An experimental study of menopause induced by bilateral ovariectomy and mechanistic effects of mesenchymal stromal cell therapy on the parotid gland of a rat model

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1. Introduction

In mammals, the saliva plays several essential roles including the protection of the oral cavity apparatus and the gastrointestinal epithelium. Moreover, it facilitates tasting, mastication, swallowing, and even digestion of food (Logemann et al., 2001). The parotid gland is one of the major salivary glands. Upon stimulation, it secretes over 50% of the total body saliva (Humphrey and Williamson, 2001). Salivary gland hypofunction is frequently observed in mammals and manifested by xerostomia (dry mouth) that leads to functional oral disorders. Xerostomia is associated with aging and many other systemic diseases (Yeh et al., 1998), and is frequent among menopausal females (Frutos et al., 2002). Additionally, it is frequently observed after radiotherapy (Jeong et al., 2013) and as a side effect of certain medications (Mortazavi et al., 2014).

The menopause is not a unique phenomenon to only human females, but it also occurs in a number of animals with longer lives, including the primates species (Walker and Herndon, 2008). Experimentally, the animal model of menopause could be achieved via bilateral ovariectomy (Arafat et al., 2016). Decline of estrogen (E2) levels during menopause is considered to be a major change that leads to many changes in the body (Messina et al., 2013). Interestingly, Rahnama et al. (2004) reported a correlation between the dryness of the mouth and the E2 deficiency, and owed it to the presence of high-affinity E2 receptors (ER), especially ER-β, in the major salivary glands of rats. Additionally, Gejima et al. (2007) detected ER-α in the parotid gland of adult female rats. The severe affection
of the parotid gland in menopausal animal models has also been documented (Kusunoki et al., 2006; Mohamed et al., 2015).

Apoptosis refers to programmed cell death that occurs in tissues to maintain homeostasis in the body. Lewis-Wambi and Jordan (2009) noticed that E2 regulates proliferation and apoptosis of many cell types. Recently, association between E2 deficiency and histological alterations induced by apoptosis in the rat’s parotid gland has been noticed (Kusunoki et al., 2004). This apoptotic induction is caused mainly by free radicals (Kusunoki et al., 2006).

All therapeutic approaches to the treating of menopausal symptoms via hormonal replacement are associated with the possibility of venous thrombotic complications (Biłkowska, 2014), breast cancer, and gynecological cancers (Bregar et al., 2014). In addition, herbal therapy also has serious side effects including gastrointestinal disorder and abnormal vaginal bleeding (Xu et al., 2012), which restrict its clinical applications and encourage researchers to find safe and effective alternative therapies for the treatment of menopausal symptoms.

In the past decade, tissue engineering, particularly stem/stromal cell regenerative medicine, has made significant advances in terms of restoration of the normal tissue functions. Mesenchymal stromal cells (MSCs) have been successfully isolated from bone marrow (BM) (Jiang et al., 2002), adipose tissue (Eirin et al., 2012), peripheral blood (PB) (Wan et al., 2006), and human umbilical cord blood (hUCB) (Hill et al., 2009). The unique feature of MSCs is the immunological property of positivity for CD105, CD73, and CD90 while lacking the expression of CD34, CD19, CD45, and HLA-DR surface molecules (Dominici et al., 2006). The hUCB-MSCs are considered as one of the most easily available, least immunogenic and legal materials (Ding et al., 2015), having the high potential for expansion and plasticity (El Maadawi and Gabr, 2011). Moreover, Doi et al. (2016) proved their safety in in vitro culture. Surprisingly, both E2 and hMSCs exhibit similar antioxidant capacity and trophic effects (Valle-Prieto and Conget, 2010). In addition, Zhang et al. (2012) and Li et al. (2015) reported that, in high glucose culture, MSCs can differentiate into steroidogenic cells which synthesize and secrete E2.

Interestingly, stromal cells have been successfully used to treat many diseases such as diabetes mellitus (Soria et al., 2001), brain injury (Bang et al., 2005), cardiovascular diseases (Kawada et al., 2004; Doi et al., 2014), and several disorders associated with ovariectomy such as myocardial ischemia (Ray et al., 2008), urinary incontinence (Lin et al., 2010), osteoporosis (Huang et al., 2016), and cerebellar disorders (Ahmed et al., 2017). Despite the severe affections of the parotid gland in menopausal animal models and side effects of its hormonal or herbal therapy, a new approach for its treatment is lacking. Therefore, the purpose of this study was to evaluate histological and immunohistochemical structural alterations in the parotid gland of a menopausal model, induced by abrupt E2 deficiency via ovariectomy in adult female albino rats and the potential therapeutic roles of hUCB-MSCs.

2. Materials and methods

2.1. Isolation and preparation of MSCs from hUCB

This isolation of cells was done in the clinical chemistry and stem cell lab, in the Medical Biochemistry & Molecular Biology Department, Faculty of Medicine, at Zagazig University, Egypt.

2.1.1. Collection of hUCB sample

Cord blood was obtained under complete aseptic conditions from the umbilical vein of six post-delivery full-term placentae at Zagazig University Hospital after getting informed consent from the husbands and after it was approved by the Institutional Review Board (IRB), Zagazig University (ZU-IRB #4626). The samples were directly collected in a sterile 50 ml Falcon tube containing 2 ml ethylene diamine tetraacetic acid (EDTA) (Lonza Bioproducts, Belgium) and 5 ml of phosphate buffer saline (PBS) of pH 7.2. Then, the samples were transported (maintaining a temperature between 15–22 °C) to the clinical chemistry-and stem cell lab for isolation of stromal cells.

2.1.2. Isolation and culture of hUCB derived MSCs

The collected blood was diluted three times with PBS. Then, 30 ml of diluted blood was carefully loaded on 10 ml of Ficoll/Paque (Lymphocyte Separation Medium 1.077, Lonza Bioproducts, Basel, Switzerland) in 50 ml centrifuge tubes. The centrifugation was done at 435 g for 30 min at 22 °C. After removal of the supernatant and careful aspiration of the mononuclear cells’ (MNCs) layer, the MNCs were washed with PBS twice and centrifuged at 20 °C for 10 min. Finally, the MNCs were isolated and the supernatant was discarded. The isolated MNCs were subcultured in DMEM (Dulbecco’s modified Eagle medium) (Cambrex Bio Science, Minnesota, USA) supplemented with 10% fetal bovine serum (FBS, Lonza Bioproducts, Basel, Switzerland) and 1% penicillin-streptomycin-amptherocin B mixture as 10 IU/25 mg/100 ml (Lonza Bioproducts, Belgium) at a concentration of 5000 cm-2 (0.2–0.3 ml media)-1. The culture was incubated at 37 °C in 5% humidified CO2 in a CO2 incubator (Heraeus, Germany). After overnight incubation (12–18 h), the media were replaced in order to eliminate non-adherent cells. The media were replaced every three days for 12–14 days until colonies of MSC were noticed as spindle-shaped fibroblastoid cells of the first passage culture (at 80–90% confluence) under the inverted microscope. At 37 °C for 5 min, colonies were released with 0.25% trypsin in 1 ml EDTA. Immediately after trypsinization and centrifugation, subculturing was done in serum-supplemented medium and incubated in 50 cm2 tissue culture flask until adherence and fusiform shape of MSCs were obtained (Bieback et al., 2004). The fourth passage of culture was used after labeling with fluorescent marker using Paul Karl Horan 26 (PKH-26) Fluorescent Cell Linker Kit (Sigma-Aldrich Chemie, Steinheim, Germany) obtained from Algomunia Co (Mohafza st., Zagazig, El Sharqia, Egypt) as per the protocol described by Haas et al. (2000).

2.1.3. Immunophenotypic characterization of hUCB-MSCs by flow cytometry applications

Based on stromal cell surface markers, we used monoclonal antibodies against human CD105 and CD90 (mesenchymal stromal cell surface marker) (20% cutoff) (Zheng et al., 2013). We also used monoclonal antibodies against human CD34 and CD45 to exclude hematopoietic, endothelial cells, and leukocytes. All the antibodies were obtained from BD Bioscience. The detached cells were washed twice with PBS and incubated with either anti-human CD105 (Cat No: 323205), CD90 (Cat No: 559869), CD34 (Cat No: 4084644), by washing the cells.
or CD45 (Cat No: 349202) according to manufacturer’s instructions, for 30 min in the dark. Cells were analyzed by FACS Scan 3 color (Becton Dickinson, Heidelberg, Germany) running Cell Quest software (BD, San Joe, USA), in the Clinical Pathology laboratories, Zagazig University.

2.2. Animals and experimental ethics

Eighteen female albino rats (6 months old) were obtained from the animal house, Faculty of Veterinary Medicine, Zagazig University, Egypt. The rats were housed in individual stainless-steel cages in a clean room with controlled temperature (23 °C) and humidity (60%), and with a 12 h dark/light cycle. The animals were given a standard diet and tap water ad-libitum. The authors adhered to the Guide for the Care and Use of Laboratory Animals of Zagazig University. The experimental protocols described in this research were approved by the Institutional Review Board (IRB), Zagazig University (ZU-IRB #4626). The rats were divided equally into three groups (n = 6): sham-operated (SHAM), ovarioctomized (OVX), and OVX + hUCB-MSCs. The OVX + hUCB-MSCs group received an injection of 4.5 × 10⁶ hUCB-MSCs dissolved in 250 μl PBS on the tail vein two months after ovarioctomy for four consecutive weeks (twice a week) (Calatrava-Ferreras et al., 2012). However, both SHAM and OVX groups were injected with vehicle (PBS) only.

2.3. Ovarioctomy procedure

The ovarioctomy was performed by a method described by Huang et al. (2016) under complete aseptic conditions ten days after acclimatization of the rats. Briefly, all rats were anesthetized by intraperitoneal injection of 1% Na pentobarbital. After the onset of anesthesia, clipping and shaving the skin was done, then aseptic scrubbing with alcohol and povidone iodine. A short dorsal midline skin incision was made halfway between the caudal edge of the rib cage and the base of the tail bilaterally with a surgical blade (No. 11). Opening the muscles and peritoneal cavity, the adipose tissue was retracted until the ovaries were identified which were then excised after ligation of their blood supply using 3-0 vicryl. At the end of the surgical procedures, the peritoneum and abdominal muscles were closed using 3-0 vicryl and the skin suture was done with 2-0 nylon. After the surgery, the rats were allowed to live in their normal environment. They were given ampicillin (4000 IU/kg, intraperitoneal) for three days. The rats were subcutaneously given a non-steroidal anti-inflammatory drug, meloxicam (0.2 mg/kg) once daily for three successive days. The wound dressing was applied every day for a week to prevent the risk of infection. For SHAM group, both ovaries were exposed and only mobilized.

2.4. Salivary flow rate

Under the effect of anesthesia (intramuscular injection of a mixture of Ketamine HCL (50 mg/kg) and Xylazine HCL (5 mg/kg)), saliva was collected from all rats two to three minutes after pilocarpine was injected subcutaneously. Saliva was flowed freely into a sterile glass pipette for a period of 30 min for each rat and was then harvested. The salivary flow rate was presented as microliters per minute.

2.5. Tissue preparation for microscopic observation

At 3 months post-ovarioctomy, rats from all experimental groups were euthanized under inhalational anesthesia. The parotid glands were dissected, weighed, and immediately fixed in 4% paraformaldehyde solution. After overnight fixation, parotid gland specimens were dehydrated in graded alcohol and embedded in paraffin. Then, 3-μm thick paraffin sections were deparaffinized, rehydrated, stained with hematoxylin and eosin (H&E), and observed under a light microscope, and photographed with the Amscope digital camera (Bancroft and Layton, 2013).

For the detection of the PKH26-labelled hUCB-MSCs homing, 3-μm thick deparaffinized sections of all groups were examined and photographed with a fluorescence microscope (Olympus BX50F4, No. 7M03285, Tokyo, Japan).

2.6. Immunohistochemistry

The immunohistochemical analyses for human CD105 and human CD34, proliferating cell nuclear antigen (PCNA), caspase 3 and single strand DNA (ssDNA), aquaporin (AQP1), α smooth muscle actin (SMA), and mouse CD34 were performed to detect hUCB-MSCs homing, proliferating cells, apoptotic cells, blood capillaries, myoepithelial cells, and endogenous hematopoietic progenitor cells, respectively (Elewa et al., 2010). In brief, antigen retrieval was done with the deparaffinized sections according to each antibody (Table 1). Then sections were incubated in methanol containing 0.3% H2O2 for 20 min at 4 °C to block the activity of endogenous peroxidase, followed by washing in distilled water, and incubation with 10% normal blocking serum for 1 h at room temperature (donkey serum for PCNA immunostaining and goat serum for staining of the other antigens). Then sections were incubated overnight with the specific primary antibody diluted in PBS (pH 7.2). The antibodies and working dilutions are shown in Table 1. For negative control, the primary antibody was replaced with only PBS. Then the sections were incubated with biotin-conjugated secondary antibody, specific to the primary one, for 30 minutes, followed by incubation for 30 min with streptavidin–peroxidase conjugate. The positive reaction was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB)–H2O2 solution, pH 7.0. Finally, the sections were washed in distilled water and counterstained with Mayer’s hematoxylin.

2.7. Histoplanimetric analysis

The number of immunopositive PCNA, ssDNA, caspase 3, and mouse-CD34 cells were counted in immunohistochemical stained sections at 400× magnification. In addition, the integrated density of AQP1 and α-SMA were evaluated using Photoshop (Adobe Systems, San Jose, Calif., USA) followed by Image J analysis software (ver. 1.32j, http://rsb.info.nih.gov/ij/). Briefly, using Photoshop we converted the RGB color images into grayscale to reduce the background color. Then, using threshold, we highlighted the signal into black color with a white background and inverted the signal color from black to white and saved the images. Subsequently, we analyzed the integrated density of the white signals on the processed images using Image J software.

2.8. Statistical analysis

All statistical analysis results were expressed as mean ± standard error (SE). The one-way ANOVA test was used to analyze the data among different groups, followed by multiple comparisons Duncan’s Post-hoc test when significant differences were observed (P < 0.05) (n = 6 per group).

3. Results

3.1. Characterization of hUCB-MSCs and homing of PKH26-labeled hUCB-MSCs in parotid gland

Morphologically, MSCs culture at day 7 of isolation showed fusiform-shaped cells (Fig. 1A). The flow cytometric analysis of the
hUCB-MSCs culture revealed that the cells had a positive expression for human CD105 (85.65%) and human CD90 (62.33%), while a negative expression for human CD34 (1.36%) and human CD45 (2.19%) were detected (Fig. 1B). To confirm the homing of PKH26-labeled hUCB-MSCs, 3-μm thick deparaffinized sections of all groups were examined. The MSCs were detected in the parotid gland sections of the OVX + hUCB-MSCs group (3 months post-ovariectomy) as red fluorescence between acini and ducts (Fig. 2A and B) but not in other groups (data not shown). Furthermore, a positive reaction for human CD105 was observed around the acini and ducts (Fig. 2C), while the human CD34 expression was not detected (Fig. 2D).

### 3.2. Salivary flow rate and weight of parotid glands

As shown in Table 2, both the salivary flow rate and the weight of the parotid glands were significantly decreased in the OVX group when compared to the SHAM group. After hUCB-MSCs injection, a significant increase was observed by comparison with that of the OVX group.

### 3.3. Histological observations

Examination of H&E stained sections revealed that the parotid gland of the SHAM group was a multilobulated organ. Each lobe was formed of a closely packed serous acini and a series of duct systems. All serous acini had very narrow lumina that could hardly be seen and were lined with pyramidal acinar cells. The acinar cell cytoplasm exhibited apical acidophilia and basal basophilia with basal rounded nuclei containing prominent nucleoli. The intralobular duct system was composed of intercalated ducts and striated ducts. Both ducts were lined with cuboidal and columnar epithelial cells, respectively, with acidophilic stained cytoplasm. The striated ducts were the predominant ducts (Fig. 3A). In the OVX group, the acini were dispersed with lightly stained cytoplasm. The acinar cells lining the acini had numerous cytoplasmic vacuoles and some crescent-shaped nuclei. The striated duct had darkly stained nuclei and an ill-defined basal striation. Additionally, an interstitial hemorrhage was pronounced (Fig. 3B). An observable improvement of the parotid gland architecture was noticed in the OVX + hUCB-MSCs group with apparently normal structures of both acinar cells and striated ducts (Fig. 3C).

### 3.4. Immunohistochemical observations

The immunohistochemical staining with anti-PCNA was performed to detect proliferating cells. The positive cells were observed in the acini of the three different experimental groups, as expected (Fig. 4A–C), while the negative control immunos-
Fig. 2. Immunofluorescence analysis. Immunofluorescence detection of the homing of the PKH26-labelled hUCB-MSCs around the acini (solid arrows) and ducts (dashed arrows) in the parotid glandular tissues of the OVX + hUCB-MSCs group (3 months post-ovariectomy) is noticed (A, B). Immunohistochemical detection of the hUCB-MSCs homing in sections of parotid glands express a positive reaction with anti-human CD 105 around acini (solid arrows) and duct (dashed arrows) (C) but a negative reaction with anti-human CD 34 (D).

Table 2

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SHAM</th>
<th>OVX</th>
<th>OVX + hUCB-MSCs</th>
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<tr>
<td>Salivary flow rate (μl/min)</td>
<td>48.08 ± 3.07(^a)</td>
<td>10.03 ± 1.10(^b)</td>
<td>43.85 ± 3.56(^a)</td>
</tr>
<tr>
<td>Parotid glands weight (mg)</td>
<td>303.05 ± 12.72(^a)</td>
<td>175.43 ± 6.49(^b)</td>
<td>280.58 ± 8.82(^a)</td>
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\(^a\) Data are expressed as the mean ± SD.
\(^b\) Different superscripts indicate significant difference at \(P < 0.05\) by one-way ANOVA followed by the multiple comparisons Duncan’s Post-hoc test for analysis of difference among different groups.

Fig. 3. Histomorphological features of the parotid glands. Representative histopathological photomicrographs of H&E stained parotid glandular tissues of the SHAM group (A), OVX group (B), and OVX + hUCB-MSCs group (C) showing serous acini (S), intercalated ducts (white arrowheads), striated duct (SD) with a prominent basal striation (solid arrows), intracytoplasmic vacuoles (black arrowheads), crescent-shaped nucleus (dashed arrow), and interstitial space with hemorrhage (Hg).

tained sections of the parotid gland of the SHAM group revealed an absence of any positive reaction (Fig. 4D). The expression of positive cells was significantly decreased in the OVX group (10.56 ± 0.63), compared to the SHAM group (30.08 ± 2.51), while they were considerably higher in the OVX + hUCB-MSCs group (23.28 ± 1.42) compared to the OVX group (Fig. 4E).

Immunohistochemical staining for caspase 3 and ssDNA were performed to observe apoptotic cell populations. The caspase 3 positive apoptotic cells were observed in the interstitial cells of the three groups (Fig. 5A–C). The negative control sections of the parotid gland of the OVX group revealed the absence of apoptotic cells (Fig. 5D). In the OVX group, there was a significant increase in caspase 3 apoptotic cells (11.17 ± 0.76), compared to the SHAM group (0.83 ± 0.17), while the frequency of such apoptotic cells was significantly decreased in the OVX + hUCB-MSCs group (3.11 ± 0.44) compared to the OVX group (Fig. 5E). The other apop-
Hormetic cell marker was ssDNA that were mainly detected in both acinar and ductal epithelium (data not shown) of the OVX group, while they were hardly to be seen in the SHAM and OVX + hUCB-MSCs groups. The OVX group showed a significant increase in ssDNA positive cells (101 ± 14.18), compared to the SHAM group (1.22 ± 0.55). On the other hand, the number of ssDNA positive cells was much lower in the OVX + hUCB-MSCs group; however, a non-significant difference was observed in the average number of the ssDNA positive cells in the OVX + hUCB-MSCs group (4.44 ± 1.3) when compared with that of the SHAM group (Fig. 5F).

The immunohistochemical staining with anti-AQP1 was performed to examine the alterations of the blood capillaries among the three different groups. The endothelial cells of blood capillaries showed positive reactions for AQP1 in all groups (Fig. 6A–C). No positive reaction was observed in the negative control sections of the parotid gland in the SHAM group (Fig. 6D). The integrated density of AQP1 positive reactions showed a significant reduction in the OVX group (15.02 × 10^6 ± 1.35), compared with the SHAM group (31.24 × 10^6 ± 3.77). However, an increase in the integrated density of AQP1 positivity was observed in the OVX + hUCB-MSCs group (29.05 × 10^6 ± 2.87), compared to the OVX group. However, there was no significant difference in the integrated density of PCNA positive reaction was observed in the OVX + hUCB-MSCs group compared to that observed in the SHAM group (Fig. 6E).

The changes in myoepithelial cell populations were investigated in different experimental groups via immunohistochemical staining with anti-α-SMA. Interestingly, the myoepithelial cell populations showed a great variations among different groups. Well-developed α-SMA positive myoepithelial cells with numerous cytoplasmic processes were observed around the acini of the parotid glands of the SHAM group (Fig. 7A), while the myoepithelial cells after ovariectomy showed fewer and shorter processes (Fig. 7B). In the OVX + hUCB-MSCs group, the cytoplasmic processes showed moderate development (Fig. 7C). The negative control sections of the parotid gland of the SHAM group showed no positive reaction (Fig. 7D). These findings were confirmed by analyzing the integrated density of immunopositive cytoplasmic processes. The integrated density of positive reactions showed a significant decrement in the OVX group (33.99 × 10^6 ± 4.48) when compared to the SHAM group (141.22 × 10^6 ± 11.55). However, following treatment with hUCB-MSCs, a higher integrated density of positive reactions

![Fig. 4. Proliferating cell populations in the parotid glands. Immunohistochemical photomicrographs showing PCNA positive cells (arrows) in the acini of SHAM group (A), OVX group (B), and OVX + hUCB-MSCs group (C). The negative control stained section of the SHAM group in which the primary antibody is replaced by PBS (D). Bar chart showing the average number of PCNA positive cells of different groups (E). Each bar carrying different superscripts letters (a, b, and c) are significantly different as analyzed by the one-way ANOVA test, followed by the multiple comparisons Duncan’s Post-hoc test (P < 0.05); n = 6 in each experimental group. Values = mean ± SE.](image)
was observed in the OVX + hUCB-MSCs group \((95.85 \times 10^6 \pm 5.82)\) compared to that of the OVX group (Fig. 7E).

Immunohistochemical staining using mouse CD34 antibody in the parotid gland sections of different experimental groups was performed to evaluate the endogenous hematopoietic progenitor cell populations (Fig. 8A–C). The negative control sections of the parotid gland of the SHAM group revealed no positive reactivity (Fig. 8D). A significant loss of CD34 positive cells was observed in the OVX group \((0.33 \pm 0.21)\) as compared with the SHAM group \((12.33 \pm 1.28)\), but their expression was markedly restored in the OVX + hUCB-MSCs group \((12.17 \pm 1.35)\) in which there was no significant difference compared to the SHAM group (Fig. 8E).

4. Discussion

During the female reproductive cycle, E2 promotes salivary glands growth and mediates changes in the saliva’s chemical composition (Valimaa et al., 2004) owing to the presence of ER-β (Rahnama et al., 2004) and ER-α (Gejima et al., 2007) in the parotid gland of adult female rats. Therefore, in the present study, we investigated the influence of abrupt E2 deficiency on the parotid gland structure via ovariectomy of adult female albino rats. Moreover, we examined the potential therapeutic roles of hUCB-MSCs on the parotid gland architecture following ovariectomy.

Both the parotid glands weight and the salivary flow rate were significantly decreased in the OVX group in comparison to the SHAM group, as previously reported (Abd El-Haleem et al., 2018). It was confirmed in the present study by our observations of the glandular architecture that showed numerous intracytoplasmic vacuoles in the acinar cells of rats of OVX group. Similar atrophic changes in the parotid glands were reported in radiated rats (Jeong et al., 2013) and in rats after bilateral ovariectomy (Parlak et al., 2014; Abd El-Haleem et al., 2018). The cellular vacuolization is considered by Myers and McGavin (2007) as an early sign of cellular degeneration. Deficiency of the trophic effects of E2 (Valimaa et al., 2004) and increment in apoptosis (Kusunoki et al. 2006) may be the causes of these structural alterations. In the present study, ovariectomy caused a statistically significant decrement of proliferative
cell populations and increment of apoptotic cells as compared to the SHAM group. The apoptotic cells expressed positive caspase 3 in the interstitial tissues and ssDNA in both acinar and ductal cells of the OVX group. On the other hand, Limesand et al. (2006) found caspase 3 activation in the acinar cells of both parotid and submandibular glands of gamma-irradiated mice. The caspase-3 is required for DNA fragmentation and some of the typical morphological alterations in cells during apoptosis (Jânicke et al., 1998). Therefore, the apoptotic cells were identified based on early events (activation of caspase-3, keratin 18 cleavage) or late events (nuclear condensation, DNA fragmentation) in the apoptosis pathway as reported by Krysko et al. (2008). We proposed that the interstitial apoptotic cells could be myoepithelial cells. Both apoptotic and proliferating myoepithelial cells were detected in the submandibular glands of atrophic rats via duct ligation (Takahashi et al., 2001). The results of the present study were reinforced by the observations of Limesand et al. (2006), who clarified that glandular homeostasis requires a balance in cell proliferation and apoptosis.

Interestingly, the ovariecomized rats showed an impairment of acinar and ductal structures with a reduction of myoepithelial cell processes, which might explain xerostomia in the menopausal human or long-lived animals.

Recently, therapeutic approaches via stromal cells have successfully been used in many disorders such as radiation-damaged rat salivary glands (Jeong et al., 2013) and damage of parotid glands in ovariecomized rats via bone marrow-MSCs (Abd El-Haleem et al., 2018). In the current work, we chose hUCB-MSCs to treat the OVX-induced damage of parotid glands due to their unique biological characteristics. The hUCB-MSCs are more advantageous than bone marrow-MSCs in this context since they are younger and have a wider differentiation capability (Zhao et al., 2016).

After hUCB-MSCs injection in this study, we observed apparently normal acinar cells and striated ducts in H&E stained sections. In addition, the increased proliferative cells and decreased apoptotic cells in the immunostained sections may suggest that MSCs may act through paracrine mechanisms via anti-apoptotic factors including cytokines (Takahashi et al., 2006), stanniocalcin-1, and...
vascular endothelial growth factor (Doorn et al., 2012) to mediate tissue repair and regeneration.

The AQP1 is a water channel found in the endothelial cell of blood capillaries. These channels play an important role in water permeability (Li et al., 1994). Therefore, decrement of AQP1 expression in our OVX group denoted a dysfunction of the blood capillaries that coincided with the noticeable interstitial hemorrhage. These findings are in support of the previous report of Jin et al. (2012) who demonstrated that AQP1 was influenced by E2 deficiency. Smith et al. (2009) added that E2 and progesterone deficiency influence the balance between vasoconstriction and vasodilatation in the submandibular gland. In our study, the improvement of AQP1 densities in the OVX + hUCB-MSCs group may be due to the neovascularization (Takahashi et al., 2006; Lim et al., 2013) via angiogenic cytokines (Takahashi et al., 2006) and endothelial cell-derived clusterin molecules (Mishima et al., 2012) secreted by h-MSCs. Jin et al. (2012) confirmed that the AQP1 plays an important role in regulating body electrolyte balance and fluid secretion. In 2016, Teos et al. (2016) and Delporte et al. (2016) demonstrated that AQP1 has a definite role in saliva secretion that was proved via increase in saliva secretion after an intraductal injection of human AQP1 incorporated with adenovirus.

Our study used integrated density of α SMA-positive area on immunostained sections as an index of the changes in myoepithelial cell populations. These densities were significantly decreased in the OVX group whereas they significantly increased after hUCB-MSCs treatment. This obvious myoepithelial loss clarifies its role in glandular hypofunction associated with ovariectomy, as previously reported by Safayi et al. (2012).

The most interesting finding is the presence of endogenous CD34+ hematopoietic progenitor cells around acini and ducts in the parotid glands of the SHAM group. CD34+ is a cell surface marker of hematopoietic stem/stromal cells and hematopoietic progenitor cells (Sidney et al., 2014). These cells are identified as side population (SP) cells (Mishima et al., 2012). Our findings revealed the ovariectomy-induced depletion of endogenous CD34+ hematopoietic progenitor cells via apoptosis. Such findings are similar to that produced by irradiation that leads to the damage of self-renewal.
property of hematopoietic stromal cell in mononuclear cells isolated from bone marrow of mice (Wang et al., 2006). In our study, the mechanism of CD34+ hematopoietic progenitor cells recovery may be via angiocrine (Kobayashi et al., 2010) and pleiotrophin (Himburg et al., 2010) growth factors that were secreted from recovered endothelial cells. Sidney et al. (2014) reported that the regenerative functions of endogenous stromal cells occur by providing molecular signals to the proliferating immature epithelial cells in the forms of basement membrane proteins, extracellular matrix, matrix metalloproteinases/proteases, and growth factors. Further investigations are required for further elucidation of the mechanism of hUCB-MSCs mediated parotid gland regeneration.

In summary, our results suggest that the bilateral ovariectomy could affect the parotid gland structure due to destruction of endothelial cells, and apoptosis of acinar and ductal cells. Interestingly, we revealed that most of the structural injuries of the parotid gland were improved by hUCB-MSCs therapy. Such improvement in mechanisms by MSCs might be via endothelial cells recovery and endogenous CD34+ hematopoietic progenitor cells rescue. Apoptosis inhibition and proliferation enhancement mechanisms of acinar and ductal cells with myoepithelial cells recovery could also be responsible. Therefore, hUCB-MSCs therapy might be a good alternative to treat parotid gland destruction, especially in menopausal cases, to evade the health risks of hormonal therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.aanat.2018.06.006.


