IR-induced injury to SGs [12]. In addition, there were already studies reporting the efficacy of MSCs treatment for IR-injured to SGs [5, 6, 9]. Thus, we opted to focus solely on the effect of the cell extract, and not on the intact cells in this study. The second limitation of this study was that no other MSC extracts were compared to LSCE. We are currently comparing the sources of different MSC extracts (e.g. adipose, labial gland, dental pulp, and periodontal ligament MSC) for efficacy in restoring functions to IR-injured SGs. Our preliminary data seem

to indicate that cell extracts from both adipose-derived MSC (ADSC-extract) and LSCE were effective and comparable in restoring salivary function, while the extracts from the dental pulp and periodontal ligament MSC were less effective (unpublished data). The third limitation of this study was that the LSCE only came from LSCs between passages 3-5, and not from later cell passages. LSCE at later cell passages, if shown as efficacious as between passages 3-5, will allow an even more ample supply of LSCE that can be stored for patients, if repeated or subsequent injections are needed. We will test LSCE from later cell passages in future studies.

Conflict of interest

The authors declare no competing financial interests.

Author Contributions

X. Su, contributed to conception, design, data acquisition, analysis, interpretation, drafted and critically revised the manuscript; Y. Liu and M. Bakkar, contributed to conception, design, data acquisition, analysis, interpretation and critically revised the manuscript; O. ElKashty, M. El-Hakim and J. Seuntjens, contributed to conception, design and critically revised the manuscript. S.D. Tran, contributed to conception, design, data acquisition, analysis, interpretation, drafted and critically revised the manuscript. All authors gave their final approval and agree to be accountable for all aspects of the work.

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Figures

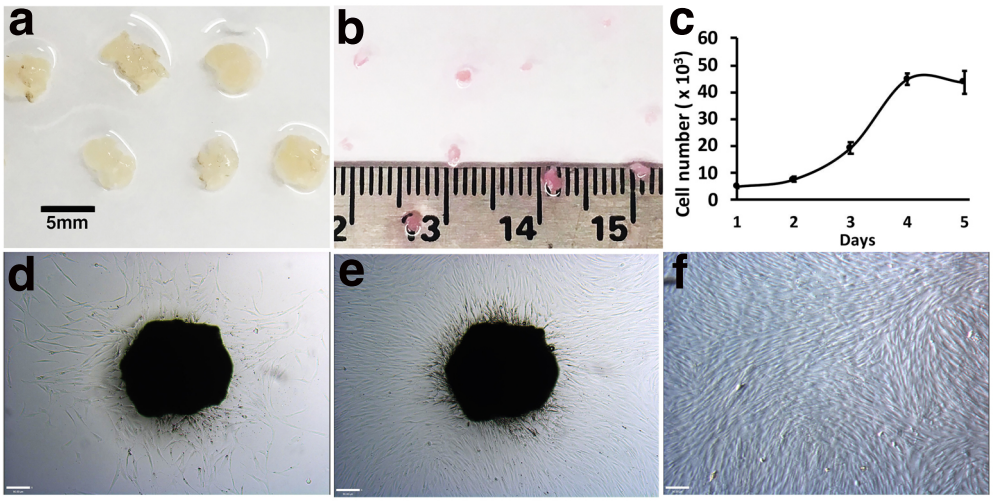

Fig.1 Culture of human labial salivary gland stem cells (LSCs). a) Human labial glands (LGs; dimension approximately 5x5mm) dissected from patient discarded tissues. b) Several 1-2mm minced pieces of human LGs were placed into a 100mm dish for explant culture at Day 0. The pink color on the picture is from the culture media. c) Cell growth curve of LSCs at passage 3. d,e) Explant culture of LSCs. The same piece of labial gland is shown in the center of the photograph (in black) at Day 7 and 14. f) LSCs at passage 3. Scale bar: 90μm.

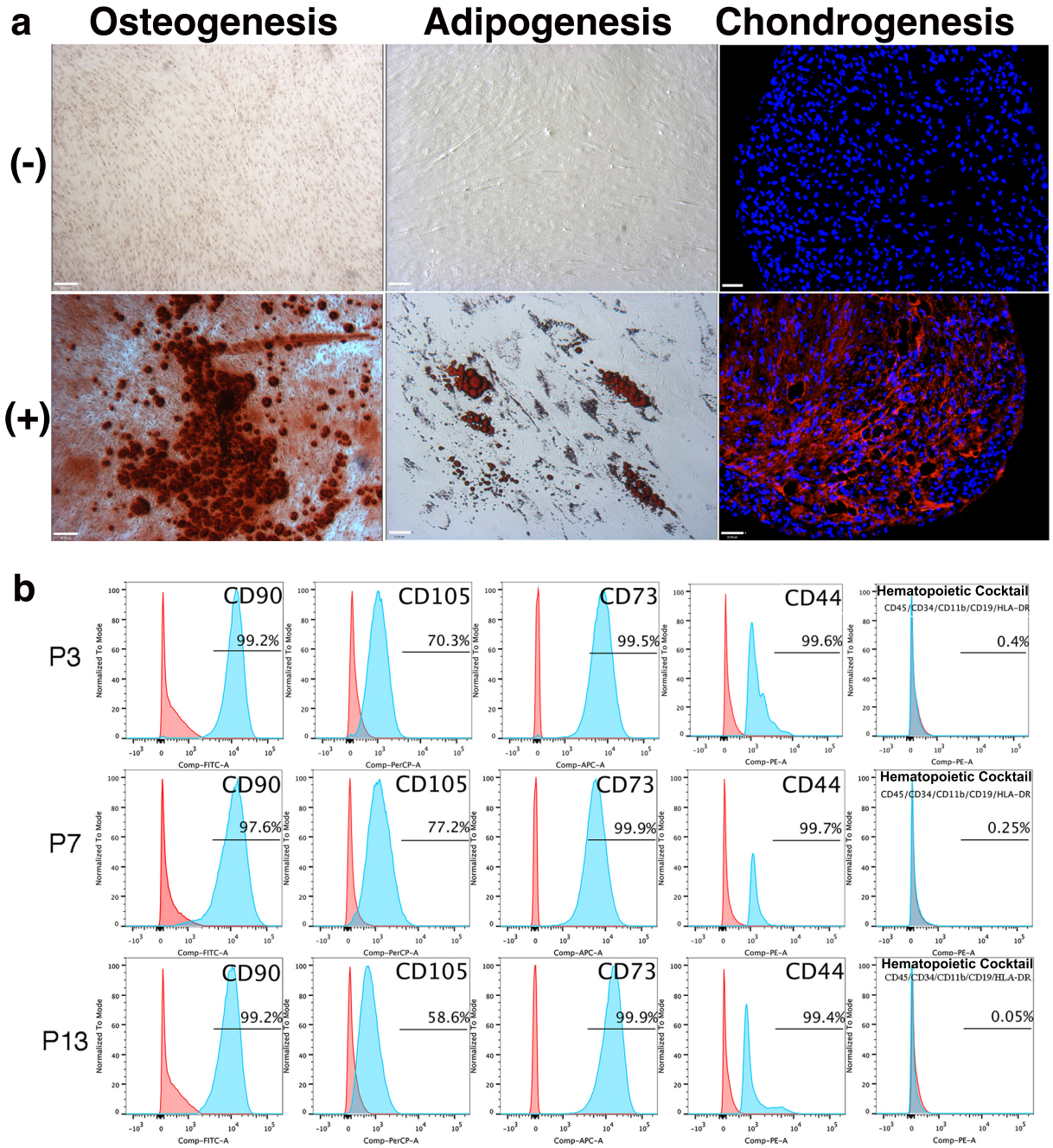

Fig. 2 Characterization of labial salivary gland stem cells. a) LSCs were induced to differentiate into three mesenchymal cell types (osteoblasts, adipocytes, or chondroblasts). Cells were stained

with either Alizarin red S (osteogenesis, day 21), oil red O (adipogenesis, day 21), or collagen type II (chondrogenesis, day 28). Scale bars: osteogenesis (90μm), adipogenesis (21μm), chondrogenesis (25μm). (-): control group with culture medium. (+): LSCs with differentiation induction medium. b) Flow cytometric analysis. LSCs at passages 3, 7 and 13 were positive for MSC markers (99.4%-99.7% CD44, 99.5%-99.9% CD73, 97.6%-99.2% CD90, 58.6%-77.2% CD105) and negative for hematopoietic cell markers (0.05%-0.4% for CD34, CD45, CD19, CD11b, HLA-DR). Red: isotype control, Blue: antibody surface marker.

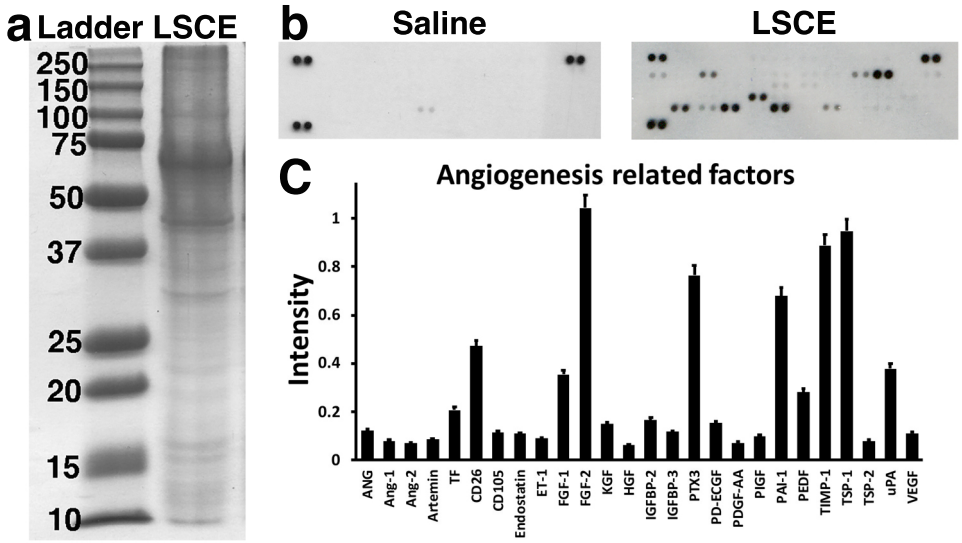

Fig. 3 Characterization of human labial stem cell extract (LSCE). a) SDS-PAGE and Coomassie blue staining were used to visualize the molecular weight distribution of proteins in human labial gland cell extract (LSCE). b) Angiogenesis-related protein arrays. Saline was used as the negative vehicle control versus LSCE. c) Relative quantification of angiogenesis-related growth factors of LSCE (data obtained from 3 experiments).

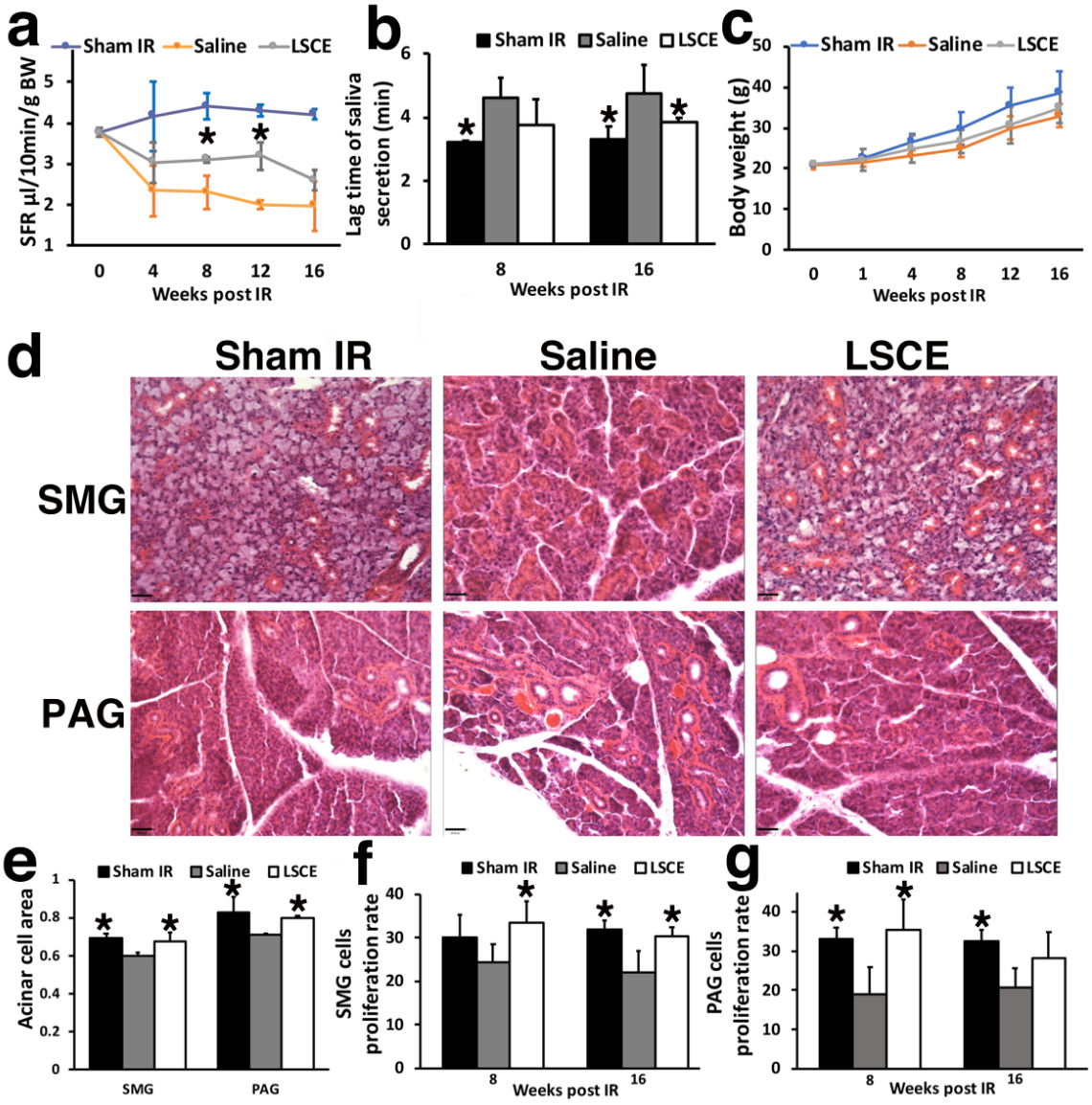
a
Sham IR
Saline
LSCE
SFR µl/10min/g BW
Weeks post IR
b
Lag time of saliva secretion (min)
c
Body weight (g)
d
Sham IR
Saline
LSCE
SMG
PAG
e
Acinar cell area
SMG
PAG
f
SMG cells proliferation rate
g
PAG cells proliferation rate

Fig. 4 Labial stem cell extract (LSCE) functionally restored irradiation-injured salivary glands. a) Salivary flow rate (SFR, μl/10min/g body weight) was measured at week 0 (before IR), 4, 8, 12 and 16 post-IR. b) Time to salivation (lag time) was measured at week 8 and 16 post-IR. c) Body weight (g) of the mouse. d) H&E staining of mouse submandibular glands (SMG) and parotid glands (PAG). Specimens were harvested at 8 weeks post-IR. Scale bar is 25μm. e) Quantification of acinar cells area in SMG and PAG. The percentage of acinar cells surface area at 200x magnification were analyzed. Image J software (NIH) was used to quantify the percentage of surface area occupied by acinar cells/total area. f,g) PCNA assay was used to calculate the salivary cell proliferation rate at week 8 and 16 post-IR. Five to eight photographs at 400X magnification were counted for each sample with Image J software (NIH). All data were presented with mean ± S.D; * $p < 0.05$ when compared to the Saline group (n=3-6).

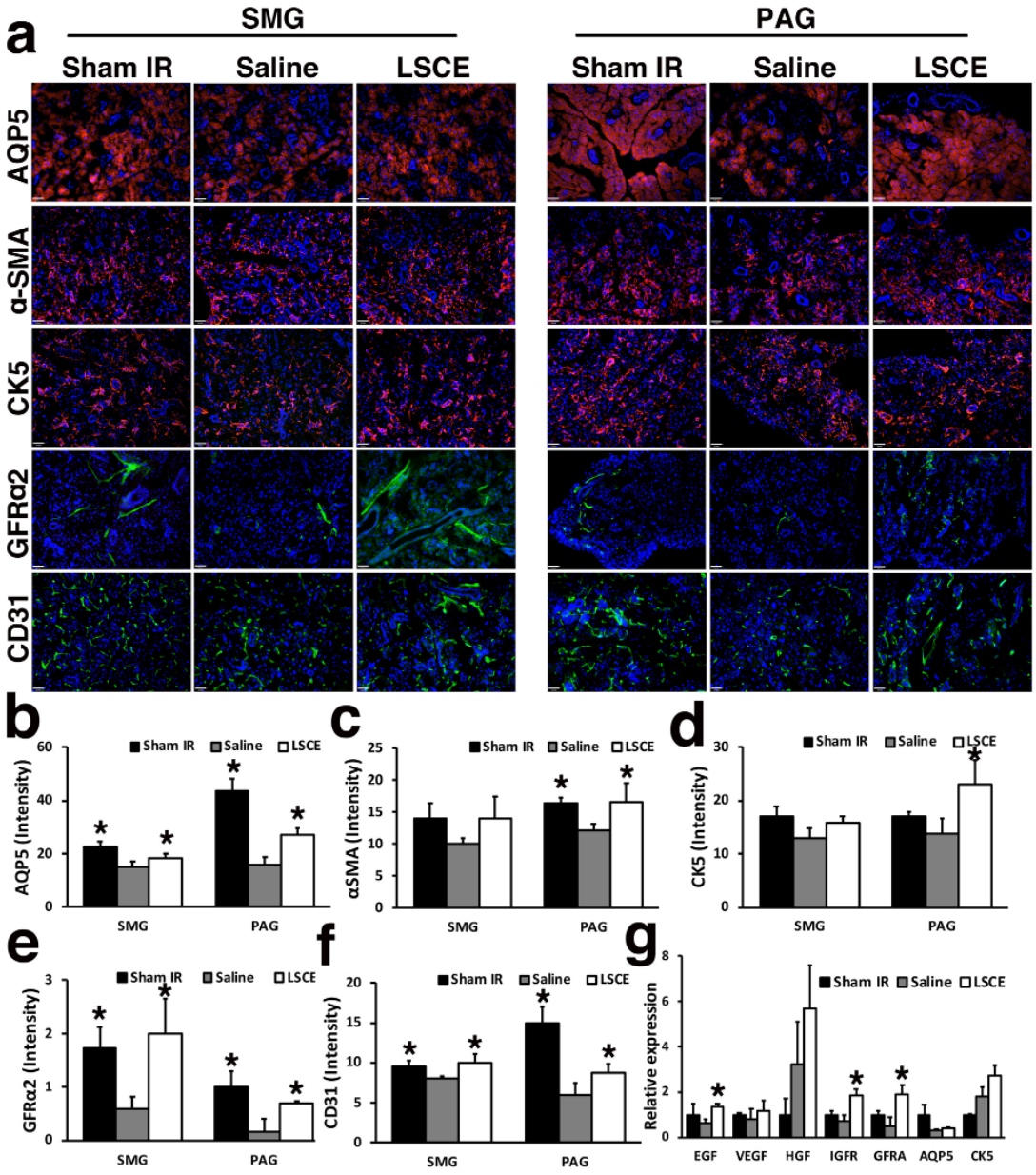

Fig. 5 Protective effects of labial stem cell extract (LSCE) on specific cell subpopulations in parotid and submandibular glands. a) Immunofluorescent staining for mouse submandibular glands and parotid glands. Positive cells of AQP5 (marker for acinar cell), α–SMA (marker for

myoepithelial cell), CK5 (marker for basal ductal cell and some myoepithelial cell), GFRα2 (marker for parasympathetic nerve) and CD31 (marker for blood vessel endothelial cell) were detected on frozen sections of salivary glands. b-f) Semi-quantification of immunofluorescent expression. Intensity of the fluorescence signal was analyzed by ImageJ software (NIH). Six fields/gland. g) Relative expression of genes related to tissue repair/regeneration was determined by quantitative real-time PCR. GAPDH was used as the endogenous reference. Three experimental replicates were performed for each sample. All data were presented as mean ± S.D; * $p < 0.05$ vs. the saline group (n=3).

Appendix

Materials and Methods:

Multilineage differentiation of LSCs

Osteogenesis differentiation LSCs were cultured with alpha-MEM with 15% FBS, 1% penicillin/streptomycin, 2 mM Glutamine, 10^{-8} M Dexamethasone sodium phosphate (D-8893, Sigma-Aldrich, St-Louis, MO, USA), 55 μM 2-Mercaptoethanol (21985-023, Gibco), 0.1 mM L-ascorbic acid phosphate (013-12061, Wako chemicals, Richmond, USA), and 2mM Beta-Glycerophosphate disodium salt hydrate (G9422, Sigma). After 4 weeks in culture, cells were characterized with Alizarin Red S (A5533, Sigma) staining.

Adipogenesis differentiation LSCs were cultured with Alpha-MEM with 15% FBS, 1% penicillin/streptomycin, 2 mM Glutamine, 0.5 mM Isobutylmethylxanthin (I5879, Sigma), 60 μM Indomethacin (I7378, Sigma), 0.5 μM Hydrocortisone (H0888, Invitrogen, San Francisco, CA, USA), 10μg/ml insulin (I-9278, Sigma), 0.1 mM L-ascorbic acid phosphate and 2-ME. Adipogenesis was characterized by Oil Red O (O1391, Sigma) staining after 21 days in culture.

Chondrogenesis differentiation LSCs were cultured with Mesenchymal Stem Cell Identification Kit (SC006, R&D Systems, MN, USA), and characterized with immunofluorescence staining for collagen type II after 28 days. Volocity Image Analysis Software TM (Version 4.5.1) was used to capture images.

Flow cytometry

Labial gland stem cells (LSCs) were harvested by Accutase (423201, Biolegend, San Diego, CA, USA); and 1 x 10^6 cells were used for each test. Cells were filtered through a 70 μM cell strainer. BD Horizon™ Fixable Viability Stain 450 (FVS450, 562247, BD Biosciences, California, USA) was added (1:1000 dilution) and incubated for 15 minutes at 4 °C. After washing twice with Staining Buffer (420201, Biolegend), cells were incubated with Fc Receptor block (564219, BD Biosciences) for 20 minutes at 4 °C. Then hMSC analysis kit (562245, BD Biosciences, San Jose, CA, USA) was used. All procedures were done according to the manufacturer's protocol. The antibodies used for flow cytometric analysis were as follow: PE Mouse Anti-Human CD44 (Clone: G44-26); FITC Mouse Anti-Human CD90 (Clone:5E10); PerCP-Cy™5.5 Mouse Anti-Human CD105 (Clone:266); APC Mouse Anti-Human CD73 (Clone: AD2); hMSC Positive Cocktail:(CD90, CD105, CD73); PE ***hMSC Negative Cocktail*** (CD34 PE (Clone 581), CD11b PE(Clone:ICRF44), CD19 PE(Clone:hib19), CD45 PE (Clone: HI30) and HLA-DR PE (Clone: G46-6)); ***hMSC Positive Isotype Control Cocktail*** (mIgG1, κ FITC (Clone: X40); mIgG1, κ PerCP-Cy5.5 (Clone: X40); mIgG1, κ APC (Clone: X40); BD Bioscience), and PE ***hMSC Negative Isotype Control Cocktail*** (mIgG1, κ PE (Clone: X40); mIgG2a, κ PE (Clone: G155-178)). The antibody was incubated with cells for 30 minutes. After washing twice with the Staining Buffer, cells were resuspended in 300 μl of Staining Buffer. Data was recorded on 3 lasers, 11 detectors LSR Fortessa (BD Biosciences) equipped with BD FACS Diva Software (v6, BD Biosciences). Post-acquisition analysis was performed using FlowJo (version 10, Tree Star Inc., OR). Isotype-matched control antibodies were used in the antibody analysis.

Salivary flow rate (SFR) and lag time

Before anesthetizing the mice, the body weight was measured. 0.3 μl/g body weight of 60 mg/ml Ketamine and 8 mg/ml Xylazine were used as the anesthetic to measure the salivary flow rate (SFR) of mice at week 4, 8, 12 and 16 post-IR. After subcutaneous injection of 0.5 mg/kg body weight of pilocarpine (P6503, Sigma-Aldrich, ST. Louis, USA), stimulated saliva secretion was collected for 10 minutes. As previously described, we assumed the density of saliva is 1g/ml, which means the weight of saliva can represent the volume, for example, 0.5g of saliva is equivalent to 0.5 ml volume. Meanwhile, the lag time of saliva was counted for each mouse according to the time from stimulation to the first drop of saliva secreted.

H&E staining

Submandibular glands and parotid glands were fixed in 4% PFA overnight at 4°C and embedded into paraffin. Specimens were sliced into 8 μm thickness followed by staining with Hematoxyline and Eosin (H&E). Image J software (NIH) was used to calculate the percentage of surface area occupied by acinar cells/total area. The magnification was 200 X, and 5-8 fields were randomly chosen for counting per gland/mouse.

PCNA staining.

Zymed PCNA staining kit (931143, Invitrogen, Carlsbad, CA, USA) was used to test the cell proliferation of SG tissue. After deparaffinization and rehydration, specimens were treated with 10 mM Citrate Buffer solution (pH 6.1) in 95°C water bath for half an hour and then cooled down to room temperature for 30 minutes. The rest of procedures followed the manufacture's instruction. The number of positive cells was counted under 400 X magnification of 5-8 fields/gland with Image J software (NIH).

Immunofluorescent staining.

Submandibular and parotid gland were embedded into optimal cutting temperature (OCT) and cut into 6-8 μm thickness frozen sections. After fixing with 4% PFA (P6148, Sigma-Aldrich, ST. Louis, USA) for 15 minutes, slides were blocked with 10% donkey serum for 1 hour. These primary antibodies were used rabbit anti-aquaporin 5 (1:200, AQP5, ab92320, Abcam, Cambridge, MA, USA); mouse anti-alpha smooth muscle actin (1:200, α -SMA, ab7817, Abcam); rabbit anti-cytokeratin 5 (1:400, CK5, Sigma-Aldrich, Oakville, ON, Canada); goat anti-GFRα -2 (1:200, AF429, R&D systems, Minneapolis, USA); goat anti-CD31 antibody (1:200, AF3628, R&D Systems, Minneapolis, USA); PBS was used as negative control. SG tissues were incubated overnight with primary antibodies or PBS at 4°C. In the next day, slides were incubated with secondary antibodies (1:200) for 1 hour at room temperature. These secondary antibodies were used: donkey anti-rabbi-Rhodamine Red™-X- conjugated, anti-mouse-Alexa Fluor® 594-conjugated, anti-goat-Alexa Fluor 488-conjugated. 4, 6-diamidino-2-phenylindole, dihydrochloride (DAPI; Invitrogen, Ottawa, ON, Canada) was used to label nucleus of the cells. Leica DM4000 fluorescent microscope were used to take 5-8 pictures for each tissue, and intensity of the fluorescence signal was analyzed by ImageJ software (NIH).

Quantitative real- time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the submandibular glands with RNeasy Plus Minikit (74134, Qiagen, Valencia, CA91355, USA). First strand cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit (4368814, ThermoFisher Scientific, MA02451, USA). qRT-PCR assays were performed by Step One Plus (Life Technologies) in TaqMan Universal Master Mix II

(4440040, Applied Biosystem, Foster City, Canada). The cycles were programmed as follow: 50°C for 2min, 95°C for 20s, then 40 cycles at 95°C for 1 s and 60°C for 20s. The probes used in this study were epidermal growth factor (EGF) (Assay ID: Mm00438696), vascular endothelial growth factor (VEGF) (Assay ID: Mm01281449), insulin-like growth factor 1 receptor (IGF-1R) (Assay ID: Mm00802841), hepatocyte growth factor (HGF) (Assay ID: Mm01135193), basic fibroblast growth factor (FGF2) (Assay ID: Mm01285715), the GDNF family receptor-α2 (GFRα2) (assay ID: Mm00433584), cytokeratin 5 (CK5) (assay ID: Mm00503549), and aquaporin-5 (AQP5) (assay ID: Mm00437578). Glyceraldehyde- 3-phosphate dehydrogenase (GAPDH, Assay ID: Mm99999915) was used as the endogenous reference. Three experimental replicates were conducted for each sample. Results were expressed as fold changes in relative gene expression.

DISCUSSION

In chapter 2, we found that freeze drying was an effective approach for storage, distribution, standardization and transportation of perishable biomaterial, such as BMCE. In our study, freeze-drying could also increase the stability of the proteins in BMCE by removing the water content. The protein array results showed that the fresh BMCE could be preserved by freeze drying without any lost or degradation of the angiogenesis-related factors and cytokines. In addition, FD-BMCE contained a slightly higher level of matrix metallo-proteinase-9 (MMP-9), platelet factor 4 (PF4) and Stromal cell-derived factor-1 (SDF-1) when compared to fresh BMCE, which indicated that freeze-drying decreased the degradation of certain proteins. For the in vivo study, as expected, FD-BMCE could functionally restore IR-injured SGs as effective as the fresh BMCE. However, the BMCE used in this study was derived from the mouse bone marrow. In order to bring this potential therapy to the clinic, a cell extract originating from "human" bone marrow needed to be tested.

To our knowledge, there is no study that has tested human BMCE as a treatment for IR-injured SGs. The main findings in Chapter 3 were 1) human BMCE was as an effective treatment as mouse BMCE in repairing irradiated-SG in a mouse model, and 2) MCE had the best therapeutic effects when compared to GCE and RBCE. According to our results, human BMCE significantly increased SG function, but the improvement of SFR and the cell proliferation rate were slightly lower than that in the mouse BMCE group. This might result from the ineffective constituents mixed with human BMCE. Assmus et al. reported that the efficacy of MC therapy was impaired by the contamination of RBCs [1]. It implied that the ineffective cell extract fraction in BMCE may reduce the benefits of treatment and "purified" BMCE would induce a better efficacy. Meanwhile, a study reported that different fractions of bone marrow cells brought different outcomes in treating the infarcted heart [2] and promoting the myocardial regeneration [3]. Hence,

there appears to be a cell extract treatment with a better efficacy from a specific cell fraction in BM, such as GCE and MCE. The separation approach would also allow choosing the best cell extract product according to their efficacy. Taken together, it is therefore necessary to separate different fractions in human BM and test the potential of these cell extracts derived from GCs, MCs and RBCs.

The therapeutic effects in different BM subpopulation cell extract groups varied in the present study. Results indicated that MCE group showed the best therapeutic effect in restoring the function of IR-damaged SGs. MC population is a heterogeneous fraction comprised of diverse cell types, including the differentiated cells (lymphocyte and monocyte) and progenitor cell populations. Research reported that instead of the small number of stem cells in MCs, the beneficial effects of MC treatment might be due to the combined effects of all mononuclear cells [4, 5]. MC treatment has benefits when compared with treatments using other cell sources. First, diverse cell types with multipotent capacities are relatively concentrated in adult BM-derived MCs, such as hematopoietic progenitor cells, endothelial progenitor cells, mesenchymal stromal cells and small embryonic-like stem cells [6-10]. Second, it can be easily harvested and used with minimal processing when compared with cultured cells [3, 11, 12]. Moreover, when incorporated with the cell-free and lyophilized strategies, MCE therapy would become a more feasible and convenient treatment for the clinical application as it would induce a less immune reaction and provide a possibility for long-term storage. With this knowledge in mind, our study proposed an effective, safe and the most readily available treatment for salivary hypofunction: MCE therapy.

However, the procedure to harvest the human BM remains invasive to some patients. Therefore, cell extract from other human tissues with a less invasive harvesting procedure was tested. In Chapter 4, the constituents and function of a cell extract isolated from human minor SGs were analyzed. To date, one research team has isolated and characterized stem cells derived from human minor SGs (labial glands, LGs), but their use has been limited to study liver regeneration [13, 14]. Our study also used human LGs and the explant culture method to expand stem cells for transplantation, as the Lu and Zhang group did [13, 14]; however, our novelty was to focus the use of these LSCs to restore function to SGs injured by IR.

In our study, human LSCs were isolated by the explant method, which provided more benefits when compared with the classical enzymatic tissue digestion procedure. First, the enzymatic digestion method needs around 1g of tissue (roughly 30-40 pieces of minor SG) for 1-2 million(s) cultured primary cells, which is not cost-effective. While an equal number of cells can be harvested from 1/10 (0.1g) of SG tissues by the explant culture method. Jing et al. reported that the cells released would be admittedly slower at the beginning without the enzymatic digestion method. However, after 10-14 days, cells migrated from the tissue became 70-80% confluent almost at the same rate as the enzymatic digestion method [15], which is in agreement with the results in our study. Jing and colleagues also revealed that explant culture method gave a higher yield of MSCs when compared with the enzymatic digestion method [15]. The second advantage of the explant culture method was the activity of cells. Enzymatic treatment loses many cells and affects cells activity [16] by affecting cell viability, cell membranes [17], and distribution of cell intra-membranous particle [18]. Furthermore, the processing time in the explant culture method is shorter than the digestion procedure, which indicates less damage for the cells. Third, MSCs

harvested by the explant method had a higher proliferation rate [15]. One reason might be that the primary cells isolated from the explant method were more homogenous [19]. The last advantage is that extracellular matrix (ECM) is not dissociated in the explant culture. ECM acts as a reservoir for cytokines and growth factors such as fibroblast growth factors (FGFs) and hepatocyte growth factor (HGF) [16, 20], and communicate with MSCs cells [16]. Taken together, these results reveal that the explant culture method is an appropriate method for isolating LSCs and indicate the clinical potential for the autologous transplantation of LSCs/LSCE.

Currently, various MSCs were reported as a regenerative therapy option to increase the salivary flow rate and thus relieving xerostomia caused by radiotherapy [21-24]. However, challenges for cell-based therapies remain. One of the challenges is the lack of easily accessed and a sufficient population of human adult MSCs. To solve these problems, the stem cell isolated from human minor SGs was reported in this study. There are a large number of minor SGs (600-1000) throughout the oral cavity [25, 26]. Because of the location and the number of minor SGs, LSCs compared with other MSCs is more accessible. In this study, LSCs were highly positive to MSCs surface markers. In addition, these homogenous cells still keep expressing MSCs surface markers during later passages. Thus, LSC is an appropriate cell source to prepare the cell extract in the present study. In addition to the accessible, the LSCE treatment is practical in the clinic. For example, little pieces of labial glands can be easily isolated from the patient/donor before radiotherapy. Expanded LSCs could be prepared into sufficient LSCE and injected into the same patient weeks later post-IR. Generally, most patients with head and neck cancer are of older age. Therefore, it is important to avoid invasive tissue harvesting procedures. Based on these

advantages of LSCs and LSCE, this cell extract therapy is a promising treatment for IR-induced SG hypofunction.

CONCLUSION AND FUTURE DIRECTION

Conclusion

Our previous studies showed that mouse bone marrow cell extract could comparably restore salivary production post-irradiation and that the native proteins in the bone marrow cell extract are the main effective ingredients for the treatment. However, protein is unstable for long-term storage and they are easy to degrade once extracted from their native cellular environment. In chapter 2 of this thesis, we reported that lyophilization is a reliable approach to protect the proteins in bone marrow cell extract from degradation. Results showed that the components in lyophilized and freshly prepared cell extract were comparable. This is because protein structure and stability can be preserved by removing water molecules. Moreover, the functionality of the freeze-dried bone marrow cell extract is also comparable to that of the freshly prepared cell extract in treating the salivary hypofunction in the mouse model. These results indicated that the lyophilization could preserve the structure and activity of the cell extract and bring many additional advantages to the utilization of cell extract treatment. For example, the lyophilization approach promoted the standardization and transportation of bone marrow cell extract. Furthermore, the protein sample in solid form can be stored under ambient conditions. Therefore, cell extract powder is reduced in volume and could be easily used in any time for the patients.

Although mouse bone marrow cell extract had already been prepared and tested, the efficacy of the human whole bone marrow cell extract remains unknown. Besides, the whole bone marrow consists of heterogeneous cells which could be categorized into three major subpopulations, including red blood cell (RBCs), granulocytes (GCs), and mononuclear cells (MCs). In Chapter 3, we prepared and tested the human BMCE in the mouse model. In addition, three cell

subpopulations of human bone marrow were isolated and prepared into cell extracts (RBCE, GCE and MCE). Results showed that growth factors in each cell extract were different and cell extract with MCE contained more angiogenesis-related growth factors in comparison with other cell extracts. For the study in vivo, human BMCE could restore the function of irradiation-induced SGs. MCE treatment provided the best therapeutic effect, while RBCE treatment did not improve the secretory production when compared to the control group. Our study narrowed down the complex of human BMCE and reduced the difficulties of identifying the effective proteins in the cell extract in future studies. Furthermore, our results indicated that MCE provided the best therapeutic effect which could be a promising alternative for cell therapy in treating the irradiation-injured SGs in the future.

The multipotent-specific stem cell isolated from human major SGs was reported to restore SG hypofunction induced by irradiation. However, cell-based therapy provides potential risks and the procedures used to harvest the major SGs are invasive and unpractical. Our study in Chapter 4 isolated an alternate cell, mesenchymal stem cell from human minor labial glands (LSC) and prepared the LSC into cell extract (LSCE). LSC was used because it is an accessible and practical cell source since it could be harvested from small pieces of human labial gland tissues by explant culture method. Results showed that the LSCE contained numerous growth factors and restored saliva secretion, protected acinar cells, blood vessels and parasympathetic nerves, promoted cell proliferation, and up-regulated the expression of tissue repair/regeneration proteins and genes.

This thesis developed a lyophilized method for BMCE preservation, isolated and analyzed the effective cell extract from the human BM cell subpopulations, and tested the effect of labial gland

stem cell extract in irradiation-injured SGs model. The findings provide the experimental basis for the future use of cell-free therapy in the clinic. However, further study is needed to optimize the cell extract application and investigate the mechanism behind the cell-free therapy.

Original Contributions

- Lyophilization is an appropriate technique to protect the proteins in the cell extract.
- Lyophilized BMCE could rescue the hypofunction of IR-injured SGs.
- The proteins in the human and mouse BMCE are different.
- Human BMCE, RBCE, GCE and MCE contain different proteins/ growth factors.
- Human BMCE can rescue the hypofunction of IR-injured SGs.
- Human MCE contains most effective proteins when compared with other cell extracts from bone marrow.
- Human MCE shows the best therapeutic effect in treating the IR-injured SGs when compared with the other cell extracts from bone marrow.
- Human GCE can rescue the hypofunction of IR-injured SGs but results in the acute inflammatory response in the mouse model.
- Human RBCE cannot rescue the IR-injured SGs.
- The gender of bone marrow donors did not influence on the effect of cell extract.
- A large number of LSCs can be harvested from small pieces of human labial gland.
- Human LSCE contains numerous growth factors.
- Human LSCE could restore the function of IR-injured SGs.

On-going project and Future plan

Ongoing work

- Comparing the ingredients in LSCE and conditioned medium of LSCs (LSC-CM), and analysis the efficacy of the LSCs, LSCE and LSC-CM in restoring the irradiation-induced SGs.
- Comparing the cell extracts of stem cells derived from various human oral tissues (dental pulp, periodontal ligament, buccal fat pad and minor salivary gland) in treating the SG hypofunction.
- Investigating the efficacy of cell extracts from human peripheral blood cell subpopulations in the SGs hypofunction mouse model.
- Developing an in vitro NSSVAC cell culture model to investigate the therapeutic effect of cell extract in vitro.
- Separating the effective proteins in cell extract by high-performance liquid chromatography and testing their effect in cell culture model in vitro. Then, the effective proteins will be identified by spectrometry.

Future plan

- Testing the effect of exosomes derived from the mesenchymal stem cells in treating the salivary hypofunction.

- Assessing the safety of each cell extract via testing the minimal and maximal dose of the cell extract in vitro with the NSSVAC model and in vivo with mouse model. In addition, the promotion tumorigenesis effect of the cell extract would be tested in the mouse model.

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