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Master Thesis

Expression of secretory leukocyte protease inhibitor (SLPI) in oral mucosal healing on the ovariectomized (OVX) mice wound

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난소절제 생쥐 구강점막 상처회복 과정에서 SLPI의 발현

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치 의 생 명 공 학 과

박 진 주

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ABBREVIATIONS

SLPI: secretory leukocyte protease inhibitor

OVX: ovariectomized

EV: estradiol valerate

PCOS: polycystic ovary syndrome

MT: Masson's Trichrome

HRT: hormone replacement therapy

LPS: lipopolysaccharide

IL: interleukin

TGF-β1: transforming growth factor-β1

MIF: migration inhibitory factor

TNF-α: tumor necrosis factor-α

MIP-1: macrophage inflammatory protein-1

MMPs: matrix metalloproteinases

TIMPs: tissue inhibitors metalloproteinases

ECM: extracellular matrix

ABSTRACT IN KOREAN

난소절제 생쥐 구강점막 상처회복 과정에서 SLPI의 발현

박 진 주

지도교수: 정 문 진

조선대학교 대학원 치의생명공학과

상처회복은 응혈, 염증, 재상피화, 육아조직의 형성, 기질 재형성의 연속적인 과정에 의해 진행 된다. 에스트로겐은 염증반응, 사이토카인의 발현, 기질 침착을 조절하여 재상피화 촉진과 혈관형성, 상처 수축을 자극하여 상처회복 과정을 증가시킨다. 난소절제 시술(ovariectomized, OVX)에 의한 에스트로겐 분비의 결여는 상처회복 과정을 지연시킨다. 분비백혈구단백분해효소억제제(secretory leukocyte protease inhibitor, SLPI)는 세린계열의 단백분해효소 억제분자로서, 상처회복 과정 동안 호중구 엘라스타제의 활성을 조절하여 과도한 염증반응을 억제하여 피부와 구강점막의 상처회복을 촉진한다. 에스트로겐에 의해 증가되는 SLPI의 발현과 분비는 염증성 사이토카인의 분비를 억제한다. 따라서, SLPI와 에스트로겐은 상처회복 과정에서 밀접한 관계를 가질 것으로 생각되고 조직형태학적 분석, 역전사 중합효소반응, 면역화학적 단백질검출법, 면역조직화학적염색, 젤라틴 자이모그래피와 엘라스타제 활성측정법 등을 이용하여 OVX

생쥐 모델에서 구강점막 상처회복 과정 동안 SLPI의 발현과 기능을 규명하고자 하였다. 본 연구에서 OVX 생쥐의 구강점막 상처는 상처 면적의 증가, 과도한 염증세포의 유입, 교원질 침착이 감소 함으로써 회복이 지연되었다. OVX 상처회복 과정 중 TGF-β1과 교원질의 발현은 낮았고 fibronectin의 발현은 정상그룹보다 높았다. 엘라스타제, MMP-2와 MMP-9의 활성도는 OVX 상처군에서 낮았고, SLPI의 발현은 증가되었다.

본 연구와 다른 연구결과들을 비교해 보면 SLPI는 OVX 구강 상처에서 과도한 염증반응과 비정상적인 교원질 침착을 억제하는 역할을 하지만 상처회복을 촉진시키기에는 충분한 역할을 하지 못했다. 따라서, 본 연구의 결과로 SLPI는 OVX 모델의 구강상처 부위의 회복 과정에서 전신적 조절 인자라기 보다는 국소적 인자로서 작용할 것으로 생각된다.

I. INTRODUCTION

Wounds are induced by trauma, microbes or foreign materials, and healed by continuous processes of coagulation, inflammation, epithelialization, granulation tissue, matrix and tissue remolding (Ashcroft et al., 2002). Inflammation is the first phase in the wound healing process and is initiated by hemostasis which includes vasoconstriction, adhesion of platelets, aggregation, and degranulation (Toriseva et al., 2009). Thromboplastic tissue products accumulate in subendothelium of lacerated vessels. Extravassive leukocytes and plasma proteins move to wound area. The apparent morphological change of keratinocytes starts within one hour of the injury at the wound margin, and the thickness of epidermis is formed by division and migration of marginal basal cells in the dermal wound. Granulation tissue, a loose provisional matrix made of fibronectin, hyaluronic acid and collagen, contains blood vessles, macrophages and fibroblasts (Bullard et al., 2003). In remodeling phase, scar is formed by synthesis, degradation, reorganization and stabilization of collagen (Ashcroft et al., 2002). Oral mucosa is generally healed faster and scar is smaller than dermis (Bullard et al., 2003). Moreover, keloid formation and chronic wounds are rare in wound healing of oral epithelium (Stephens, 2004). Recovery of both oral mucosa and dermal wound proceeds through hemostasis, inflammation, proliferation and remodeling of collagen phases (Sciubba et al., 1978; Walsh et al., 1996). The difference between oral and dermal

wound healing is the presence of scar formation and speed of healing (Szpaderska et al., 2003).

Estrogen plays an important role in dermal healing, which in its absence leads to delayed wound healing through the recruitment of increased leukocyte in the early phases of wound. Estrogen accelerates cutaneous wound healing process by enhanced matrix deposition, rapid epithelialization, and inhibited inflammation response (Ashcroft et al., 1999). Estrogen is known to significantly reduce wound size and to increase collage amount and wound stiffness (Peter et al., 2008). The lack of estrogen causes thinning of epidermis and further causing reduced barrier and moisture-retaining function (Stevenson, 2007). Deprivation of estrogen in postmenopausal women is one of the main causes on the poor wound healing, skin wrinkling, dryness, atrophy, laxity, hot flushes and vulvar atrophy (Hall et al., 2005).

Secretory leukocyte protease inhibitor (SLPI) is 11.7 kD consisting of 130 amino acids and plays an important role in protecting oral mucosa and dermis from serine proteinases (Williams et al., 2006). SLPI inhibits serine proteinases such as, neutrophils, pancreatic acinar cells, and proteinases secreted by mast cells (Hiemstra, 2002). SLPI is expressed in mucosal tissues, and also expressed in cells that affect immune system such as monocytes, macrophages, and neutrophils (Thuraisingam et al., 2006). Furthermore, SLPI inhibits bacterial infection and accelerates epithelial cell proliferation, and has anti-inflammatory function in early

inflammatory response of odontoblasts (Choi et al., 2009). SLPI is known to inhibit serine proteinases in wound healing process (Angelov et al., 2004). SLPI plays an important role in controlling the function of neutrophil elastase that degrades proteins such as collagen and fibronectin in impaired cutaneous wound (Kafienah et al., 1998; Angelov et al., 2004).

Estradiol valerate (EV) induced polycystic ovary syndrome (PCOS) rats has morphologically abnormal ovary with ovarian cysts and hyperthecosis, reduced estrogen phase, anovulation, and increased androgen synthesis (Lara et al., 2000). Cutaneous wound healing is significantly delayed in ovariectomized (OVX) rat without ovary to produce the estrogen (Holcomb et al., 2009). In OVX animal with absence of estrogen, MMP-2, MMP-9, type-I and type-III collagen expression and gelatinoytic activity are significantly low, interfering extracellular matrix turnover and further reducing alveolar wound healing and new bone formation (Zecchin et al., 2005; Pereira et al., 2007).

SLPI promotes wound healing of dermis and oral mucosa (Ashcroft et al., 2000; Angelov et al., 2004), and estrogen regulate the expression of SLPI (King et al., 2003; Chen et al., 2004). SLPI is not expressed in the uterus tissue of OVX rat but expressed after injection of estrogen (Chen et al., 2004).

Therefore, SLPI and estrogen may have a close relation on the expression and function in wound healing process. Previous study reported on the function and expression of SLPI in cutaneous wound healing of PCOS with reduced estrogen

concentration. But the relationship between SLPI and estrogen in oral mucosa healing of OVX animals, with their estrogen secretion completely blocked, is unknown. Therefore the aim of this study is to identify oral mucosal healing process and expression and function of SLPI in OVX animal model.

II. MATERIALS AND METHODS

II-1. Induction of the ovariectomized (OVX) condition

Six week old virgin adult female ICR mice (Samtako Bio, KOR), weighing 90 ~100 g, were used. A total 61 mice was experiment. The mice underwent bilateral OVX operations under general anesthesia with intramuscular injections of 2% Rompun (Bayerkorea, KOR) and 10% ketamine (Yuhan, KOR). After OVX, the temperature and humidity was maintained at 23 ± 2°C and 60 ± 10%, respectively. The animals were kept on a 12 h light, 12 h dark photoperiod and allowed free access to pelleted mice chow and tap water during 60 days. All animal studies were approved by the 'Institutional Animal Care and Use Committees' at Chosun University and animal care was carried out in SPF level systems following by the 'Guide for the Care and Use of Laboratory Animals'.

II-2. Induction of the oral mucosa wound

A total 112 mice was divided equally into the normal and OVX group. The mice were anesthetized with a mixing solution of 0.2 ml/100g Ketamine (Yuhan, KOR) and 0.08 ml/100g Rompun (Bayerkorea, KOR) as a single intramuscular injection. Oral wounds were created in the buccal mucosa with a 1.5 mm dermal

biopsy punch (Miltex Instrument Company, USA) without perforation of the dermis, and allowed to heal by secondary intention. After wounding, the mice were sacrificed and the wound was excised at day 1, 3, 5, and 7, respectively. A total 28 mice was divided equally into the normal and OVX group and these two groups did not make wound for using as the control, respectively.

II-3. Histology and histomorphometrical analysis

Excised oral wounds were fixed in 10% buffered neutral formalin and incubated overnight at 4°C. The tissues were washed in 1 × PBS for 2 hours and dehydrated by sequential washing in 70%, 80%, 90%, 100% I, 100% II, 100% III, and finally 100% IV ethanol. The clearing process was performed using xylene, and the tissues were embedded in paraffin. The paraffin blocks were cut into 6 μm thick sections using a Motorized Rotary Microtome MT990 (RMC products, USA) and the sections were placed on a 37°C slide warmer overnight after attaching them onto a slide glass coated with 3-(Trimethoxysilyl) propyl methacrylate (SIGMA, USA). Hematoxylin & Eosin (H&E) staining was performed to observe the difference in the histological changes and measure the wound area. The inflammatory cells were counted in Giemsa stained tissues. Masson's Trichrome (MT) and Picrosirius Red staining were carried out to assess collagen deposition during matrix remodeling. Wound distance was measured as the length of the between wound edges. The inflammatory cells were counted in three distinct

areas (left, middle, and right wound area). The stained tissues were observed by optical microscopy (Carl Zeiss, GER) and the tissues stained with Picrosirius Red were observed by polarizing microscopy (Carl Zeiss). Histomorphological analysis was performed using AxioVision LE release 4.6 (Carl Zeiss) program.

II-4. Extraction of total RNA and reverse transcription and polymerase chain reaction

The tissues were ground after being deep-frozen in liquid NO_2 and the total RNA was extracted with Tri reagent (MRC Inc, USA) according to the manufacturer's instructions. A 1 μg sample of the total RNA was used to synthesize the complementary DNA (cDNA). The synthesis of cDNA was performed on the AccuPower RT Premix (Bioneer, KOR). The PCR reaction was carried out in a thermal cycler (Takara, JPN) after adding a 1 μl of cDNA to the AccuPower PCR Premix (Bioneer). The following primers (Bioneer) were used for RT-PCR analysis: 1) mouse SLPI Forward: 5'-CGG AAT TCC AGA GCT CCC CTG CCT TC-3', Reverse: 5'-GCT CTA GAC ATA GAG AAA TGA ATG CGT TT-3'; 2) mouse TGF- β1 Forward: 5'-CTC TCC ACC TGC AAG ACC AT-3', Reverse: 5'-CTG CCG TAC AAC TCC AGT GA-3'; 3) mouse type I collagen Forward: 5'-TTC TGG ATC AAG TGG TGA AC-3', Reverse: 5'-GTT CCA CTG TCC CGT ATT T-3'; 4) mouse fibronectin Forward: 5'-CCA AGA TCT GAT AAC GTC CC-3', Reverse:

5'-CTG TTG TGC CGT CAG AGG AT-3'; 5) mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) Forward: 5'-CCA TGG AGA AGG CTG GG-3' and Reverse: 5'-CAA AGT TGT CAT GGA TGA CC-3'. GAPDH was used as the internal control for RT-PCR. The PCR condition were as follows: 1) 33 cycles, 94°C 40 sec, 66°C 40 sec and 72°C 40 sec for SLPI, 2) 35 cycles, 94°C 30 sec, 60°C 30 sec and 72°C 30 sec for TGF-β1, 3) 35 cycles, 94°C 30 sec, 49°C 30 sec and 72°C 30 sec for type I collagen, 4) 35 cycles, 94°C 30 sec, 58°C 30 sec and 72°C 30 sec for fibronectin and 5) 30 cycles, 94°C 30 sec, 56°C 30 sec and 72°C 30 sec for GAPDH. After the last cycle, all samples were incubated for an additional 5 min at 72°C. The products were electrophoresed on a 2% agarose gel buffered with 0.5× Tris-Borate-EDTA and stained with ethidium bromide after amplification. The staining bands were visualized by Gel-Doc (BioRad Laboratories, USA). The primer sets specifically recognized only the genes of interest, as indicated by the amplification of a single band of the expected size (676 bp for SLPI, 699 bp for TGF-β1, 191 bp for type I collagen, 276 bp for fibronectin and 199 bp for GAPDH) according to the nucleotide sequence of SLPI (Genbank # NM_003064), TGF-β1 (Genbank # NM_021578), type I collagen (Genbank # NM_60424), fibronectin (Genbank # NM_010233) and GAPDH (Genbank # M33197). The intensity of the bands was measured using a Science Lab Image Gauge (FUJI FILM, JPN).

II-5. Western blotting

Western blotting was performed to examine the expression of SLPI, TGF-β1 and β-actin protein. The tissues were ground after being deep-frozen by liquid NO_2 and incubated with NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-Cl (pH 7.4), 2 mM Na_3VO_4, 2 mM $Na_4P_2O_7$, 50 mM NaF, 2 mM EDTA (pH 7.4) 0.1 μg/ml, leupeptin and 1 μg/ml aprotinin) on ice for 30 min. After protein extraction, the concentration in 30 μg was determined using a *Dc* protein assay kit (BioRad Laboratories) and electrophoresed onto 12% SDS polyacrylamide gel. After electrophoresis, the protein was transferred to a nitrocellulose membrane and blocked with 5% non-fat dry milk or 5% BSA for 1 hour at room temperature. The membrane was blotted with 1:200 of anti-rabbit SLPI (14), 1:1,000 of anti-rabbit TGF-β1 (Santa Cruz Biotechnology, USA) and 1:1,000 of anti-mouse β-actin (Santa Cruz Biotechnology) for 16 hours at 4°C. After washing, the membrane was blotted with 1:5,000 of HRP-conjugated goat anti-rabbit or mouse-IgG (SantaCruz Biotechnology) and developed using chemiluminescence film (Amershamphamacia) after being treated with an ECL solution (Amershamphamacia). The size of the SLPI, TGF-β1 and β-actin bands was a 12 kDa, 25 kDa, and 42 kDa, respectively. The density of expressed bands was measured using a Science Lab Image Gauge (FUJI FILM).

II-6. Gelatin zymography

Gelatin zymography was used to identify MMP-2 and MMP-9 in the wound tissues. The total protein (30 μg) of the tissues and HT-1080 cells was electrophoresed on SDS 10% - zymogram gel (Invitrogen, USA). The conditioned media from the HT-1080 human fibrosarcoma cells were loaded as a size marker for MMP-2 and MMP-9. After electrophoresis, the gels were incubated three times in a zymogram renaturing buffer (Invitrogen) at room temperature for 20 min, and then in a zymogram developing buffer (Invitrogen) for 30 min. The gels were then incubated at 37°C for 16 hours after being transferred to the same developing buffer. After developing, the gels were stained with 0.2% Coomassie brilliant blue (BioRad Laboratories) for 1 hour and incubated with a destaining solution (10% acetic acid, 40% methanol in distilled water) for 30 min. The density of the MMP-2 and MMP-9 bands was measured using a Science Lab Image Gauge (FUJI FILM).

II-7. Immunohistochemistry

The sample was deparaffinized sequentially in xyline I, II and III for 5 min, respectively, and hydrated sequentially in 100%, 90%, 80%, and 70% ethanol for 5 min. For SLPI antibody, the sample was washed a three times in 1× PBS for 5 min and primary blocked with 0.6% H_2O_2 in methanol for 20 min at room temperature.

For Ly-6G and Mac-3 antibodies, the sample was washed a three times in 1× PBS for 5 min and primary blocked with 3% H_2O_2 in methanol for 20 min at room temperature. After washing with 1× PBS, the tissues were incubated 0.01% citrate buffer (pH 7.4) at 99°C for 20 min. Normal goat serum (15 μl in 1 ml PBS, Vector Lab, USA) was used for secondary blocking. After washing, the sample was incubated with anti-rabbit SLPI (1:1,000), anti-mouse TGF-β1 (1:2,500), anti-mouse Ly-6G (1:100, BD Biosciences Phamingen, USA) or anti-mouse Mac-3 (1:100, BD Biosciences Phamingen, USA) diluted in fresh normal goat serum for 16 hours at 4°C. The negative control sample was not incubated with primary antibodies. Goat-anti-rabbit or mouse IgG (1:200, Vector Lab) was used for the secondary antibodies for 20 min at room temperature. After washing twice with PBS for 10 min, the sample was incubated with an ABC reagent for 20 min and developed for 1min and 40 sec using the 0.05% DAB (Deaminobenzidine Tetrahydrochloride, Vector Lab). After washing twice with ddH_2O for 10 min, the sample was stained with hematoxylin as a counterstain and dehydration, clearing and mounting was then performed. A pixel value represented the expression intensity of the SLPI and TGF-β1 protein in the wound area. The Mac-3 and Ly-6G positive cells were counted in the wound area. A pixel value and number of macrophages and neutrophils positive cells were quantified and calculated using Axiovision LE release 4.6 software (Carl Zeiss).

II-8. Elastase activity

For detection of elastase activity in the wound tissue, 30 μg of total protein was used. The elastase activity was performed using EnzCheck Elastase Assay kit (Molecular probes, USA) according to the manufacturer's instructions. The activity was measured by fluorescence microplate reader GENios FL (TECAN, CHE).

II-9. Statistical analysis

All experiments were carried out at least in triplicate. The data is reported as the mean and standard deviation determined using Excel 2007 statistical software (Microsoft, USA). The significant differences (*, $p<0.05$) were determined using a Student's t-test.

III. RESULTS

III-1. Wound distance

H&E staining was used to examine the area of healing in normal and OVX group after wounding on day 1, 3, 5, and 7. The stained wound tissues showed that the OVX wound area was wider than that of the normal group from day 1 to 7 (Fig. 1A). The wound distance of the OVX group was 1.4, 1.1, and 1.2 times wider than that of the normal group on day 3, 5, and 7 (Fig. 1B).

III-2. Infiltrated inflammatory cells

The difference between normal and OVX group in the number of inflammatory cells recruited in the wound area was observed in the Giemsa stained tissues (Fig. 2A). The number of inflammatory cells recruited in the OVX group was 1.1, 1.1, 1.1, and 1.2 times higher than that of normal group at day 1, 3, 5, and 7, respectively (Fig. 2B).

III-3. Collagen deposition

Newly formed collagen in the wound area was shown by Masson's Trichrome (MT) staining. More collagen deposition was observed in the healing area of the

normal group than the OVX group from day 1 to 7 (Fig. 3A). To confirm the result of MT staining, the same tissues were stained with Picrosirius Red and the level of collagen accumulation was quantified (Fig. 3B and C). The intensity of collagen deposited in the normal group was 1.8, 1.5, 1.9, and 1.6 times higher than that of OVX group at day 1, 3, 5, and 7, respectively (Fig. 3C).

III-4. Differential expression of type I collagen and fibronectin mRNA

In the unwounded tissue (control), the type I collagen mRNA expression of OVX group was 2.3 times lower than the normal group. After wounding, type I collagen mRNA was lower in the OVX group than the normal group from day 1 to 7 (Fig. 4A). The expression intensity of type I collagen mRNA in the OVX group was 0.2, 0.6, 0.8, and 0.6 times lower than normal group on day 1, 3, 5, and 7, respectively (Fig. 4B). Fibronectin mRNA was higher in the OVX group than the normal group in the control. After wounding, fibronectin mRNA was higher in the OVX group than the normal group from day 1 to 7 (Fig. 4C). The expression intensity of fibronectin mRNA in the OVX group was 2.7, 1.5, and 1.3 times higher than the normal group on day 1, 3, and 5, respectively (Fig. 4D).

III-5. Expression of MMP-2 and MMP-9

To detect the MMP activity, a zymography assay was conducted in the wound tissues. Pro- and active MMP-2 and -9 were elevated in the normal and OVX wound compared to that of the control (Fig. 5). Active form MMP-9 in OVX was 0.6, 0.08, and 0.1 times lower than the normal group on day 1, 5, and 7 except day 3 (Fig. 5B). Active form MMP-2 in the OVX group was 0.3, 0.4, and 0.1 times lower than that of the normal group on day 1, 5, and 7 except day 3 (Fig. 5C).

III-6. Differential expression of SLPI mRNA and protein

In the control, the mRNA expression of SLPI was no difference between OVX and normal group. After wounding, the mRNA expression of SLPI in the OVX group was higher than the normal group from day 1 to 7 (Fig. 6A). Intensity of expression of SLPI mRNA in the OVX group was 1.9, 2.5, 5.4, and 2.4 times higher than normal group on day 1, 3, 5, and 7, respectively (Fig. 6B). Expression of SLPI protein in the OVX group was 2.4 times higher than the normal group in the control. After wounding, the level of the SLPI protein in the OVX group was 2.2, 1.9, 3.1, and 14.3 times higher than the normal group on day 1, 3, 5, and 7, respectively (Fig. 6C and D). Immunohistochemistry was performed for SLPI protein in the wound tissues. Expression of SLPI protein in the dermal area of the OVX group was higher than the normal group (Fig. 6E).

III-7. Expression of TGF-β1 mRNA and protein

In the control, the level of TGF- β1 expression was lower in the OVX than the normal group. After wounding, the increased mRNA and protein expression of TGF-β1 was lower in the OVX group than that of the normal group from day 1 to 7 (Fig. 7A and C). The expression intensity of TGF-β1 mRNA in the OVX group was 0.1, 0.8, 0.8, and 0.9 times lower than that of the normal group at day 1, 3, 5, and 7, respectively. The level of the TGF-β1 protein in the OVX group was 0.3 and 0.2 times lower than that of the normal group at day 3 and 5 (Fig. 7B and D).

IV. DISCUSSION

Sex steroids can influence in several common pathological conditions including in inflammatory response on wound healing or cancer progression. Among these steroids, estrogens play an important role in the development of sexual dimorphism for reproduction in a female animal. During menopause, skin undergoes profound changes, including a decrease in dermal collagen and reduced thickness but hormone replacement therapy (HRT) has the reversible effect on estrogen deprivation (Schmidt et al., 1994). Treatment of topical estrogens increase the cutaneous wound healing by modulating the inflammatory response, cytokine expression and matrix deposition, promoting re-epithelialization, stimulating angiogenesis and wound contraction (Ashcroft et al., 1997; 1999). Ovariectomy is the systemic depletion of estrogen level and delayed alveolar wound healing as the decreased epithelial coverage and promoting fraction of collagen after molar extractions in rats (Pereira et al., 2007). SLPI null mice showed that more delayed oral mucosa and skin wound healing compared that of normal mice (Ashcroft et al., 2000; Angelov et al., 2004). Previous study showed that treatment of estrogen increased the secretion of SLPI but decreased lipopolysaccharide (LPS)-induced cytokine secretion such as interleukin-6 (IL-6) and IL-8 in human uterine epithelial cells (Fahey et al., 2008). SLPI was not expressed in the uterine tissues of the Ovariectomized (OVX) rat, but increased

after the estrogen treatment (Chen et al., 2004). Estrogen increased the SLPI mRNA expression and secretion in endocervix of postmenopausal women (Kumar et al., 2011). Expression of transforming growth factor-β1 (TGF-β1) is increased in wounded corneal fibroblasts on the wound edge (Song et al., 2000). Blocked TGF-β1 by adding neutralizing TGF-β1 antibody inhibits migration of corneal fibroblasts in wound healing assay (Wang et al., 2011). Also, decreased expression TGF-β1 by infection of *pseudomonas aeruginosa* was delayed wound healing of female rat (Zhang et al., 2011). Estrogen increased the secretion and expression of TGF-β1 in young and old female human dermal fibroblasts and this regulation accelerates wound healing (Ashcroft et al., 1997). Therefore, we speculated that estrogen depletion may cause the decreasing of SLPI and TGF-β1expression in unwounded OVX oral mucosa and during wound healing. Our previous study showed that a decrease of the estrogen level by induction of PCOS with enlarged wound area of rat skin compared to that of normal wound (Jeong et al., 2011). In this study, wound healing of OVX mice oral mucosa is delayed due to large wound area, increased infiltration of inflammatory cells and inhibition of matrix degradation compared to that of normal oral wound. SLPI expression was increased in wounded skin and ovary, and high in unwounded skin of PCOS rat compared that of normal in our previous study (Jeong et al., 2011; Park et al., 2011). TGF-β1 expression was low in unwounded and wounded skin of PCOS rat (Jeong et al., 2011). In present study is also showed that the increased expression

of SLPI and lower expression of TGF-β1 during healing of oral wound in OVX mice as well as unwounded tissue in consistent with the results of PCOS wound healing. Therefore, SLPI may regulate oral mucosa wound healing in locally by estrogen independently and TGF-β1 may be controlled by estrogen dependently in oral mucosa wound healing of OVX mice.

Neutrophil and macrophage release the protease for phagocytosis of debris, microbial agents and degraded matrix components in inflammatory phase during wound healing process. Among this protease, elastase is released from neutrophil and degrades firbronection in wounds of aged humans (Herrick et al. 1996). Fibronectin is one of the major components of extracellular matrix (ECM) released from fibroblasts and an important role for cell migration and adhesion (Kurkinen et al. 1980). In wound healing, fibronectin induces keratinocyte migration, increasing collagen deposition and stimulating wound contraction (Clark, 1988). Estrogens modulate the inflammatory response by inhibiting neutrophil chemotaxis, decreasing the migration of cells from the vasculature into injured tissues (Ashcroft et al., 1999). In a skin wound of OVX mice, estrogen treatment inhibits of macrophage migration inhibitory factor (MIF) expression and decreases the infiltration of macrophages (Ashcroft et al., 2003; Hardman et al., 2008). Elastase activity is increased in wrinkled skin of OVX mice compared to that of normal (Tsukahara et al., 2004). Estrogen treatment accelerated the skin wound healing in elderly males and females with a decrease in wound size and increased collagen

and fibronectin levels (Ashcroft et al., 1999). SLPI is capable of inhibiting various serine proteases, including neutrophil elastase, cathepsin G, mast cell chymase, and chymotrypsin (Thompson et al., 1986; Vogelmeier et al., 1991). SLPI regulates inflammation and promotes wound healing and also exhibits antibacterial properties and antagonizes LPS-induced inflammatory response induced by monocytes and macrophages (Jin et al., 1997; 1998). SLPI is also expressed in inflammatory cells, such as mast cell, neutrophil and macrophage and reduces neutrophil immigration throughout the decreasing of tumor necrosis factor-α (TNF-α) and macrophage inflammatory protein-1 (MIP-1) secretion in damaged lung and liver (Lentsch et al., 1999; Mihaila et al., 2001). The infiltration of neutrophil and macrophage increased and elastase activity and fibronectin expression are increased in SLPI null mice oral wound (Angelov et al., 2004). In our previous report, the number of infiltrated inflammatory cells and macrophages in the skin wound area was high in PCOS compared to the normal group (Jeong et al., 2011). In this study, mRNA and protein expression of SLPI is also increased in unwounded and wounded oral tissue of OVX mice. Moreover, the number of infiltrated inflammatory cells such as neutrophil and macrophage are increased and elastase activity was decreased in oral wound tissue of OVX mice compared with normal wound. The expression of fibronectin is increased in unwounded and wounded oral tissue of OVX mice compared to that of normal mice. Therefore, these results suggest that increased SLPI expression might be caused by

excessive infiltrated inflammatory cells, resulting in increasing SLPI may upregulate expression of fibronectin associated with reduced elastase activity during oral wound healing of OVX mice.

Matrix metalloproteinase (MMP) is zinc-dependent proteinases essential for several physiological and pathological events, such as embryonic development, angiogenesis, wound repair, and metastasis (Birkedal-Hansen, 1993; Chavey et al., 2003). Tissue inhibitors of metalloproteinases (TIMPs) play an important role in preventing excessive ECM degradation by MMPs (Li et al., 2000). In wound remodeling phase of healing, MMPs and elastase regulates collagen synthesis, degradation and reorganization with counteracting molecules such as TIMP and SLPI (Ashcroft, 2003). Estrogen inhibits collagen degradation during the remodeling phase of wound healing by inhibiting MMP-2 and -9 secretions in fibroblasts (Moalli et al., 2002). Also, 17β-estradiol treatment increases collagen and fibronectin in wound areas and reduces MMP-mediated collagenolysis with increasing TIMP in rabbit uterine fibroblasts (Sato et al., 1991; Ashcroft et al., 1999; Pirila et al., 2001). OVX rat wound healing showed a decreasing in collagen deposition and MMP-2 and -9 were activated significantly by the sera from PCOS women compared to those normal women (Ashcroft et al., 2003; Tan et al., 2011). In other study, MMP-2, -9 and type I collagen expression were decreased in OVX rat molar extraction wounds (Zecchin et al., 2005). In the skin wound of SLPI deficient mice, the level of MMP-2 and -9 secretions and type I collagen

expression were higher and lower than that of a normal mice wound (Angelov et al., 2004). The expression of MMP-9 increased during the inflammatory response but decreased in bronchial epithelial cells after the SLPI treatment (Qu et al., 2008). In PCOS unwounded and wounded rat skin, SLPI expression was increased but MMP-2 and -9 expressions were low compared with normal rat unwounded and wounded skin (Jeong et al., 2011). And also SLPI and MMP-2 expression was high and low in polycystic ovaries compared to normal ovaries (Park et al., 2011). In consistent with the result of PCOS wound healing, expression of SLPI was higher in OVX oral wound than that of normal wound. MMP-2 and -9 activities were significantly lower in both the unwounded and wounded OVX oral than in normal oral tissue. Also, collagen deposition and expression were decreased in both the unwounded and wounded OVX oral compared with normal oral tissue. Therefore, SLPI might be a substitutive molecule instead of estrogen, acting mainly as a protease inhibitor in matrix remodeling on the estrogen deficiency condition.

TGF-β1 is major contributor to matrix production, which induces synthesis and inhibits degradation of ECM and stimulates granulation tissue formation and collagen deposition (Branton et al., 1999; Niesler et al., 2001). TGF-β1 stimulates production of fibronectin and collagen, ECM components, and overexpression of TGF-β1 could induce the fibrotic lung diseases (Roberts et al., 1986; Sporn et al., 1986; Khalil et al., 1991). MMP-2 and MMP-9 are induced by TGF-β1 in human

monocytic cells (Lee et al., 2005). Estrogen also increases the level of TGF-β1 from osteoblasts (Oursler et al., 1991) and an ovariectomy down-regulates TGF-β 1 transcription in bone (Ikeda et al., 1993). Estrogen increases TGF-β1 secretion and expression in *in vivo* and *in vitro* dermal fibroblasts (Ashcroft et al., 1997). Also, stimulation of TGF-β1 secretion by estrogen increases collagen deposition during the wound remodeling phase (Ashcroft, 2003). SLPI reduces the TGF-β1 activity and overexpression of SLPI decreased the TGF-β1 expression (Ashcroft et al., 2000; Zhang et al., 2002). In PCOS skin wound, the collagen deposition in a wound area was low in the PCOS skin compared to the normal during healing period in spite of the high level of SLPI mRNA and protein expression whereas TGF-β1 of mRNA and protein was low (Jeong et al., 2011). In this study, collagen deposition was low in OVX oral wound compared to normal and TGF-β1 expression was low in OVX oral unwounded and wounded tissue. Therefore, our result suggests that increased expression of SLPI and low expression of TGF-β1 by estrogen deficiency might lead to low collagen deposition in an oral wound.

In summary, oral mucosa wound healing in OVX mouse is delayed as showed that increased wound area, excessive infiltration of inflammatory cell, decreased collagen deposition. The expression of TGF-β1 and collagen was low and fibronectin was high in OVX wound compared that of normal group. The activity of elastase, MMP-2, and MMP-9 was low and SLPI expression was up-regulated in OVX wound. Therefore, on the basis of previous and our results, the increased

SLPI in the OVX oral wound is associated with the prevention of an excessive inflammatory response and aberrant collagen deposition but it is not enough to accelerate OVX oral wound healing. In conjunction with our and other reports, it was known that SLPI promotes wound healing. Therefore, these SLPI may act as a local rather than a systemic modulating molecule in OVX mouse oral wounds.

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FIGURE LEGENDS

Figure 1. Difference in the oral wound area between normal and OVX group. (A) Difference in wound area between normal and OVX group at the oral wound. The wound area of the OVX group was wider than the normal. (B) The wound distance rate of the OVX group was wider than that of the normal group from days 1 to 7. All scale bars are 100 μm. The arrows demarcate wound edge. OVX, ovariectomized.

Figure 2. Infiltrated inflammatory cells in the wound area of normal and OVX group. (A)(B) The number of inflammatory cells in the OVX wound area was increased from days 1 to 7 compared to that of the normal group. All tissues were stained with Giemsa. OVX, ovariectomized.

Figure 3. Difference in the accumulated collagen between normal and OVX oral wound. (A)(B) Difference in accumulated collagen (blue color, bright red color) in the healing area between the normal and OVX group at the oral wound with Masson's Trichrome and Picrocirius Red staining. (C) The collagen positive intensity in the wound area was low in the OVX group from days 1 to 7. All scale bars indicate 100 μm. The arrows demarcate the wound edge. OVX, ovariectomized.

Figure 4. Differential expression of type I collagen and fibronectin mRNA in the oral wounds of normal and OVX group. (A)(B) Type I collagen mRNA expression was low in the OVX compared to the normal group. (C)(D) Fibronectin mRNA expression was higher in the OVX group than the normal group. Type I collagen and fibronectin mRNA were normalized to the corresponding GAPDH, respectively. M, size marker; N, normal; O, OVX.

Figure 5. Differential expression of MMP-2 and MMP-9 in the oral wounds of the normal and OVX group. (A) Pro- and active MMP-2 and -9 were detected with zymography. (B)(C) Active MMP-2 and -9 in OVX group were significantly lower than that of the normal group. N, normal; O,OVX

Figure 6. Differential expression of SLPI in the oral wound of normal and OVX group. (A)(C) SLPI mRNA and protein expression in the normal and OVX oral wound. (B)(D) Increased SLPI mRNA and protein expression was high in the OVX group than the normal group. (E) Intensity of SLPI was high in OVX wound in immunohistochemistry. SLPI mRNA and protein were normalized to the corresponding GAPDH and β-actin, respectively. M, size marker; N, normal; O, OVX. The arrows demarcate wound edge. All scale bars are 100 μm.

Figure 7. The expression of TGF-β1 in the oral wound of normal and OVX group.

(A)(C) TGF-β1 mRNA and protein expression in the normal and OVX oral wound. (B)(D) TGF-β1 mRNA expression and protein were low in the OVX oral wound compared to the normal group. TGF-β1 mRNA and protein were normalized to the corresponding GAPDH and β-actin, respectively. M, size marker; N, normal; O, OVX.

FIGURES

Figure 1.

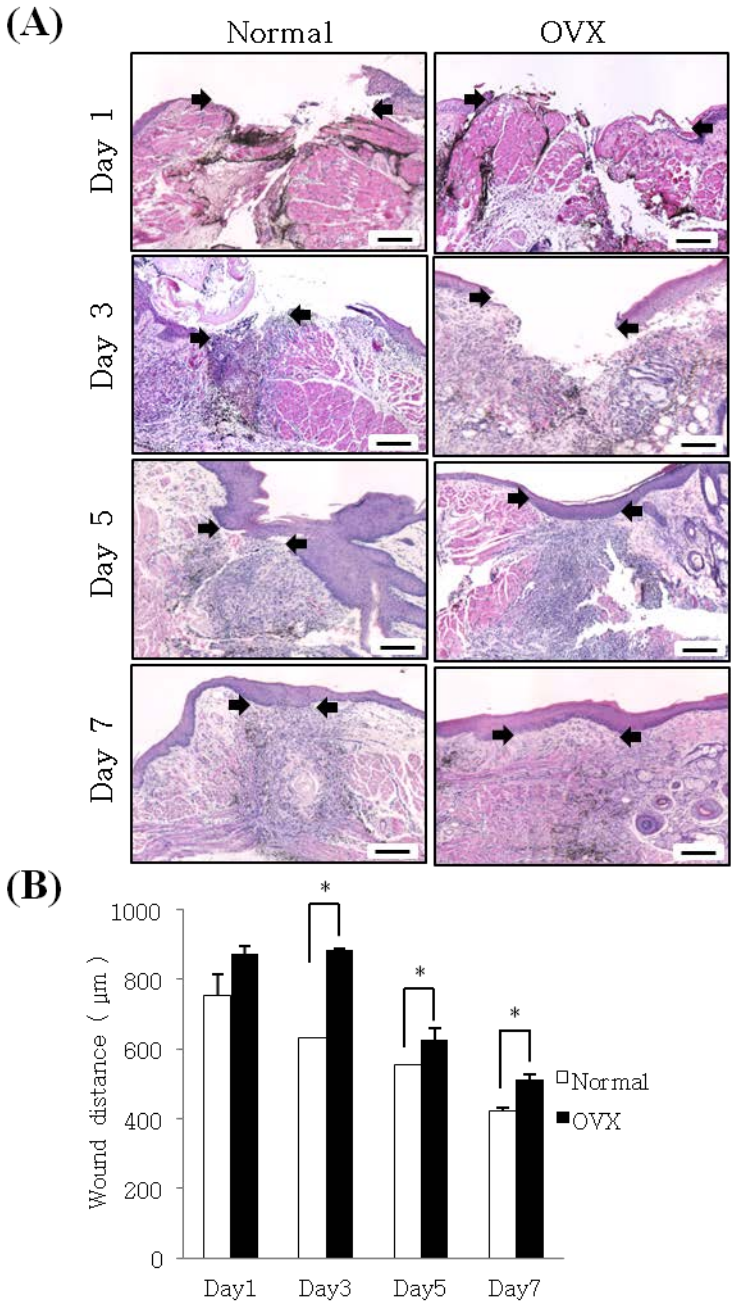


Figure 2.

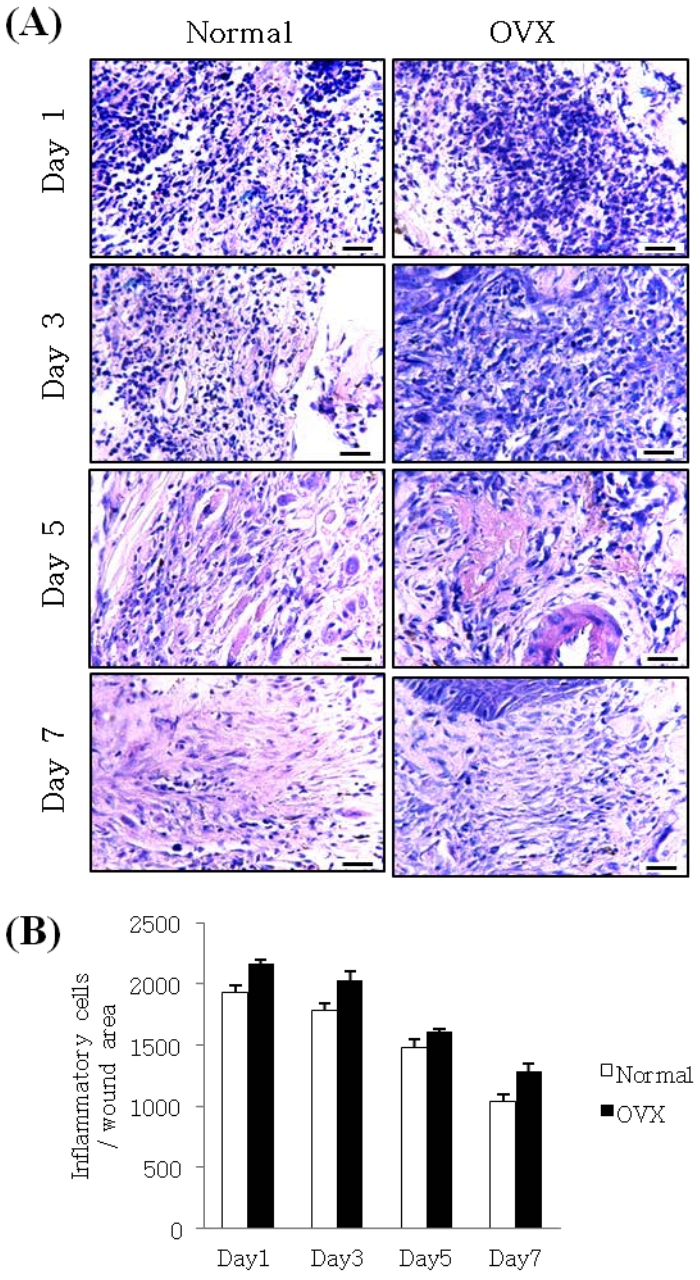

(A)
Normal
OVX
Day 1
Day 3
Day 5
Day 7
(B)
Inflammatory cells / wound area
2500
2000
1500
1000
500
0
Day1
Day3
Day5
Day7
Normal
OVX

Figure 3.

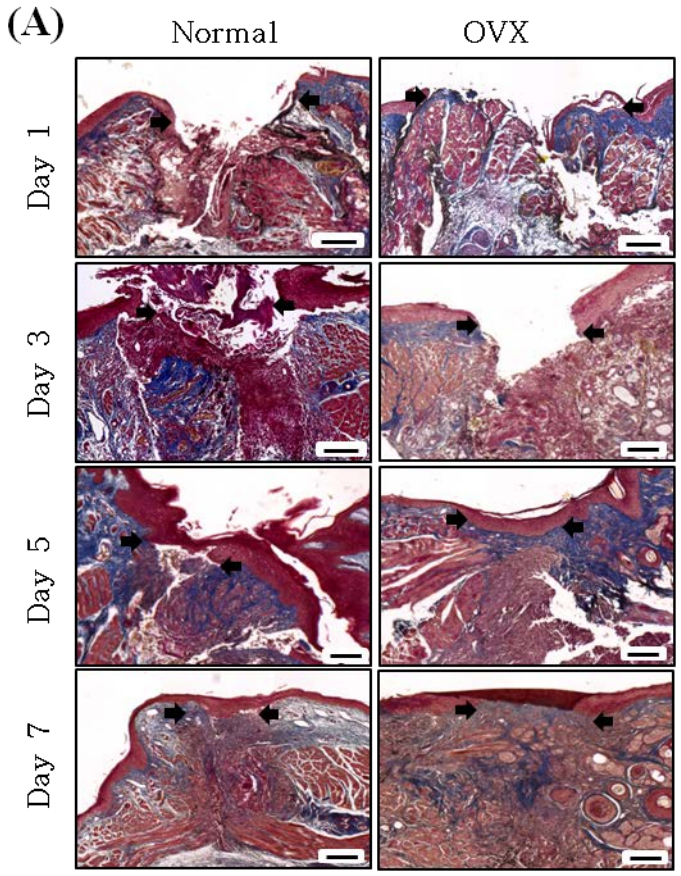


Figure 3.

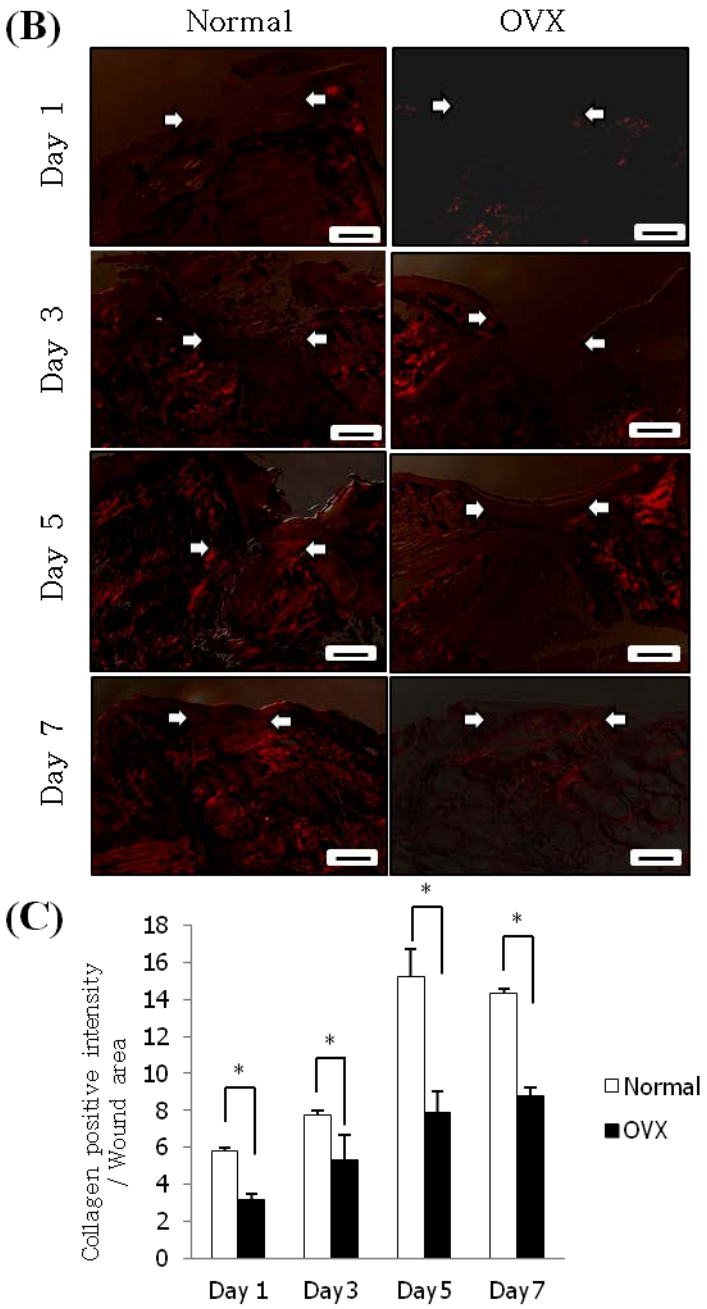

(B)
Normal
OVX
Day 1
Day 3
Day 5
Day 7
(C)
Collagen positive intensity / Wound area
18
16
14
12
10
8
6
4
2
0
*
Day 1
Day 3
Day 5
Day 7
Normal
OVX

Figure 4.

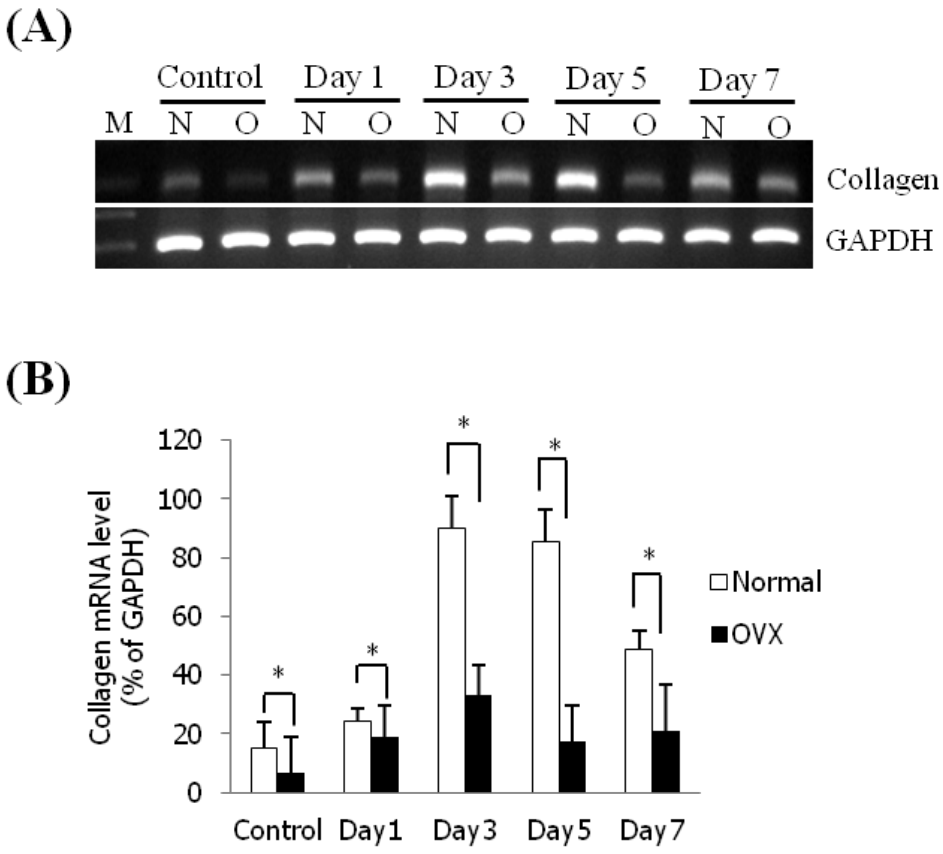

(A)
Control
Day 1
Day 3
Day 5
Day 7
M
N
O
N
O
N
O
N
O
N
O
Collagen
GAPDH
(B)
Collagen mRNA level
(% of GAPDH)
120
100
80
60
40
20
0
*
*
*
*
*
Normal
OVX
Control
Day1
Day3
Day5
Day7

Figure 4.

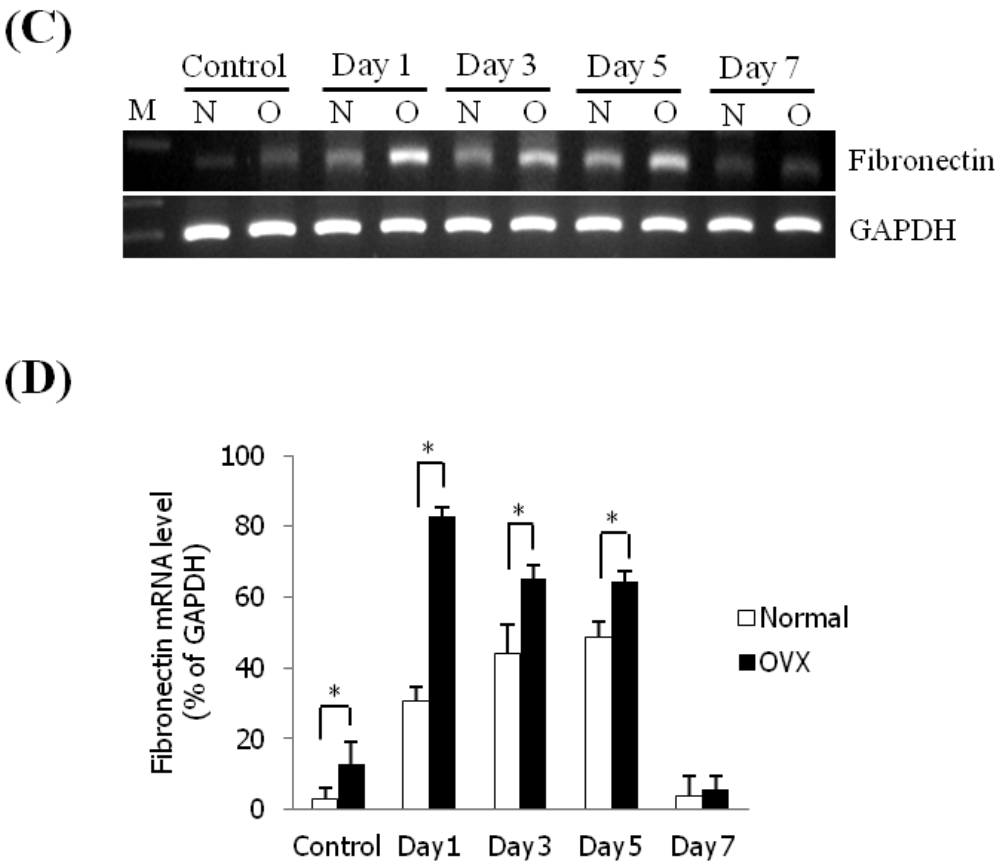

(C)
Control
Day 1
Day 3
Day 5
Day 7
M
N
O
N
O
N
O
N
O
N
O
Fibronectin
GAPDH
(D)
Fibronectin mRNA level
(% of GAPDH)
100
80
60
40
20
0
*
*
*
*
Normal
OVX
Control
Day1
Day3
Day5
Day7

Figure 5.

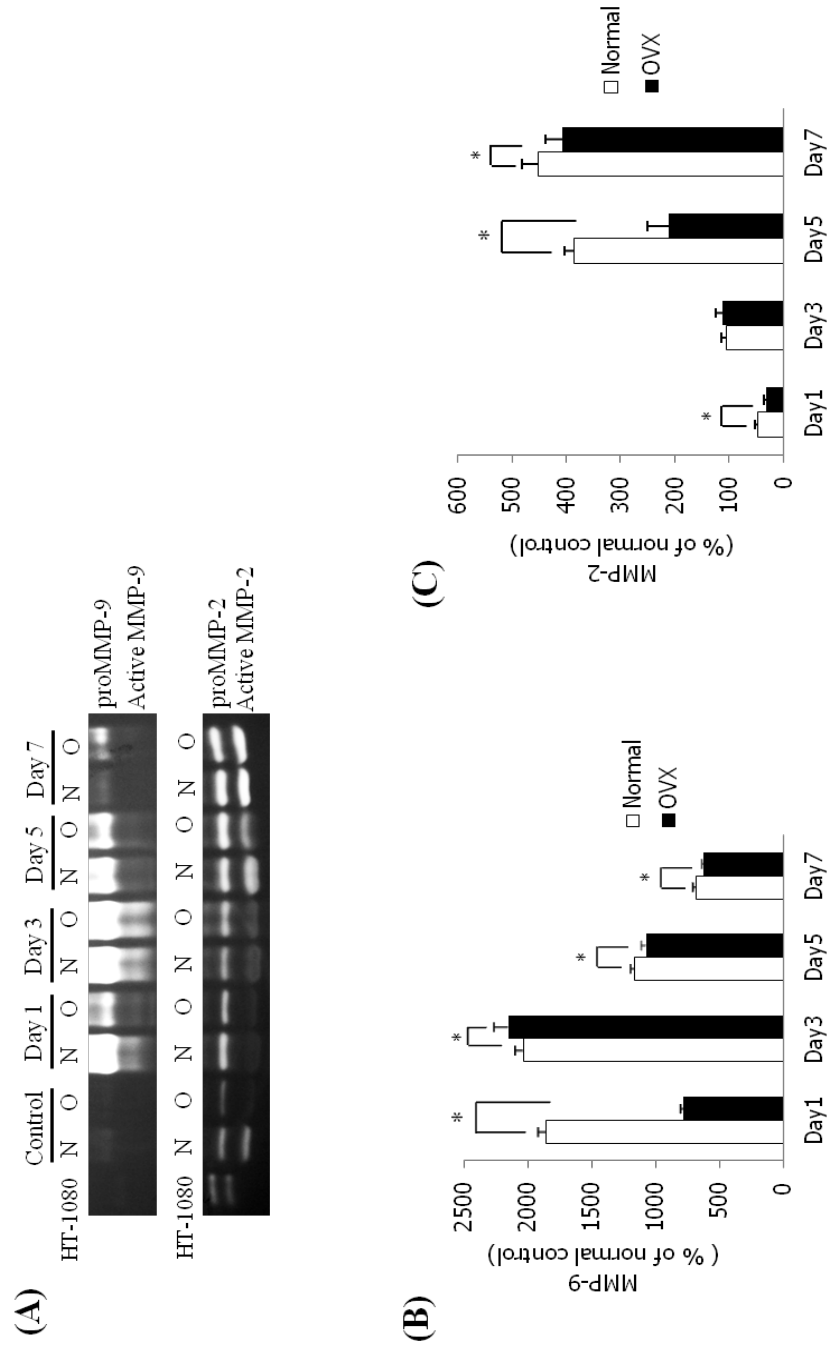

(A)
Control
Day 1
Day 3
Day 5
Day 7
N O N O N O N O N O
HT-1080
proMMP-9
Active MMP-9
HT-1080
proMMP-2
Active MMP-2
(B)
MMP-9
(% of normal control)
2500
2000
1500
1000
500
0
Day1 Day3 Day5 Day7
Normal
OVX
(C)
MMP-2
(% of normal control)
600
500
400
300
200
100
0
Day1 Day3 Day5 Day7
Normal
OVX

Figure 6.

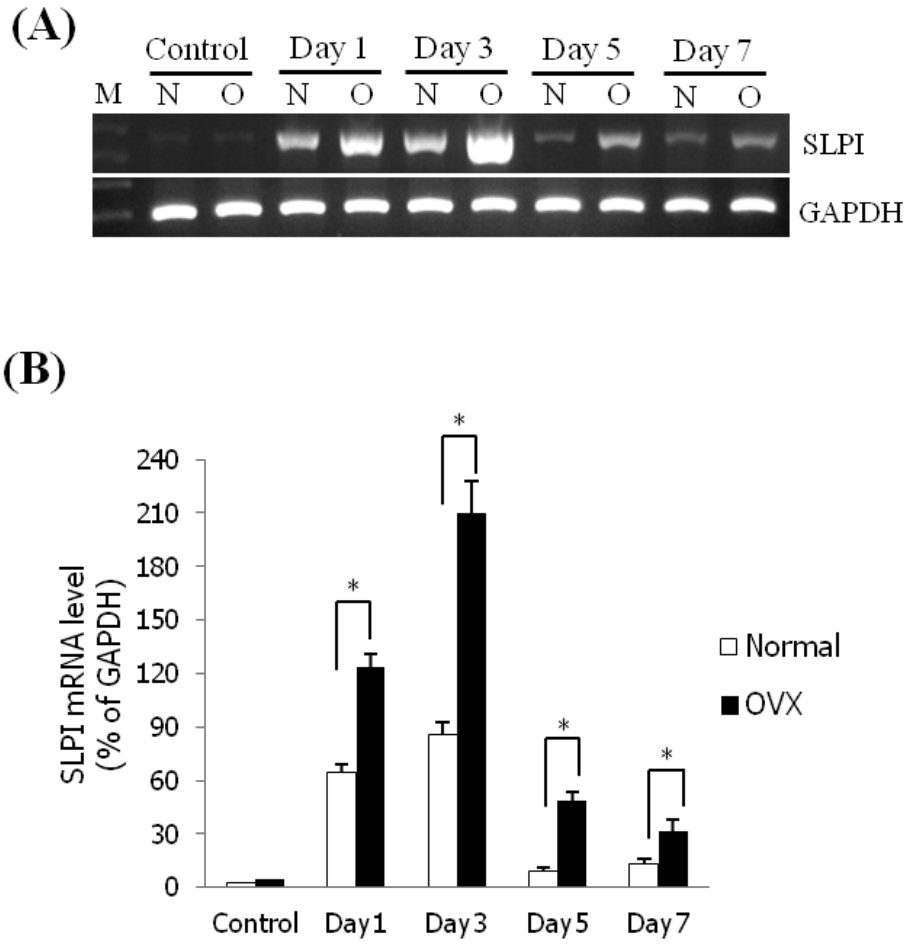

(A)
Control
Day 1
Day 3
Day 5
Day 7
M
N
O
N
O
N
O
N
O
N
O
SLPI
GAPDH
(B)
SLPI mRNA level
(% of GAPDH)
240
210
180
150
120
90
60
30
0
*
*
*
*
Control
Day1
Day3
Day5
Day7
Normal
OVX

Figure 6.

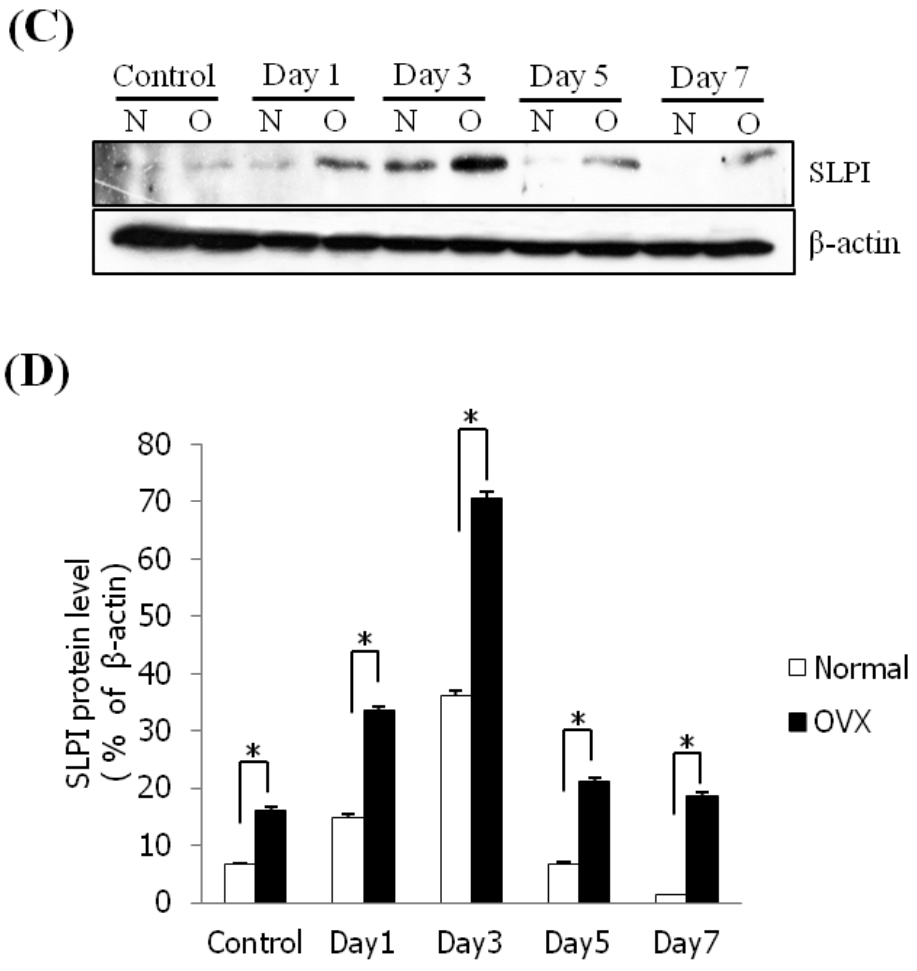

(C)
Control
Day 1
Day 3
Day 5
Day 7
N O N O N O N O N O
SLPI
β-actin
(D)
SLPI protein level
(% of β-actin)
80
70
60
50
40
30
20
10
0
Control
Day1
Day3
Day5
Day7
Normal
OVX

Figure 6.

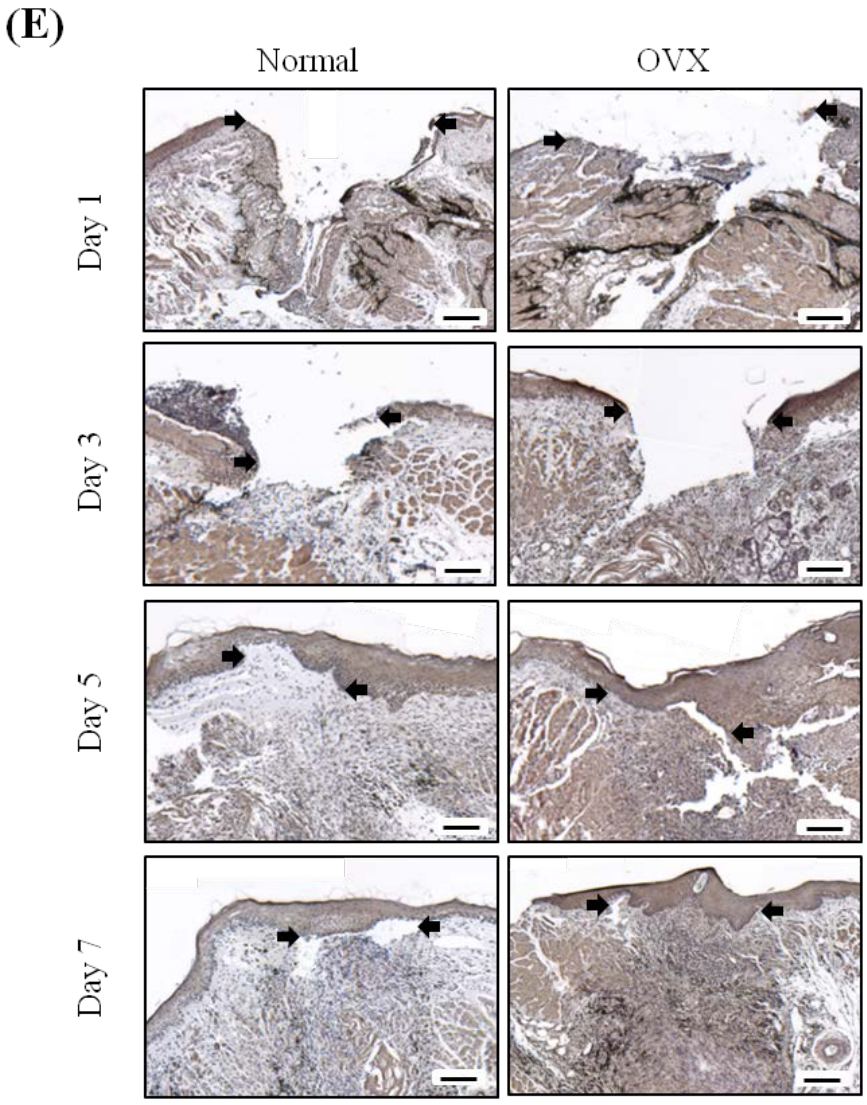


Figure 7.

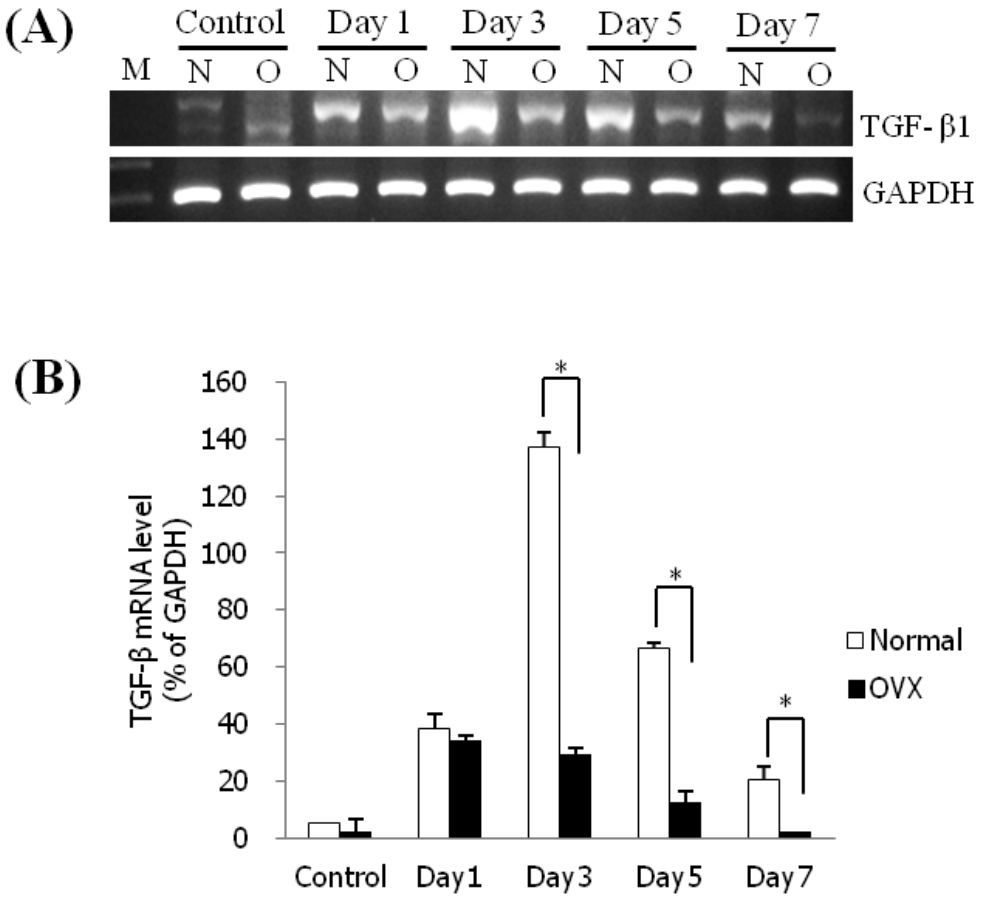
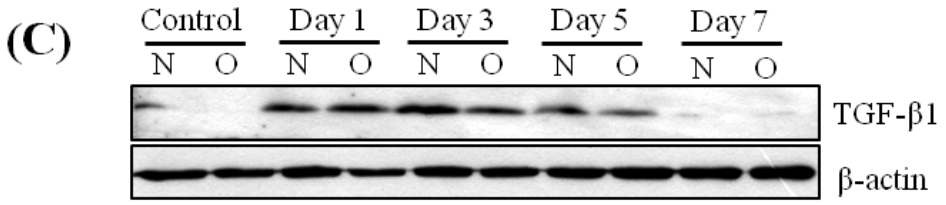
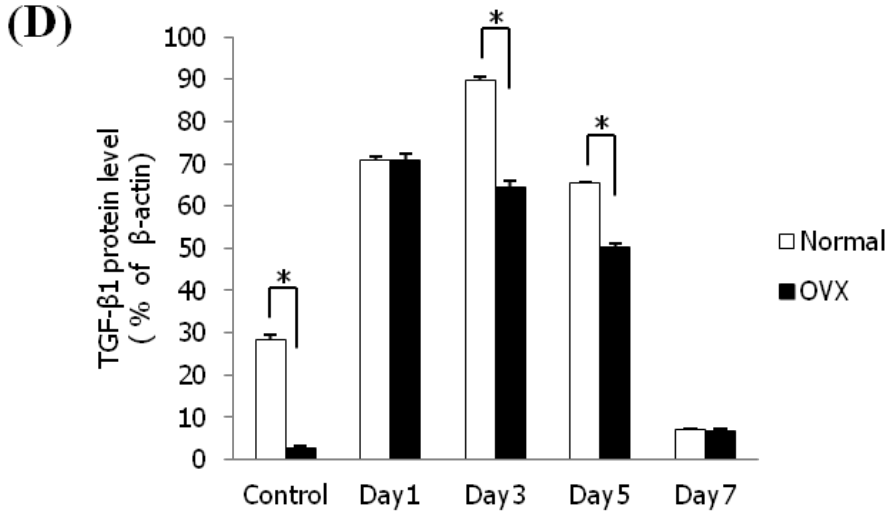

(A)
Control
Day 1
Day 3
Day 5
Day 7
M
N
O
N
O
N
O
N
O
N
O
TGF-β1
GAPDH
(B)
TGF-β mRNA level
(% of GAPDH)
160
140
120
100
80
60
40
20
0
*
*
*
Normal
OVX
Control
Day1
Day3
Day5
Day7

Figure 7.


(C)
Control
Day 1
Day 3
Day 5
Day 7
N O N O N O N O N O
TGF-β1
β-actin


(D)
TGF-β1 protein level
(% of β-actin)
100
90
80
70
60
50
40
30
20
10
0
*
*
*
Control
Day1
Day3
Day5
Day7
Normal
OVX

ABSTRACT

Expression of secretory leukocyte protease inhibitor (SLPI) in oral mucosal healing on the ovariectomized (OVX) mice wound

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Wound healing consists of continuous processes including coagulation, inflammation, re-epithelialization, granulation tissue, matrix remolding, sequentially. Estrogen increases wound healing by modulating the inflammatory response, cytokine expression and matrix deposition, promoting re-epithelialization, stimulating angiogenesis and wound contraction. The systemic depletion of estrogen level such as ovariectomy (OVX) leads to delayed wound healing. Secretory leukocyte protease inhibitor (SLPI) is serine protease inhibitor, which promotes wound healing of skin and oral mucosa by controlling the activity of neutrophil elastase for inhibition of excessive inflammatory response during wound healing processes. SLPI expression and secretion is increased by estrogen

and this regulation inhibits the secretion of pro-inflammatory cytokines. Therefore, we hypothesized that SLPI and estrogen may have a close relation on wound healing and investigated the expression and function of SLPI during oral mucosa healing process of OVX animal model using the histomorphometrical analysis, RT-PCR, western blotting, immunohistochemistry, gelatin zymography and elastase activity.

As a result, oral mucosa wound healing in OVX mouse is delayed as showed that increased wound area, excessive infiltration of inflammatory cell, decreased collagen deposition. The expression of TGF-β1 and collagen was low and fibronectin was high in OVX wound compared that normal group. The activity of elastase, MMP-2, and MMP-9 was low and SLPI expression was up-regulated in OVX wound. On the basis of previous and our results, the increased SLPI in the OVX oral wound is associated with the prevention of an excessive inflammatory response and aberrant collagen deposition but it is not enough to accelerate OVX oral wound healing. In conjunction with our and other reports, it is known that SLPI promotes wound healing. Therefore, SLPI may act as a local rather than a systemic modulating molecule in OVX mouse oral wounds.

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