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**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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Abstract:

The current study was conducted on a menopause rat model induced by ovariectomy to assess the histological and immunohistochemical alterations in the parotid glands and to verify the efficiency of human umbilical cord derived-mesenchymal stromal cell (hUCB-MSCs) in treating this condition. Eighteen adult female rats were equally divided into three groups: sham-operated (SHAM), ovariectomized (OVX) and OVX injected with hUCB-MSCs (OVX + hUCB-MSCs). At 3 months post-ovariectomy, the salivary flow rate and size of the parotid glands were measured. The parotid glands were histologically investigated via H&E stained sections. Furthermore, immunohistochemical analysis for human CD105, human CD34, proliferating cell nuclear antigen (PCNA), single strand DNA (ssDNA), Caspase 3, aquaporin (AQP)1, α -Smooth Muscle Actin (α -SMA) and mouse CD34 were performed. The OVX group showed interstitial hemorrhage, dispersed acini and intracytoplasmic vacuoles in the acinar cells. Furthermore, immunohistochemical staining revealed a significant decrement in the number of ssDNA positive apoptotic cells, but a significant increment of PCNA positive proliferating cells, AQP1 positive blood capillaries, α -SMA positive myoepithelial cells and endogenous CD34 positive hematopoietic progenitor cells in the OVX + hUCB-MSCs group as compared with the OVX group. These findings suggest a potential regenerative therapy of MSCs to injured parotid gland structures. However, further investigations are required to illustrate the mechanism of hUCB-MSCs mediated parotid gland regeneration.

Keywords: Parotid gland; ovariectomized; mesenchymal stromal cells; immunohistochemistry

1 **Abbreviations:**

2 α -SMA: Alpha-Smooth Muscle Actin

3 AQP1: Aquaporin1

4 Er: Estrogen receptor

5 hUCB: human Umbilical Cord Blood

6 hUCB-MCs: human Umbilical Cord Blood-Derived Mesenchymal Stromal Cells

7 MSCs: Mesenchymal Stromal Cells

8 OVX: Ovariectomized

9 PCNA: Proliferating Cell Nuclear Antigen

10 SHAM: Sham-operated

11 ssDNA: Single Stranded DNA

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17 **1. Introduction**

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

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

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

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

46 In the past decade, tissue engineering, particularly stem/stromal cell regenerative medicine, has made
47 significant advances in terms of restoration of the normal tissue functions. Mesenchymal stromal cells
48 (MSCs) have been successfully isolated from bone marrow (BM) (Jiang et al.,2002), adipose tissue (Eirin et
49 al.,2012), peripheral blood (PB) (Wan et al.,2006), and human umbilical cord blood (hUCB) (Hill et
50 al.,2009). The unique feature of MSCs is the immunological property of positivity for CD105, CD73, and
51 CD90 while lacking the expression of CD34, CD19, CD45, and HLA-DR surface molecules (Dominici et al.,
52 2006). The hUCB-MSCs are considered as one of the most easily available, least immunogenic and legal
53 materials (Ding et al., 2015), having the high potential for expansion and plasticity (El Maadawi and Gabr,

54 2011). Moreover, Doi et al. (2016) proved their safety in *in vitro* culture. Surprisingly, both E2 and hMSCs
55 exhibit similar antioxidant capacity and trophic effects (Valle-Prieto and Conget, 2010). In addition, Zhang
56 et al., (2012); Li et al. (2015) reported that, in high glucose culture, MSCs can differentiate into
57 steroidogenic cells which synthesize and secrete E2.

58 Interestingly, stromal cells have been successfully used to treat many diseases such as diabetes mellitus
59 (Soria et al., 2001), brain injury (Bang et al., 2005), cardiovascular diseases (Kawada et al., 2004; Dosi et al.,
60 2014), and several disorders associated with ovariectomy such as myocardial ischemia (Ray et al., 2008),
61 urinary incontinence (Lin et al., 2010), osteoporosis (Huang et al., 2016), and cerebellar disorders (Ahmed et
62 al., 2017). Despite the severe affection of the parotid gland in menopausal animal models and side effects of
63 its hormonal or herbal therapy, a new approach for its treatment is lacking. Therefore, the purpose of this
64 study was to evaluate histological and immunohistochemical structural alterations in the parotid gland of a
65 menopausal model, induced by abrupt E2 deficiency via ovariectomy in adult female albino rats and the
66 potential therapeutic roles of hUCB-MSCs.

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73 **2. Material and methods**

74 **2.1. Isolation and preparation of MSCs from hUCB**

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

77 *Collection of hUCB sample*

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

85 *2.1.1. Isolation and Culture of hUCB derived MSCs*

86 The collected blood was diluted three times with PBS. Then, 30 ml of diluted blood was carefully loaded on
87 10 mL of Ficoll/Paque (Lymphocyte Separation Medium 1.077, Lonza Bioproducts, Basel, Switzerland) in
88 50 mL centrifuge tubes. The centrifugation was done at 435 g for 30 min at 22°C. After removal of the
89 supernatant and careful aspiration of the mononuclear cells' (MNCs) layer, the MNCs were washed with
90 PBS twice and centrifuged at 20°C for 10 min. Finally, the MNCs were isolated and the supernatant was

91 discarded. The isolated MNCs were subcultured in DMEM (Dulbecco's modified Eagles medium) (Cambrex
92 Bio Science, Minnesota, USA) supplemented with 10% fetal bovine serum (FBS, Lonza Bioproducts, Basel,
93 Switzerland) and 1% penicillin-streptomycin-amphotericin B mixture as 10 IU /25 mg/100 ml (Lonza
94 Bioproducts, Belgium) at a concentration of 5000 cm⁻² (0.2–0.3 ml media)⁻¹. The culture was incubated at
95 37°C in 5% humidified CO₂ in a CO₂ incubator (Heraeus, Germany). After overnight incubation (12-18
96 hours), the media were replaced in order to eliminate non-adherent cells. The media were replaced every 3
97 days for 12–14 days until colonies of MSC were noticed as spindle-shaped fibroblastoid cells of the first
98 passage culture (at 80-90% confluence) under the inverted microscope. At 37°C for 5 min, colonies were
99 released with 0.25% trypsin in 1 ml EDTA. Immediately after trypsinization and centrifugation, subculturing
100 was done in serum-supplemented medium and incubated in 50 cm² tissue culture flask until adhesiveness and
101 fusiform shape of MSCs were obtained (Bieback et al., 2004). The fourth passage of culture was used after
102 labeling with fluorescent marker using Paul Karl Horan 26 (PKH-26) Fluorescent Cell Linker Kit
103 (Sigma-Aldrich Chemie, Steinheim, Germany) obtained from Algomhuria Co (Mohafza st., Zagazig, El
104 Sharqia, Egypt) as per the protocol described by Haas et al. (2000).

105 2.1.2. *Immunophenotypic characterization of hUCB-MSCs by flow cytometry applications*

106 Based on stromal cell surface markers, we used monoclonal antibodies against human CD105 and CD90
107 (mesenchymal stromal cell surface marker) (20% cutoff) (Zheng et al.,2013). We also used monoclonal
108 antibodies against human CD34 and CD45 to exclude hematopoietic, endothelial cells, and leukocytes. All
109 the antibodies were obtained from BD Bioscience. The detached cells were washed twice with PBS and

110 incubated with either anti-human CD105 (Cat No: 323205), CD90 (Cat No: 559869), CD34 (Cat No:
111 4084644), or CD45 (Cat No: 349202) according to manufacturer's instructions, for 30 min in the dark. Cells
112 were analyzed by FACS Scan 3 color (Becton Dickinson, Heidelberg, Germany) running Cell Quest
113 software (BD, San Joe, USA), in the Clinical Pathology laboratories, Zagazig University.

114 **2.2. Animals and experimental ethics**

115 Eighteen female albino rats (6-months old) were obtained from the animal house, Faculty of Veterinary
116 Medicine, Zagazig University, Egypt. The rats were housed in individual stainless-steel cages in a clean
117 room with controlled temperature (23°C) and humidity (60%), and with a 12 h dark/light cycle. The animals
118 were given a standard diet and tap water ad-libitum. The authors adhered to the Guide for the Care and
119 Use of Laboratory Animals of Zagazig University. The experimental protocols described in this research
120 were approved by the Institutional Review Board (IRB), Zagazig University (ZU-IRB #4626). The rats were
121 divided equally into three groups (n=6): sham-operated (SHAM), ovariectomized (OVX), and OVX +
122 hUCB-MSCs. The OVX + hUCB-MSCs group received an injection of 4.5×10^6 hUCB-MSCs dissolved in
123 250 μ L PBS on the tail vein two months after ovariectomy for four consecutive weeks (twice in a week)
124 (Calatrava-Ferreras et al., 2012). However, both SHAM and OVX groups were injected with vehicle (PBS)
125 only.

126 **2.3. Ovariectomy procedure**

127 The ovariectomy was performed by method described by Huang et al. (2016) under complete aseptic
128 conditions ten days after acclimatization of rats. Briefly, all rats were anesthetized by intraperitoneal

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

140 **2.4. Salivary flow rate**

141 Under the effect of anesthesia (intramuscular injection of a mixture of ketamine Hcl (50 mg/kg) and xylazine
142 Hcl (5 mg/kg)), saliva was collected from all rats two to three minutes after pilocarpine was injected
143 subcutaneously. Saliva was flowed freely into a sterile glass pipettes for a period of 30 min for each rat and
144 was then harvested. Salivary flow rate was presented as microliters per minute.

145 **2.5. Tissue preparation for microscopic observation**

146 At 3 months post-ovariectomy, rats from all experimental groups were euthanized under inhalational
147 anesthesia. The parotid glands were dissected, weighed, and immediately fixed in 4% paraformaldehyde

148 solution. After overnight fixation, parotid gland specimens were dehydrated in graded alcohol and embedded
149 in paraffin. Then, 3- μ m thick paraffin sections were deparaffinized, rehydrated, stained with hematoxylin
150 and eosin (H&E), and observed under light microscope, and photographed with the Amscope digital camera
151 (Bancroft and Layton, 2013).

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

155 **2.6. Immunohistochemistry**

156 The immunohistochemical analyses for human CD105 and human CD34, proliferating cell nuclear antigen
157 (PCNA), caspase 3 and single strand DNA (ssDNA), aquaporin (AQP)1, α smooth muscle actin (SMA), and
158 mouse-CD34 were performed to detect hUCB-MSCs homing, proliferating cells, apoptotic cells, blood
159 capillaries, myoepithelial cells, and endogenous hematopoietic progenitor cells, respectively (Elewa et al.,
160 2010). In brief, antigen retrieval was done with the deparaffinized sections according to each antibody (Table
161 1). Then sections were incubated in methanol containing 0.3% H₂O₂ for 20 minutes at 4°C to block the
162 activity of endogenous peroxidase, followed by washing in distilled water, and incubation with 10% normal
163 blocking serum for 1 h at room temperature (donkey serum for PCNA immunostaining and goat serum for
164 staining of the other antigens). Then sections were incubated overnight with the specific primary antibody
165 diluted in PBS (pH 7.2). The antibodies and working dilutions are shown in Table 1. For negative control,
166 the primary antibody was replaced with only PBS. Then the sections were incubated with biotin-conjugated

167 secondary antibody, specific to the primary one, for 30 min, followed by incubation for 30 min with
168 streptavidin-peroxidase conjugate. The positive reaction was visualized with 3, 3'-diaminobenzidine
169 tetrahydrochloride (DAB)-H₂O₂ solution, pH 7.0. Finally, the sections were washed in distilled water and
170 counterstained with Mayer's hematoxylin.

171 **2.7. Histoplanimetric analysis**

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

179 **2.8. Statistical analysis**

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

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187 **3. Results**

188 **3.1. Characterization of hUCB-MSCs and homing of PKH26-labeled hUCB-MSCs in parotid gland**

189 Morphologically, MSCs culture at day 7 of isolation showed fusiform-shaped cells (Fig. 1A). The flow
190 cytometric analysis of the hUCB-MSCs culture revealed that the cells had a positive expression for human
191 CD105 (85.65%) and human CD90 (62.33%), while a negative expression for human CD34 (1.36%) and
192 human CD45 (2.19%) were detected (Fig. 1B). To confirm the homing of PKH26-labeled hUCB-MSCs,
193 3- μ m thick deparaffinized sections of all groups were examined. The MSCs were detected in the parotid
194 gland sections of the OVX + hUCB-MSCs group (3 months post-ovariectomy) as red fluorescence between
195 acini and ducts (Figs. 2A and B) but not in other groups (data not shown). Furthermore, a positive reaction
196 for human CD105 was observed around the acini and ducts (Fig. 2C), while the human CD34 expression was
197 not detected (Fig. 2D).

198 **3.2. Salivary flow rate and weight of parotid glands**

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

202 **3.3. Histological observations**

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

216 **3.4. Immunohistochemical observations**

217 The immunohistochemical staining with anti-PCNA was performed to detect proliferating cells. The positive
218 cells were observed in the acini of the three different experimental groups, as expected (Figs. 4A, B, and C),
219 while the negative control immunostained sections of the parotid gland of the SHAM group revealed absence
220 of any positive reaction (Fig. 4D). The expressions of positive cells were significantly decreased in the OVX
221 group (10.56 ± 0.63), compared to the SHAM group (30.08 ± 2.51), while they were considerably higher in
222 the OVX + hUCB-MSCs group (23.28 ± 1.42) compared to the OVX group (Fig. 4E).

223 Immunohistochemical staining for caspase 3 and ssDNA were performed to observe apoptotic cell
224 populations. The caspase 3 positive apoptotic cells were observed in the interstitial cells of the three groups
225 (Figs. 5A, B, and C). The negative control sections of the parotid gland of the OVX group revealed the
226 absence of apoptotic cells (Fig. 5D). In the OVX group, there was a significant increase in caspase 3
227 apoptotic cells (11.17 ± 0.76), compared to the SHAM group (0.83 ± 0.17), while the frequency of such
228 apoptotic cells was significantly decreased in the OVX + hUCB-MSCs group (3.11 ± 0.44) compared to the
229 OVX group (Fig. 5E). The other apoptotic cell marker was ssDNA that were mainly detected in both acinar
230 and ductal epithelium (data not shown) of the OVX group, while they were hardly to be seen in the SHAM
231 and OVX + hUCB-MSCs groups. The OVX group showed a significant increase in ssDNA positive cells
232 (101 ± 14.18), compared to the SHAM group (1.22 ± 0.55). On the other hand, the number of ssDNA
233 positive cells was much less in the OVX + hUCB-MSCs group; however, a non-significant difference was
234 observed in the average number of the ssDNA positive cells in the OVX + hUCB-MSCs group (4.44 ± 1.3)
235 when compared with that of the SHAM group (Fig. 5F).

236 The immunohistochemical staining with anti-AQP1 was performed to examine the alterations of the blood
237 capillaries among the three different groups. The endothelial cells of blood capillaries showed positive
238 reactions for AQP1 in all groups (Figs. 6A, B, and C). No positive reaction was observed in the negative
239 control sections of the parotid gland in the SHAM group (Fig. 6D). The integrated density of AQP1 positive
240 reactions showed a significant reduction in the OVX group ($15.02 \times 10^6 \pm 1.35$), compared with the SHAM
241 group ($31.24 \times 10^6 \pm 3.77$). However, an increase in the integrated density of AQP1 positivity was observed

242 in the OVX + hUCB-MSCs group ($29.05 \times 10^6 \pm 2.87$), compared to the OVX group. However, there was
243 no significant difference in the integrated density of AQP1 positivity was observed in the OVX +
244 hUCB-MSCs group compared to that observed in the SHAM group (Fig. 6E).

245 The changes in myoepithelial cell populations were investigated in different experimental groups via
246 immunohistochemical staining with anti- α -SMA. Interestingly, the myoepithelial cell populations showed a
247 great variation among different groups. Well-developed α -SMA positive myoepithelial cells with numerous
248 cytoplasmic processes were observed around the acini of the parotid glands of the SHAM group (Fig. 7A),
249 while the myoepithelial cells after ovariectomy showed fewer and shorter processes (Fig. 7B). In the OVX +
250 hUCB-MSCs group, the cytoplasmic processes showed moderate development (Fig. 7C). The negative
251 control sections of the parotid gland of the SHAM group showed no positive reaction (Fig. 7D). These
252 findings were confirmed by analyzing the integrated density of immunopositive cytoplasmic processes. The
253 integrated density of positive reactions showed a significant decrement in the OVX group ($33.99 \times 10^6 \pm$
254 4.48) when compared to the SHAM group ($141.22 \times 10^6 \pm 11.55$). However, following treatment with
255 hUCB-MSCs, a higher integrated density of positive reactions was observed in the OVX + hUCB-MSCs
256 group ($95.85 \times 10^6 \pm 5.82$) compared to that of the OVX group (Fig. 7E).

257 Immunohistochemical staining using mouse CD34 antibody in the parotid gland sections of different
258 experimental groups was performed to evaluate the endogenous hematopoietic progenitor cell populations
259 (Figs. 8A, B, and C). The negative control sections of the parotid gland of the SHAM group revealed no
260 positive reactivity (Fig. 8D). A significant loss of CD34 positive cells was observed in the OVX group (0.33

261 ± 0.21) as compared with the SHAM group (12.33 ± 1.28), but their expression was markedly restored in the
262 OVX + hUCB-MSCs group (12.17 ± 1.35) in which there was no significant difference compared to the
263 SHAM group (Fig. 8E).

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278 **4. Discussion**

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

285 Both parotid glands weight and salivary flow rate were significantly decreased in the OVX group in
286 comparison to the SHAM group, as previously reported (Abd El-Haleem et al.,2018). It was confirmed in the
287 present study by our observations of the glandular architecture that showed numerous intracytoplasmic
288 vacuoles in the acinar cells of rats of OVX group. Similar atrophic changes in the parotid glands were
289 reported in radiated rats (Jeong et al., 2013) and in rats after bilateral ovariectomy (Parlak et al., 2014, Abd
290 El-Haleem et al., 2018). The cellular vacuolization is considered by Myers and McGavin (2007) as an early
291 sign of cellular degeneration. Deficiency of the trophic effects of E2 (Valimaa et al., 2004) and increment in
292 apoptosis (Kusunoki et al. 2006) may be the causes of these structural alterations. In the present study,
293 ovariectomy caused a statistically significant decrement of proliferative cell populations and increment of
294 apoptotic cells as compared to the SHAM group. The apoptotic cells expressed positive caspase 3 in the
295 interstitial tissues and ssDNA in both acinar and ductal cells of the OVX group. On the other hand, Limesand

296 et al. (2006) found caspase 3 activation in the acinar cells of both parotid and submandibular glands of
297 gamma-irradiated mice. The caspase-3 is required for DNA fragmentation and some of the typical
298 morphological alterations in cells during apoptosis (Jänicke et al., 1998). Therefore, the apoptotic cells were
299 identified based on early events (activation of caspase-3, keratin 18 cleavage) or late events (nuclear
300 condensation, DNA fragmentation) in the apoptosis pathway as reported by Krysko et al. (2008). We
301 proposed that the interstitial apoptotic cells could be myoepithelial cells. Both apoptotic and proliferating
302 myoepithelial cells were detected in the submandibular glands of atrophic rats via duct ligation (Takahashi et
303 al., 2001). The results of the present study were reinforced by the observations of Limesand et al. (2006),
304 who clarified that glandular homeostasis requires a balance in cell proliferation and apoptosis.

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

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

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

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

331 Our study used integrated density of α SMA-positive area on immunostained sections as an index of the
332 changes in myoepithelial cell populations. These densities were significantly decreased in the OVX group

333 whereas they significantly increased after hUCB-MSCs treatment. This obvious myoepithelial loss clarifies
334 its role in glandular hypofunction associated with ovariectomy, as previously reported by Safayi et al.
335 (2012).

336 The most interesting finding is the presence of endogenous CD34⁺ hematopoietic progenitor cells around
337 acini and ducts in the parotid glands of SHAM group. CD34⁺ is a cell surface marker of hematopoietic
338 stem/stromal cells and hematopoietic progenitor cells (Sidney et al., 2014). These cells are identified as side
339 population (SP) cells (Mishima et al., 2012). Our findings revealed the ovariectomy-induced depletion of
340 endogenous CD34⁺ hematopoietic progenitor cells via apoptosis. Such findings are similar to that produced
341 by irradiation that leads to the damage of self-renewal property of hematopoietic stromal cell in mononuclear
342 cells isolated from bone marrow of mice (Wang et al., 2006). In our study, the mechanism of CD34⁺
343 hematopoietic progenitor cells recovery may be via angiocrine (Kobayashi et al., 2010) and pleiotrophin
344 (Himburg et al., 2010) growth factors that were secreted from recovered endothelial cells. Sidney et al.
345 (2014) reported that the regenerative functions of endogenous stromal cells occur by providing molecular
346 signals to the proliferating immature epithelial cells in the forms of basement membrane proteins,
347 extracellular matrix, matrix metalloproteinases/proteases, and growth factors. Further investigations are
348 required for further elucidation of the mechanism of hUCB-MSCs mediated parotid gland regeneration.

349 In summary, our results suggest that the bilateral ovariectomy could affect the parotid gland structure due to
350 destruction of endothelial cells, and apoptosis of acinar and ductal cells. Interestingly, we revealed that most
351 of the structural injuries of the parotid gland were improved by hUCB-MSCs therapy. Such improvement in

352 mechanisms by MSCs might be via endothelial cells recovery and endogenous CD34⁺ hematopoietic
353 progenitor cells rescue. Apoptosis inhibition and proliferation enhancement mechanisms of acinar and ductal
354 cells with myoepithelial cells recovery could also be responsible. Therefore, hUCB-MSCs therapy might be
355 a good alternative to treat parotid gland destruction, especially in menopausal cases, to evade the health risks
356 of hormonal therapy.

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554 **Figure Legends**

555 **Fig. 1** Identification of the hUCB-MSCs. A photomicrograph of the culture at day 7 of isolation
556 showing a fusiform-shaped morphology of cells (solid arrows) (A). Histograms of flow cytometric
557 analysis of the hUCB-MSCs culture, showing positive expressions for human CD105 (85.65%) and
558 human CD90 (62.33%) depending on 20% cutoff, while negative expressions for human CD34
559 (1.36%) and human CD45 (2.19%). Open histogram indicates the negative control background
560 signal; positive reactivity is displayed in shaded histogram (B).

561 **Fig. 2** Immunofluorescence analysis. Immunofluorescence detection of the homing of the
562 PKH26-labelled hUCB-MSCs around the acini (solid arrows) and ducts (dashed arrows) in the
563 parotid glandular tissues of the OVX + hUCB-MSCs group (3 months post-ovariectomy) is noticed

564 (A, B). Immunohistochemical detection of the hUCB-MSCs homing in sections of parotid glands
565 express a positive reaction with anti-human CD 105 around acini (solid arrows) and duct (dashed
566 arrows) (C) but a negative reaction with anti-human CD 34 (D).

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

573 **Fig. 4** Proliferating cell populations in the parotid glands. Immunohistochemical photomicrographs showing
574 PCNA positive cells (arrows) in the acini of SHAM group (A), OVX group (B), and OVX + hUCB-MSCs
575 group (C). The negative control stained section of the SHAM group in which the primary antibody is
576 replaced by PBS (D). Bar chart showing the average number of PCNA positive cells of different groups (E).
577 Each bar carrying different superscripts letters (a, b, and c) are significantly different as analyzed by the
578 one-way ANOVA test, followed by the multiple comparisons Duncan's Post-hoc test ($P < 0.05$); $n = 6$ in
579 each experimental group. Values = mean \pm SE.

580 **Fig. 5** Apoptotic cell populations in the parotid glands. Immunohistochemical photomicrographs showing
581 Caspase 3 positive cells (arrows) in the interstitial tissues of SHAM group (A), OVX group (B) and OVX +
582 hUCB-MSCs group (C). The negative control stained section of the OVX group in which the primary

583 antibody is replaced by PBS (D). Bar charts showing the average number of Caspase 3 positive (E) and
584 ssDNA positive apoptotic cells in different groups (F). Each bar carrying different superscripts letters (a, b
585 and c) are significantly different as analyzed by the one-way ANOVA test, followed by the multiple
586 comparisons Duncan's Post-hoc test ($P < 0.05$); $n = 6$ in each experimental group. Values = mean \pm SE.

587 **Fig. 6** Blood capillary populations in the parotid glands. Immunohistochemical photomicrographs showing
588 AQP1 positive blood capillaries in between the acini (arrows) and around striated ducts (arrowheads) of the
589 SHAM group (A), OVX group (B), and OVX + hUCB-MSCs group (C). The negative control stained
590 section of the SHAM group in which the primary antibody is replaced by PBS (D). Bar chart showing the
591 integrated density ratio of AQP1 positive blood capillaries in different groups (E). Each bar carrying different
592 superscripts letters (a, b and c) are significantly different as analyzed by the one-way ANOVA test, followed
593 by the multiple comparisons Duncan's Post-hoc test ($P < 0.05$); $n = 6$ in each experimental group. Values =
594 mean \pm SE.

595 **Fig. 7** Myoepithelial cell populations in the parotid glands. Immunohistochemical photomicrographs
596 showing α -SMA positive cells with their cytoplasmic processes around the acini (arrows) and intercalated
597 duct (arrowhead) of SHAM group (A), OVX group (B), and OVX + hUCB-MSCs group (C). The negative
598 control stained section of the SHAM group in which the primary antibody is replaced by PBS (D). Bar chart
599 showing the integrated density ratio of distribution of α -SMA positive cells in different groups (E). Each bar
600 carrying different superscripts letters (a, b and c) are significantly different as analyzed by the one-way

601 ANOVA test, followed by the multiple comparisons Duncan's Post-hoc test ($P < 0.05$); $n = 6$ in each
602 experimental group. Values = mean \pm SE.

603 **Fig. 8** Endogenous hematopoietic progenitor cell populations in the parotid glands. Immunohistochemical
604 photomicrographs showing anti-mouse CD34 positive cells surrounding acini (arrows) and striated ducts
605 (arrowheads) of SHAM group (A), OVX group (B), and OVX + hUCB-MSCs group (C). Insets indicate
606 immunopositive reaction of hematopoietic progenitor cells that have large nucleus with little cytoplasm. The
607 negative control stained section of the SHAM group in which the primary antibody is replaced by PBS (D).
608 Bar chart showing the average number of mouse CD34 positive cells in different groups (E). Each bar
609 carrying different superscripts letters (a, b and c) are significantly different as analyzed by the one-way
610 ANOVA test, followed by the multiple comparisons Duncan's Post-hoc test ($P < 0.05$); $n = 6$ in each
611 experimental group. Values = mean \pm SE.

Figure

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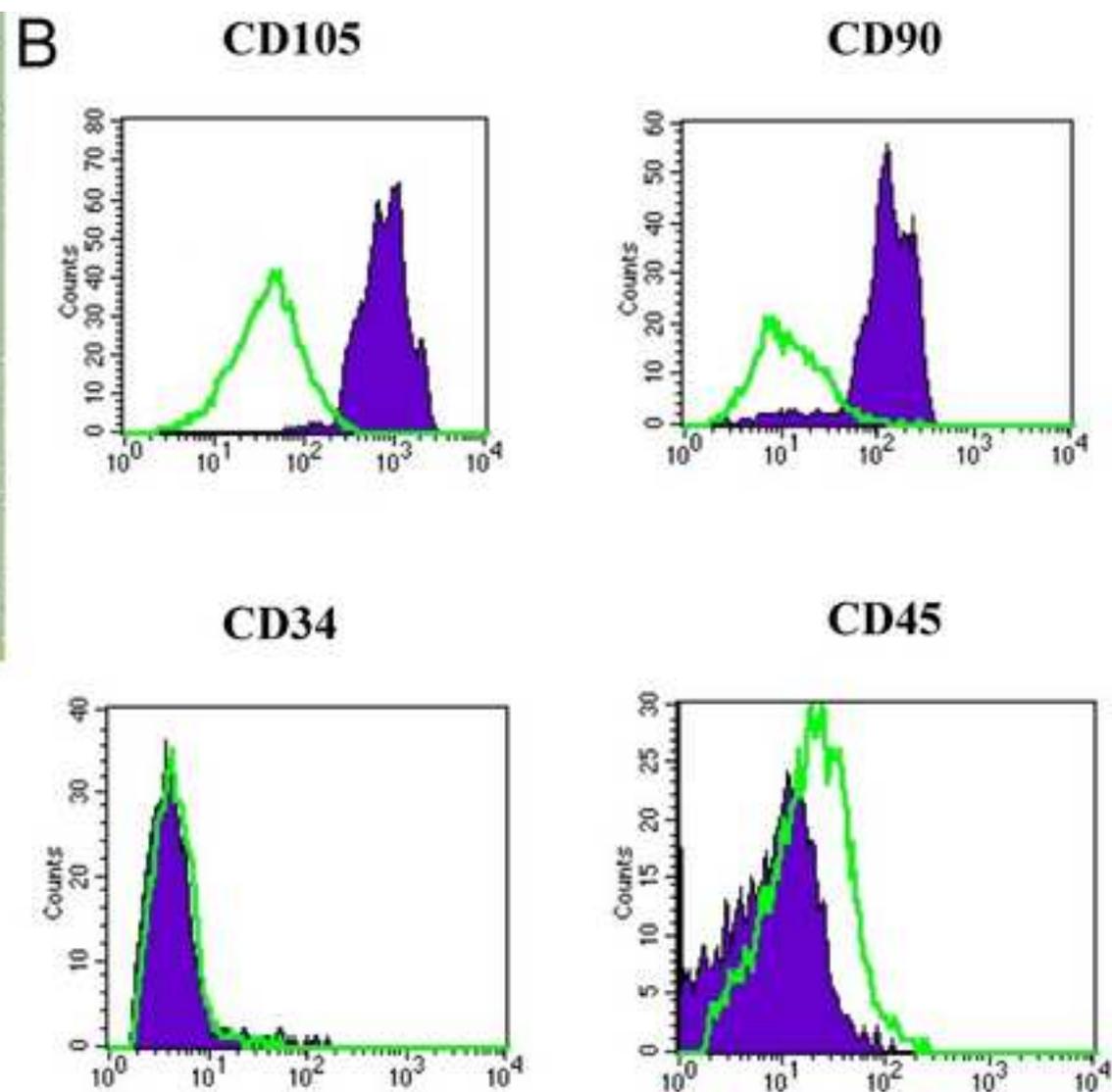
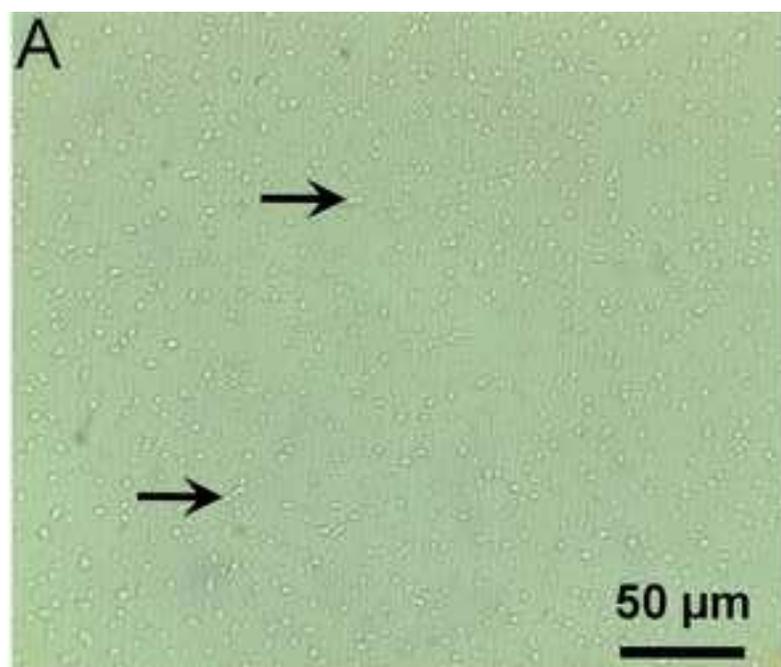
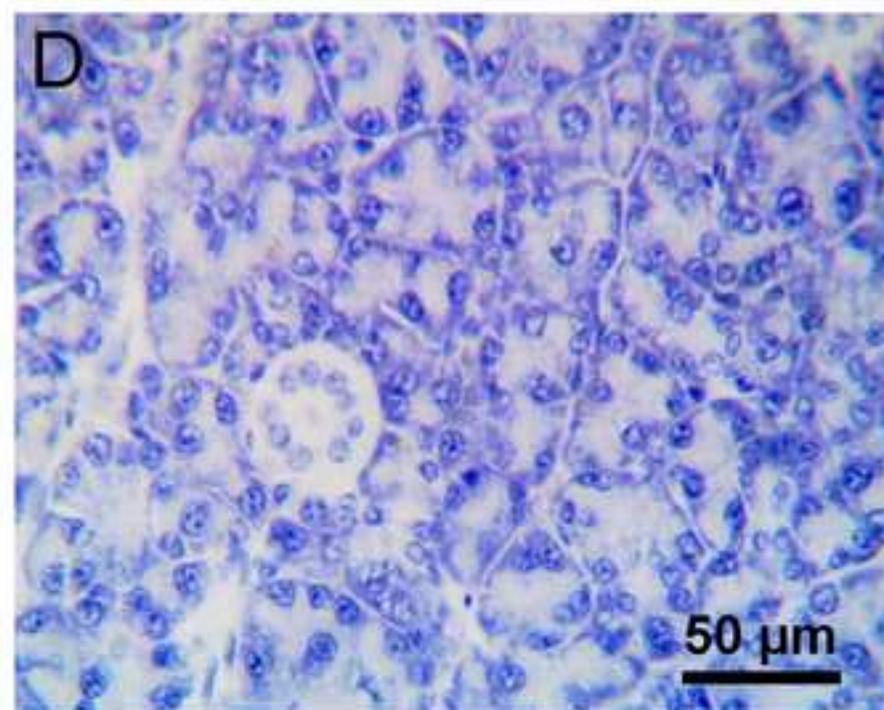
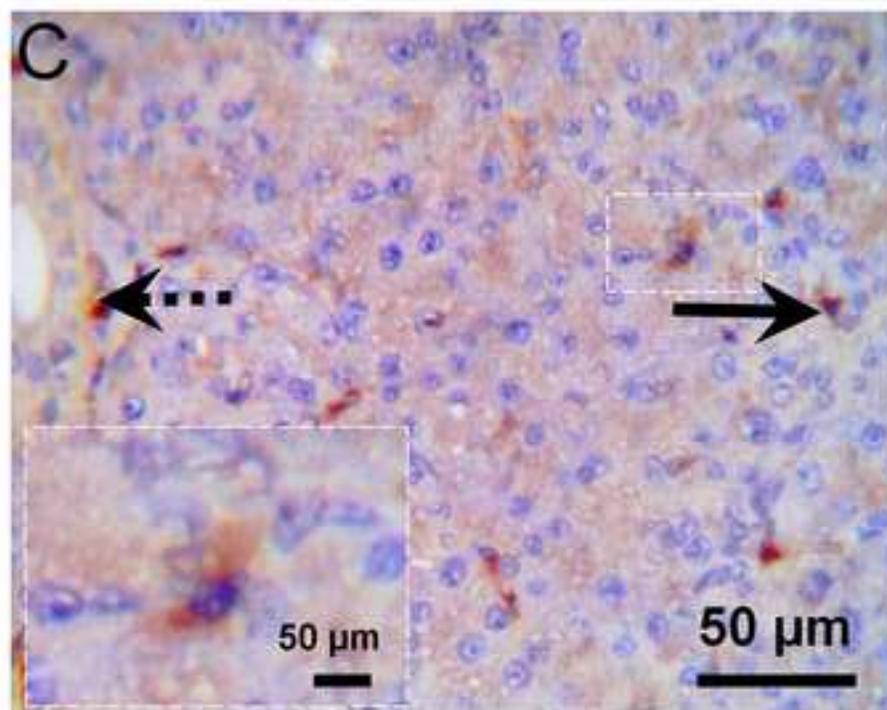
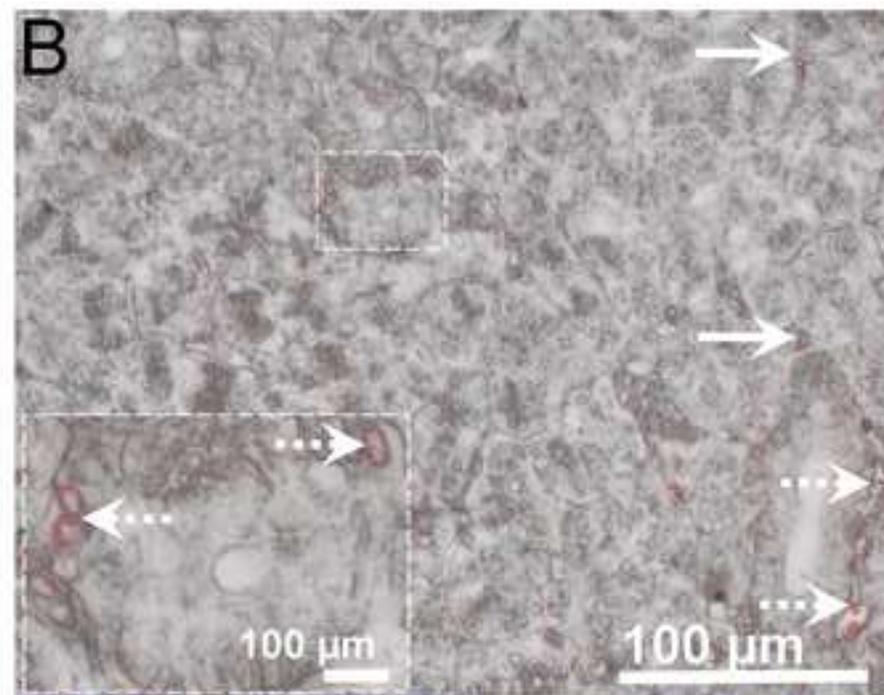
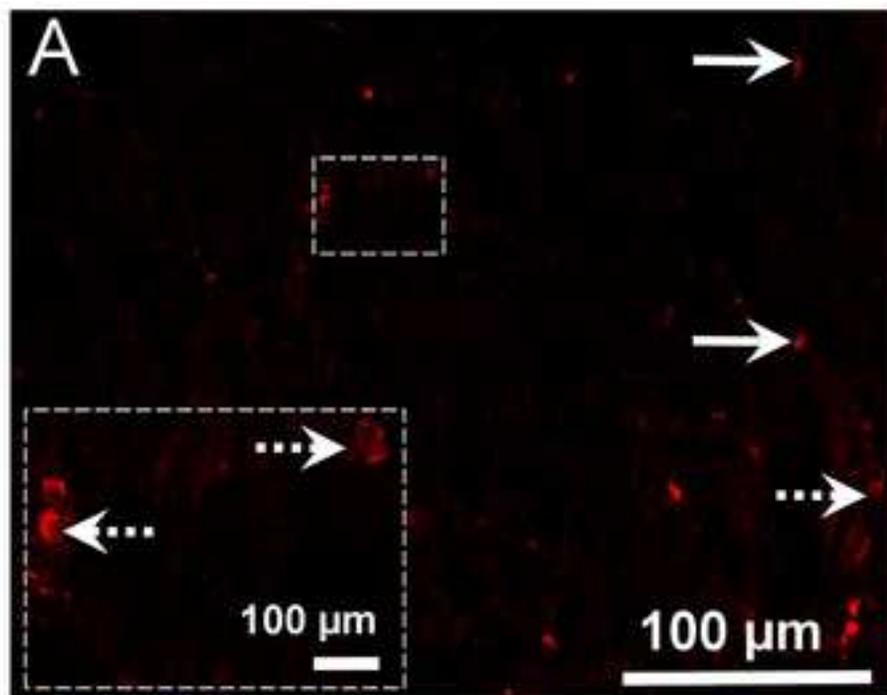


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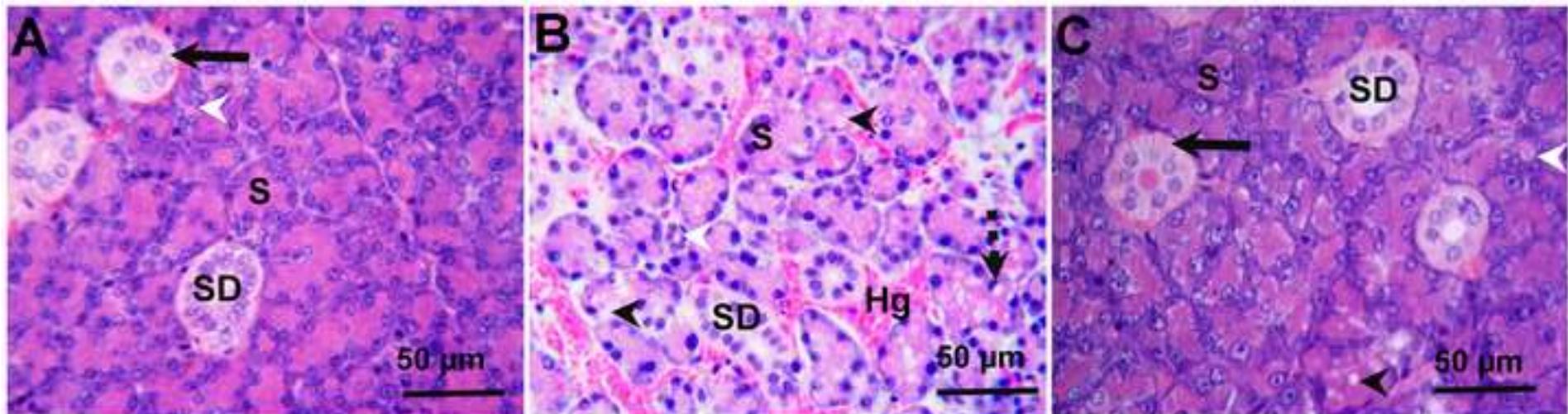
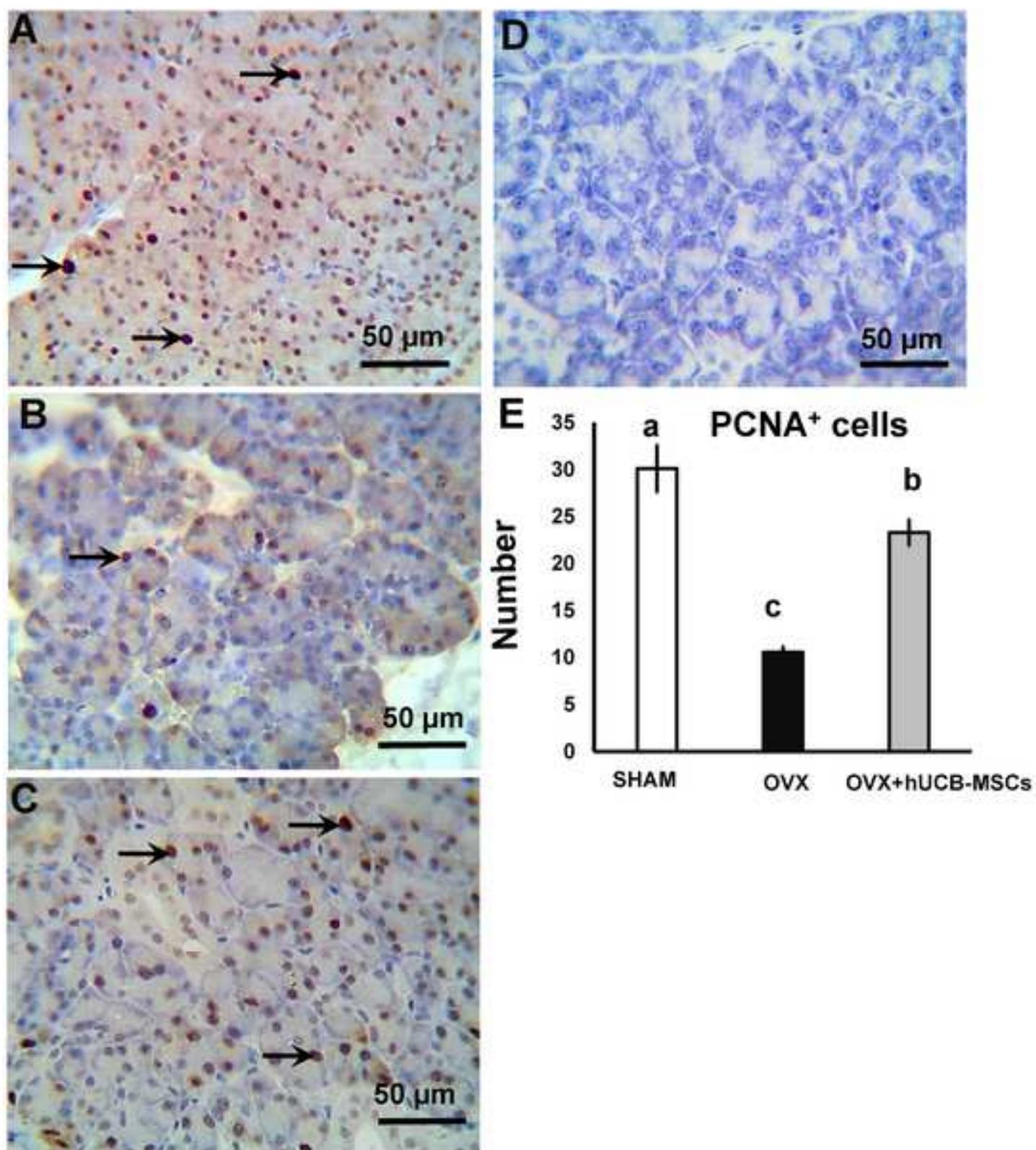
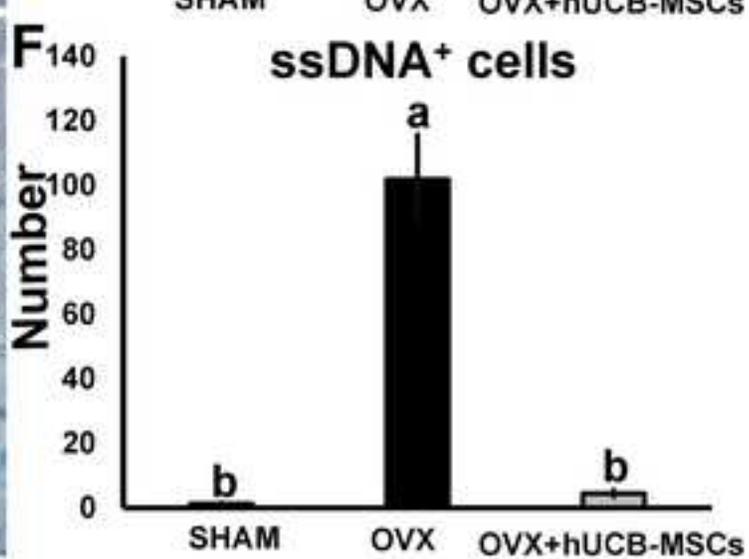
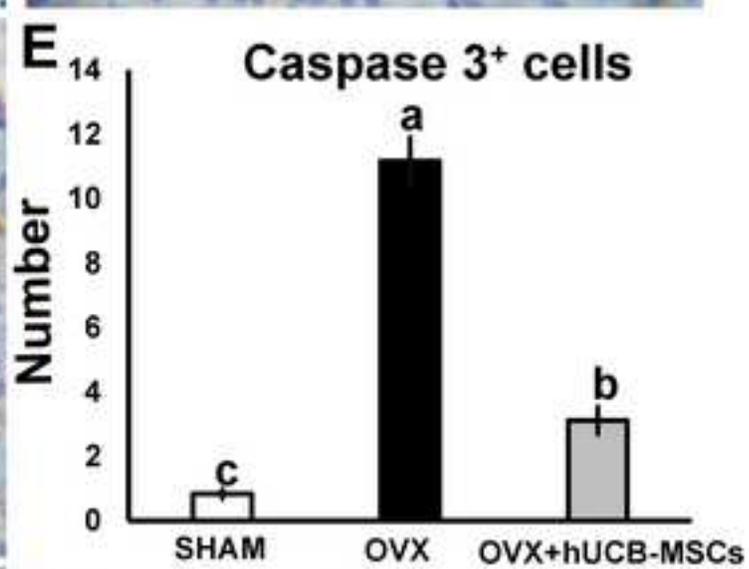
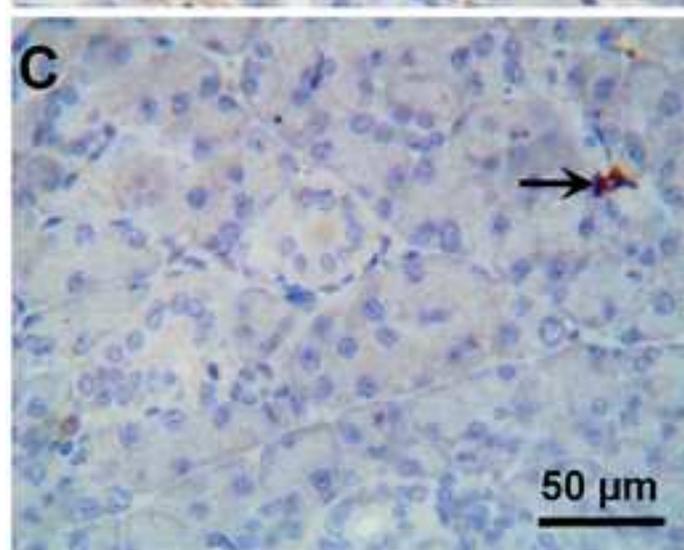
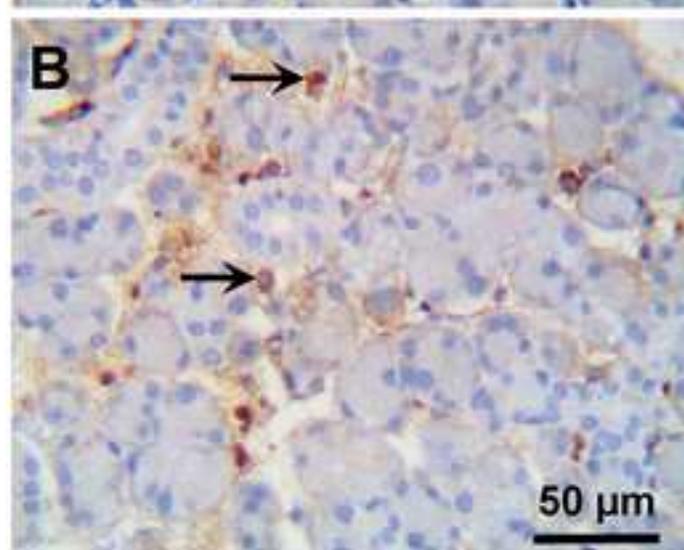
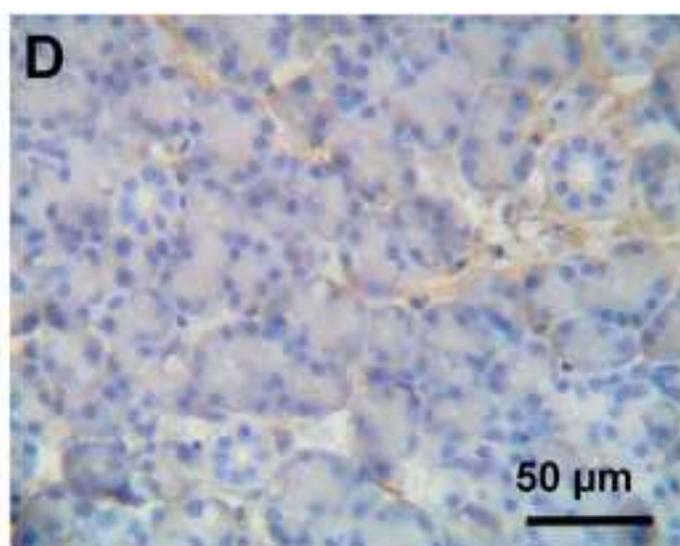
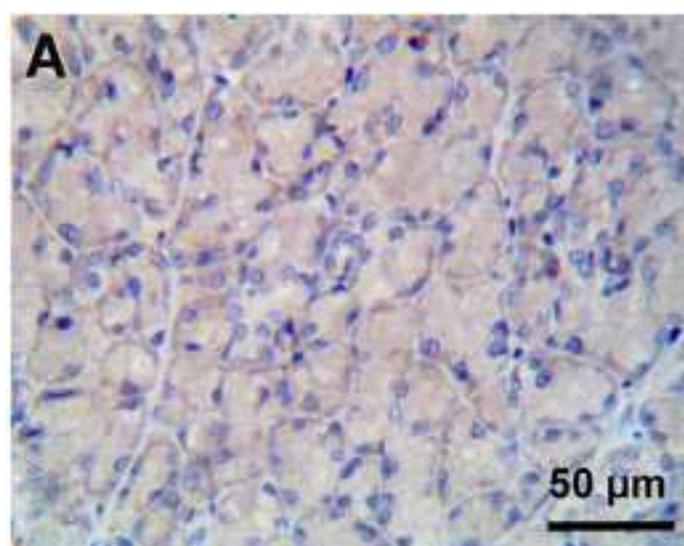


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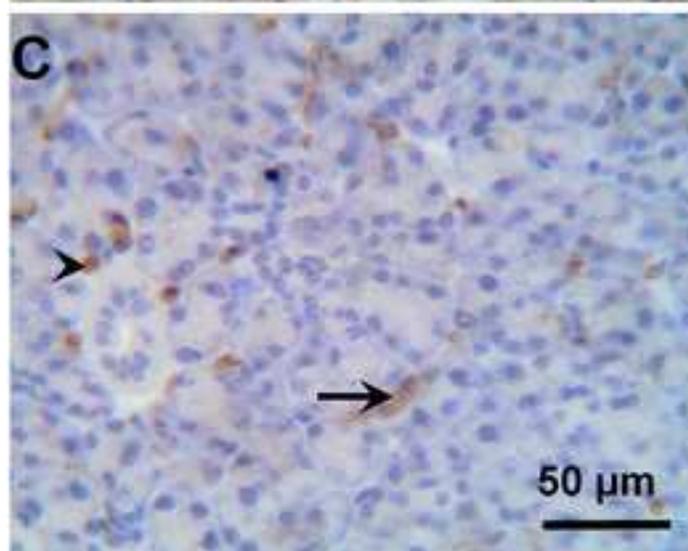
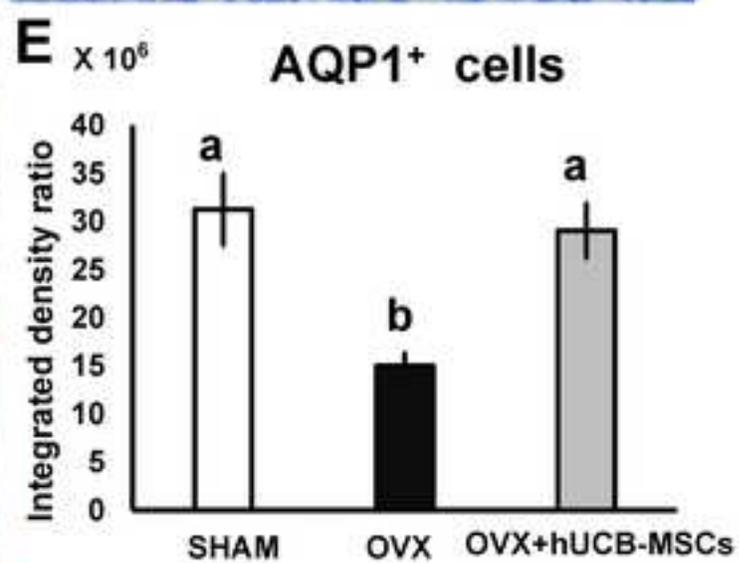
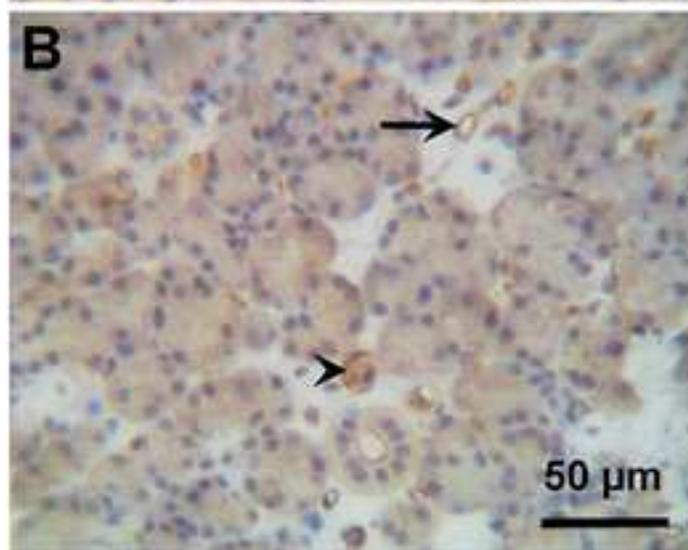
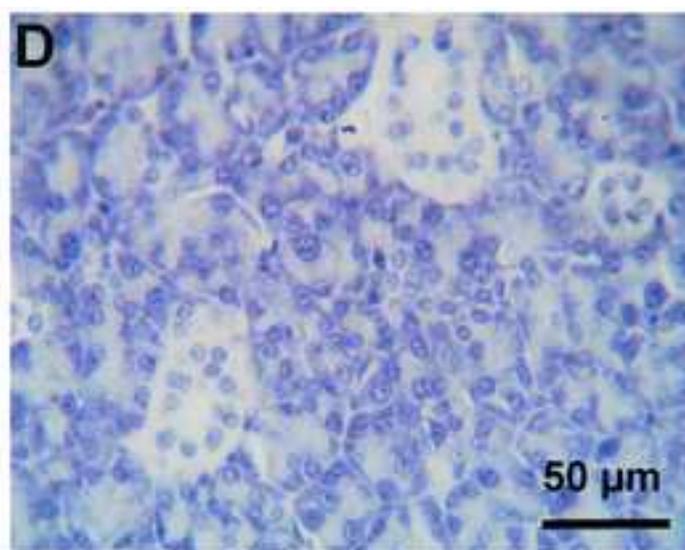
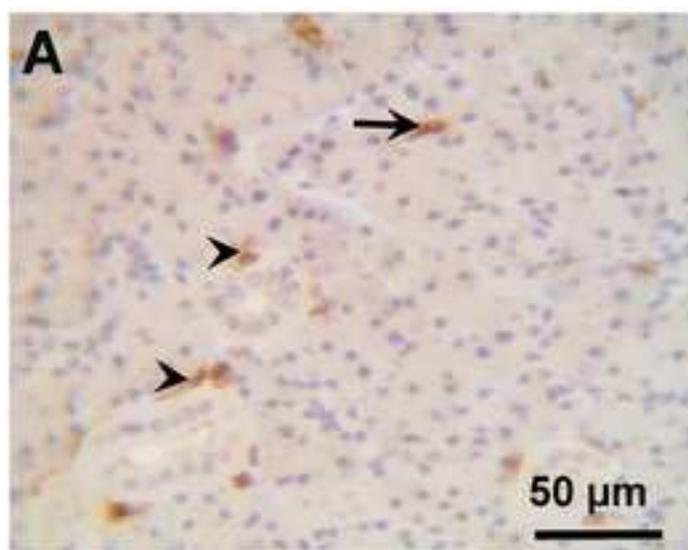


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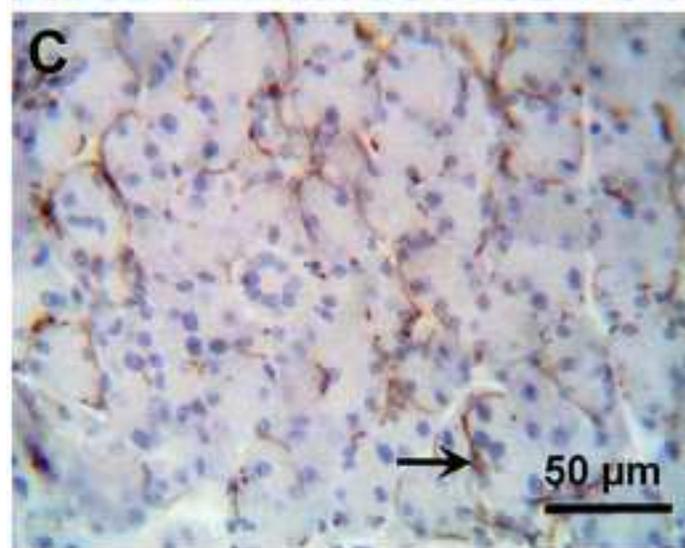
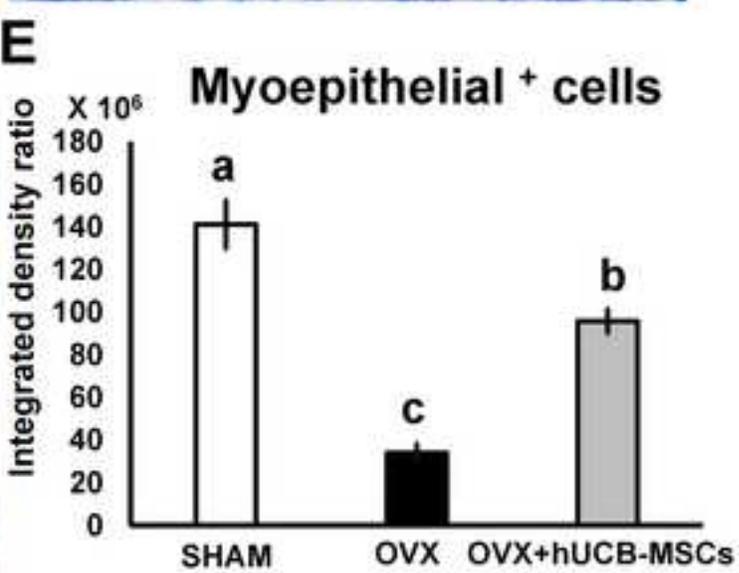
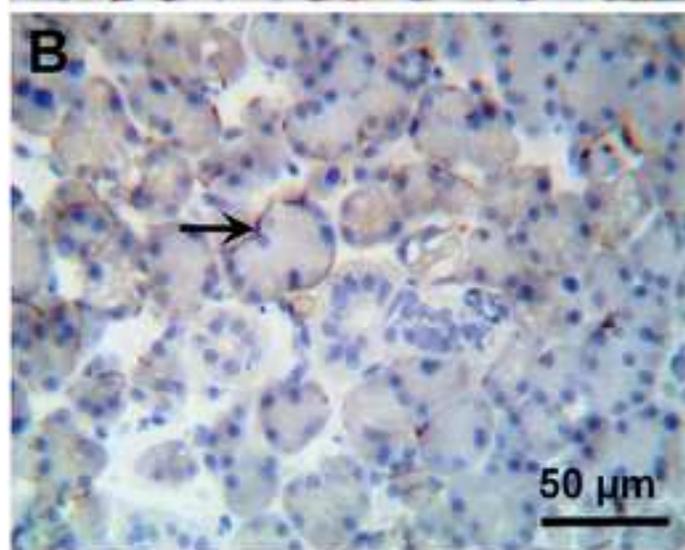
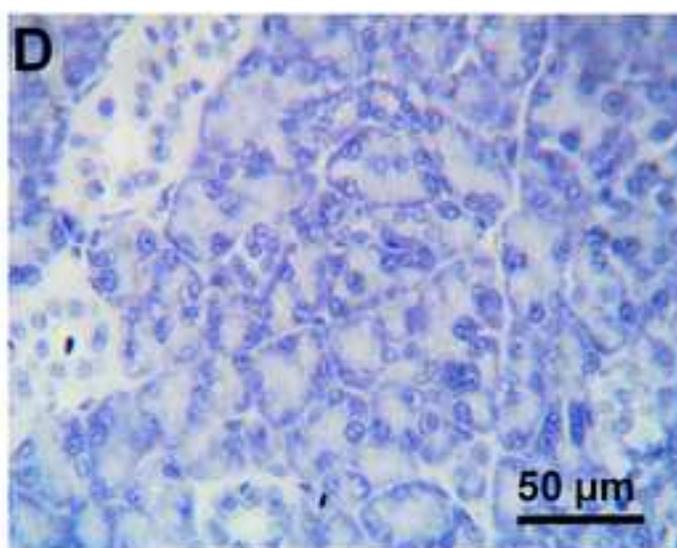
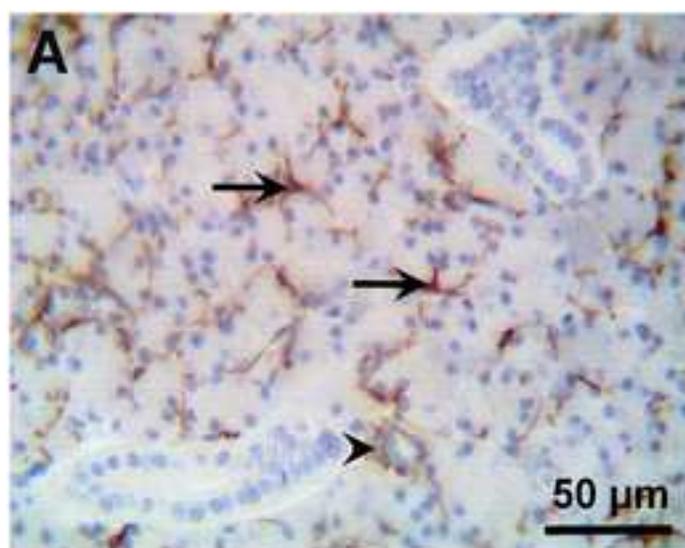


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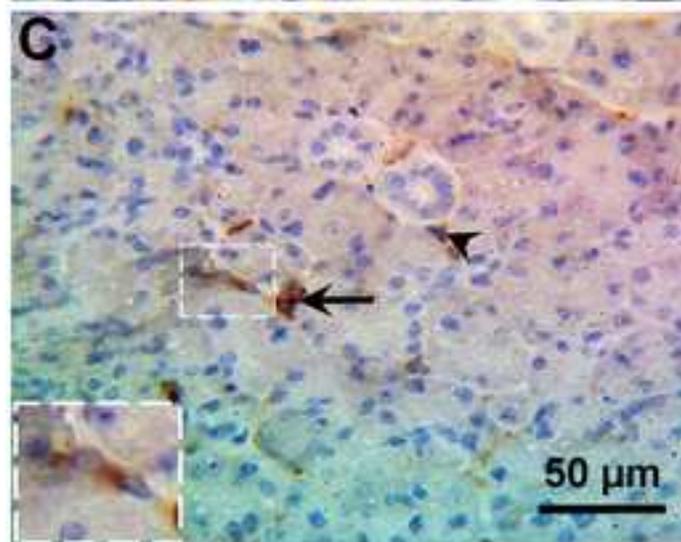
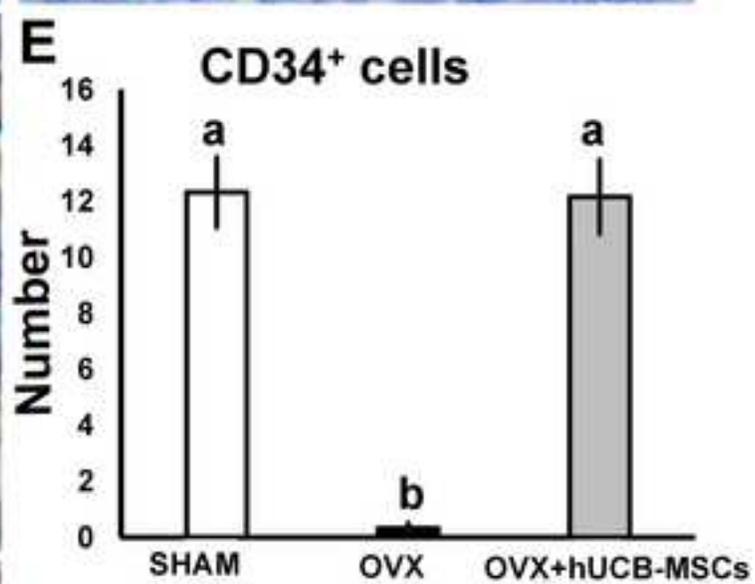
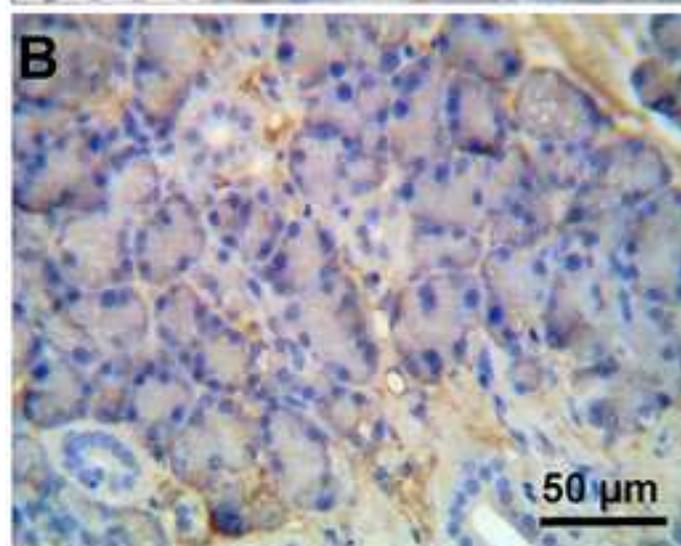
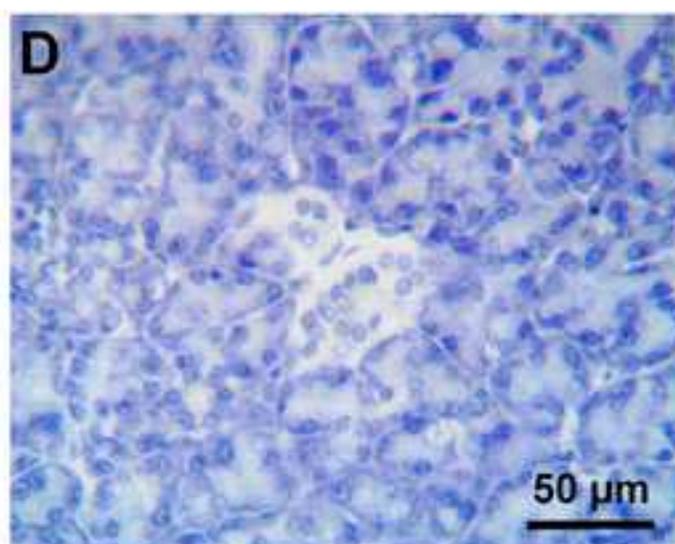
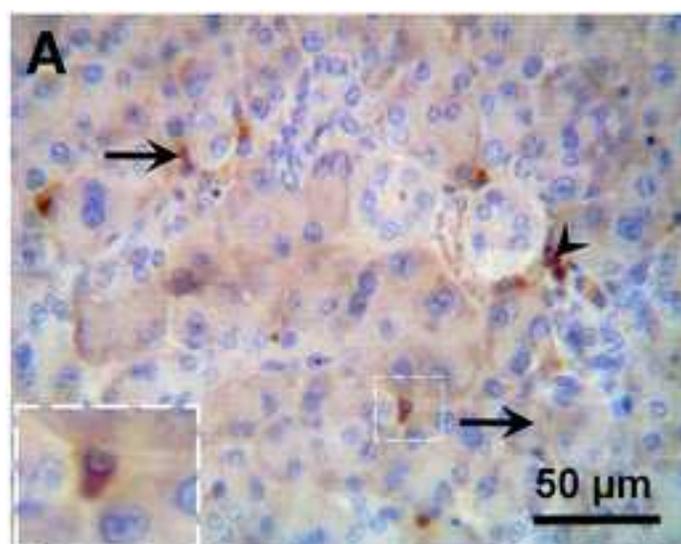


Table 1 A list of antibodies, sources, working dilutions, and antigen retrieval.

| Antibodies | Sources | Dilution | Antigen retrieval, temperature, time |
|------------------------------|---|----------|--|
| Anti-human CD105: SN6h | Gennova Scientific SLc | 1:200 | Citrate buffer (pH 6.0),95°C, 20 min. |
| Anti-humanCD34: QBEnd/10 | Gennova Scientific SLc | 1:400 | Citrate buffer (pH 6.0),105°C, 20 min. |
| Monoclonal anti-goat PCNA | Santa Cruz Biochemistry, (Santa Cruz, USA) | 1:2000 | Dako, 105°C, 20 min. |
| Anti-rabbit ssDNA | IBL – Fujioka, japan | 1:200 | No antigen retrieval used, don't Autoclave. |
| Anti-rabbit AQP1 | Santa Cruz | 1:50 | 2XSSC, 105°C, 20 min. |
| Anti-rabbit α -SMA | Abcam, Cambridge, UK | 1:3000 | Citrate buffer (pH 6.0),105°C, 20min. |
| Anti-rabbit Caspase 3 | IMGENEX | 1:800 | 2XSSC, 105°C, 20min. |
| Anti-mouse CD34 | Abcam | 1:400 | Citrate buffer,105°C 20min. |

Table 2 Statistical analysis of salivary flow rate and weight of parotid glands in all investigated groups.

| Parameters | SHAM | OVX | OVX+hUCB-MSCs |
|---|---------------------------------|--------------------------------|--------------------------------|
| Salivary flow rate (μl/min) | 48.08 \pm 3.07 ^a | 10.03 \pm 1.10 ^b | 43.85 \pm 3.56 ^a |
| parotid glands' weight (mg) | 303.65 \pm 12.72 ^a | 175.43 \pm 6.49 ^b | 280.58 \pm 8.82 ^a |

Data are expressed as the mean \pm SD.

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.