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ORIGINAL ARTICLE

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Low-dose estrogen release from silastic capsule enhanced flap wound healing in an animal model

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Abstract

Background: Deep and extensive wounds usually cannot be closed directly by suturing or skin grafting. Flap transplantation is typically used to reconstruct large wounds clinically. The flap survival is based on a stable blood perfusion. It is established that estrogen promotes wound healing and angiogenesis, and regulates the inflammatory response, leading to enhanced flap survival after transplantation. However, estrogen concentrations administered in previous studies were significantly higher than physiological levels, potentially causing systemic side effects. Estrogen-sustained-release silastic capsules can maintain blood serum estrogen closer to physiological levels. This study aimed to investigate whether administering estrogen at a lower concentration, closer to physiological levels, could still enhance flap survival.

Materials and Methods: This study was performed in a random skin flap model in ovariectomized (OVX) mice. Sustained-release estrogen silastic capsules were implanted into OVX mice to determine the functional role of estrogen in wound healing after flap transplantation. Flap blood perfusion was

Abbreviations: OVX, Ovariectomized; CD31, Cluster of differentiation 31; HIF-1α, Hypoxia-inducible factor 1 alpha; α-SMA, Alpha–smooth muscle actin; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end-labelling; IGF-1, Insulin-like growth factor 1; TGF-β, Transforming growth factor beta; LSCI, Laser speckle contrast imaging; IACUC, Institutional Animal Care and Use Committee; SC, Sesame oil capsule; E2, Estradiol; ERα and ERβ, Estrogen receptors alpha and beta; GPR30, G protein-coupled receptor 30; RNA, Ribonucleic acid; IHC, Immunohistochemistry; SD, Standard deviation.

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analysed using a colour laser Doppler scanner. Immunohistochemical staining of CD31, hypoxia-inducible factor 1 alpha (HIF-1α), alpha–smooth muscle actin (α-SMA), cleaved caspase 3 and apoptotic terminal dUTP nick end-labelling stain was used to investigate flap angiogenesis, tissue hypoxia, wound healing and cell death in the flap tissue, respectively.

Results: We observed that administering estrogen at a lower concentration enhanced superficial blood perfusion while reducing the flap's ischemic area and tissue necrosis. HIF-1α expression was significantly decreased in the dermis layer but not in the fascia, whereas cleaved caspase 3 levels decreased in the fascia but remained unchanged in the dermis. Additionally, there was no significant difference in CD31and α-SMA expression between the groups.

Conclusion: In summary, the study showed that an estrogen silastic capsule maintained physiological estrogen levels and improved superficial perfusion, thereby reducing dermal hypoxia, and cell death in a mouse random pattern skin flap model. Although no significant promotion of angiogenesis was observed, the study suggests that appropriate estrogen supplements could enhance flap wound recovery.

KEYWORDS

flap transplantation, estrogen supplementation, estrogen-sustained-release capsules

Key Messages

- Estrogen silastic capsule maintained physiological estrogen levels increased flap blood perfusion, reducing ischemia and tissue necrosis.
- Low-dose estrogen did not significantly promote angiogenesis.
- Appropriate estrogen supplements could enhance flap wound recovery.

1 | INTRODUCTION

Flap transplantation is a prevalent clinical therapeutic modality for the reconstruction of extensive tissue defects. The term 'flap' encompasses skin and underlying tissues, such as subcutaneous adipose tissue, muscular components and occasionally partial bone. This anatomical unit is characterized by primary arterial and venous conduits that support the principal blood circulation. Upon grafting a flap to the recipient site, reestablishing viable blood perfusion between the flap and the host tissue becomes imperative for its enduring viability. The flap is inherently endowed with one to two major vascular pedicles, facilitating its intrinsic vascular supply beyond the primary blood flow. However, the network of microvascular anastomoses at the interface between the flap tissue and the recipient site must be rebuilt. Failure to promptly reestablish microvascular circulation can lead to ischemic necrosis, starting from the marginal regions of the flap tissue.[1] Reconstructing microvascular circulation is affected by various factors, including smoking, local blood circulation insufficiency due to oedema or compression, malnutrition, inflammation, infection and vascular alterations caused by chronic conditions, such as diabetes and hypertension.[2] These pathophysiologic influences can significantly impede angiogenesis and vascular anastomotic integration.[3,4] Identifying methods that stimulate angiogenesis or mitigate tissue necrosis is paramount to enhancing the success rate of flap transplantation surgery and promoting postoperative wound healing.

The natural wound healing process involves three successive and overlapping stages: haemostasis or inflammation, proliferation and remodelling.[5,6] Acute wounds heal more slowly in males than in females, with different responses in both sexes. Many factors, including sex hormones, influence wound healing.[7,8] Hormone therapy, particularly estrogen, is promising in reversing delayed wound healing, particularly in ischemic ulcers and diabetic wounds.[9,10] Estrogen, a female sex hormone, exhibits excellent healing effects during wound repair.[10] Estrogen promotes cell proliferation and angiogenesis and inhibits apoptosis in tissues.[11] Fibroblast proliferation and extracellular matrix are crucial for wound healing. Estrogen can increase the proliferation of fibroblasts and the synthesis of collagen, TGF-β and IGF-1.[12]

Insufficient blood supply causes tissue necrosis and hinders wound healing.[13,14] Estrogen can improve vascular network formation and prevent tissue necrosis in a mouse model of skin ischemia.[15,16] Previous studies have shown that both single and three doses of estrogen had a protective effect after flap ischemia–reperfusion injury in male Wistar rats and inhibited apoptosis by altering the p38 mitogen-activated protein kinase and MKP-1/ERK signalling pathway.[16,17]

Females post-menopause and those with estrogen deficiency face wound healing challenges, notably inflammation and re-granulation, reversible by exogenous estrogen treatment.[18] Menopause can be mimicked in animal models by surgically removing the ovaries. Moreover, 17β-estradiol replacement therapy in ovariectomized (OVX) mice is a common technique used to determine specific hormone effects. However, these studies were complicated by the non-monotonic dose response of estradiol when administered as therapy.[19,20] Previous study has shown that implanted estradiol pellets 0.1 mg or 0.25 mg (releasing estradiol 80 and 200 μg kg^{-1} per day) prevented skin flap necrosis by protecting the vascular network from reperfusion injury in a mice ischemia model.[15] Commercially prepared hormone pellets deliver consistent and uniform subcutaneous hormone released for a specified time. However, the serum hormone levels are elevated more than projected, and hormone delivery is inconsistent.[21,22] High estrogen levels could decelerate wound healing by dampening the inflammatory response.[23] Moreover, recent evidence suggests that the effects of estrogens may follow hormetic principles on many systems, where physiological concentrations are protective, while higher and prolonged concentrations can be harmful.[19] Therefore, in promoting flap survival, using estrogen at concentrations closer to physiological levels may reduce negative systemic effects.

Silastic capsules used for hormone delivery are considered stable drug release systems and are commonly used in various research applications.[24,25] These capsules are superior to other methods such as pellets and injections in consistently producing the desired hormone levels within the physiological range.[26] Estrogen within silastic capsules can be stably released into the bloodstream.[27] For physiological concentrations in mice, a range of 18–36 μg/mL of estradiol in sesame oil within a silastic capsule was recommended. Therefore, we opted for a silastic implant containing estradiol to maintain the estrogen concentration at a physiological level.

This study aimed to utilize a stable delivery system for estrogen to investigate whether administering concentrations closer to physiological levels, lower than those used in previous studies, can still enhance flap survival.[15–17,21,22] We used a subcutaneous estrogen silastic capsule for a mice model of a random pattern skin flap to investigate its impact on blood perfusion, angiogenesis and prevention of cell death during wound healing. Our findings suggest that appropriate estrogen supplements might enhance flap transplantation recovery and help avoid the potential side effects associated with high dosage of estrogen administration.

2 | MATERIALS AND METHODS

2.1 | Ovariectomized animal model

Eighteen female BALB/c (weight, 16–18 g; age, 9 weeks old) was purchased from Bio-LASCO Co., Ltd. (Taipei City, Taiwan). This animal study was performed with the approval of the appropriate Animal Care and Use Committee (IACUC-20-215). After a 1-week acclimatization period in the animal centre, the mice underwent bilateral ovariectomies to induce a menopausal state[28] before the start of the study. To minimize potential confounders, we assigned unique numbers to the tails of the mice and corresponding cage cards. Postoperatively, the mice received intramuscular injections of ketoprofen (2.5 mg/kg; Astar, Hsinchu, Taiwan) and cefazolin (25 mg/kg; China Chemical & Pharmaceutical, Taipei City, Taiwan) for 7 days. On the 28th day after oophorectomy, all OVX mice were randomized into three groups for the dorsal skin flap model ($n = 6$ mice/group): control (C), sesame oil capsule (SC) and estradiol (E2) capsule. Skin samples will not be included if they show indications of infection or have been bitten by mice; however, there were no exclusions in this study. This work has been reported in accordance with the ARRIVE (Animals in Research: Reporting In Vivo Experiments) guidelines.[29]

2.2 | In vivo delivery of estrogens using silastic tubing

Silastic implants containing estradiol (E8875; Sigma-Aldrich, St. Louis, MO, United States) were prepared as described previously.[30] Two-centimetre segments of silastic tubes (inner/outer diameter: 1.57/3.57 mm) (Instech Laboratories, Paolo Alto, United States) were used as capsules. These capsules contained sesame oil (S3547; Sigma-Aldrich, St. Louis, MO, United States) with or without estradiol at 25 μg/mL. Before the implant surgery, all capsules were incubated in sesame oil with or without estradiol at 37°C overnight. The capsules were then implanted under the right flank of mice in the SC and E2 groups.

2.3 | Random pattern skin flap in mice

The random pattern skin flap surgery was performed 7 days after silastic capsule implant surgery, as described in a previous study with mild modifications.[31,32] A 3-sided skin flap (length: 3.0, width: 1.0 cm) was created on each mouse from caudal to cranial, and wound edges were sealed with a 3M™ vetbond™ tissue adhesive (3 M, St Paul, MN, United States). Skin flap wounds were photographed every 3 days, and blood perfusion in the island flap was analysed on day 7 using a laser speckle contrast image system (Moor FLPI-2, Moor Instruments Ltd., Axminster, United Kingdom). On day 9, six mice from each group were euthanized, and the wound flap and surrounding healthy areas were harvested for sectioning and histological staining.

2.4 | Haematoxylin–eosin staining

Haematoxylin–eosin (H&E) staining was conducted to assess the necrotic area and inflammatory cell infiltration. Microscopic examination revealed that necrotic areas exhibited characteristics of tissue swelling, anucleated cells and eosinophilic-like cells (cells appearing pink), while inflammatory cells were identified by small, darkly stained nuclei surrounded by pale-staining cytoplasm. All sections were analysed using a whole-slide scanning system (AxioScan Z1; Zeiss) and Zen software to delineate and quantify necrotic areas.

2.5 | Immunohistochemistry stain

Harvested skin flap tissues were fixed in 4% paraformaldehyde. Paraffin-embedded tissue sections (5 μm) were used for tissue staining as previously described.[33] Sections were blocked in goat serum (S-1000, Vector Laboratories, Inc., California, United States) and incubated with primary antibody overnight at 4°C. The following primary antibodies were used for immunohistochemistry (IHC): rabbit anti-hypoxia-inducible factor 1 alpha (HIF-1α) (#PAI-16601, 1:500, Invitrogen), mouse anti-CD31 (#3528, 1:500, Cell Signalling Technology) and rabbit anti-*cleaved caspase 3* (GTX86952, 1:500, GenTex). After removing the immersion solution and washing thrice with phosphate-buffered saline, the sections were incubated with a secondary antibody containing biotin (BA-1100, BA-2000; Vector Laboratories, Inc., California, United States) for 1 h. After washing, the sections were soaked in an Avidin-biotin complex kit solution (#PK-6100, Vector Laboratories, Inc., California, United States) for 1 h and then incubated with diaminobenzidine solution (#D5637; Sigma-Aldrich, St. Louis, Missouri, United States) for 10 min. All tissue sections were counterstained with haematoxylin and imaged using a whole-slide scanning machine (AxioScan Z1; Zeiss). ImageJ software was used to analyse protein expression in the images; the quantified data were calculated to the related fold.

2.6 | Terminal dUTP nick end-labelling assay

Terminal dUTP nick end-labelling (TUNEL) staining was used to detect apoptotic cells in the skin flap tissue. After dewaxing, rehydrating and removing endogenous peroxidase, the TUNEL staining was performed following the manufacturer's instructions (ab206386, Abcam). Subsequently, all sections were observed using a whole-slide scanning machine (AxioScan Z1; Zeiss).

2.7 | Statistical analyses

All the descriptive statistics are expressed as means and standard deviations (SD). The one-way analysis of variance (ANOVA) and the Tukey's multiple comparisons test were used to calculate statistical differences with p-values of <0.05 considered significant. Data were analysed using Microsoft Excel (version 16.43; Microsoft, Redmond, Washington, United States) and GraphPad Prism (version 5.0; GraphPad Software, San Diego, California, USA).

3 | RESULTS

3.1 | OVX mice had lower blood perfusion and estradiol levels after random pattern skin flap surgery

After random skin flap surgery, blood perfusion of the flap in each OVX mouse was measured using a MoorFLPI-2 blood flow imager (LSCI) on postoperative days 1–7. LSCI images depicted skin blood perfusion levels, where blue and red represented low and high blood perfusion, respectively. On postoperative day 1, the random skin flap had lower blood perfusion. However, blood perfusion in the skin flap started to increase on postoperative days 2–7 (Figure 1A). We collected blood samples using submandibular venipuncture to ensure that blood estradiol levels were reduced in OVX mice and recovered by estradiol capsule implantation.[34,35] The serum estradiol levels of the normal group (blood collected before oophorectomy), OVX group (blood collected 28th day after oophorectomy) and OVX mice

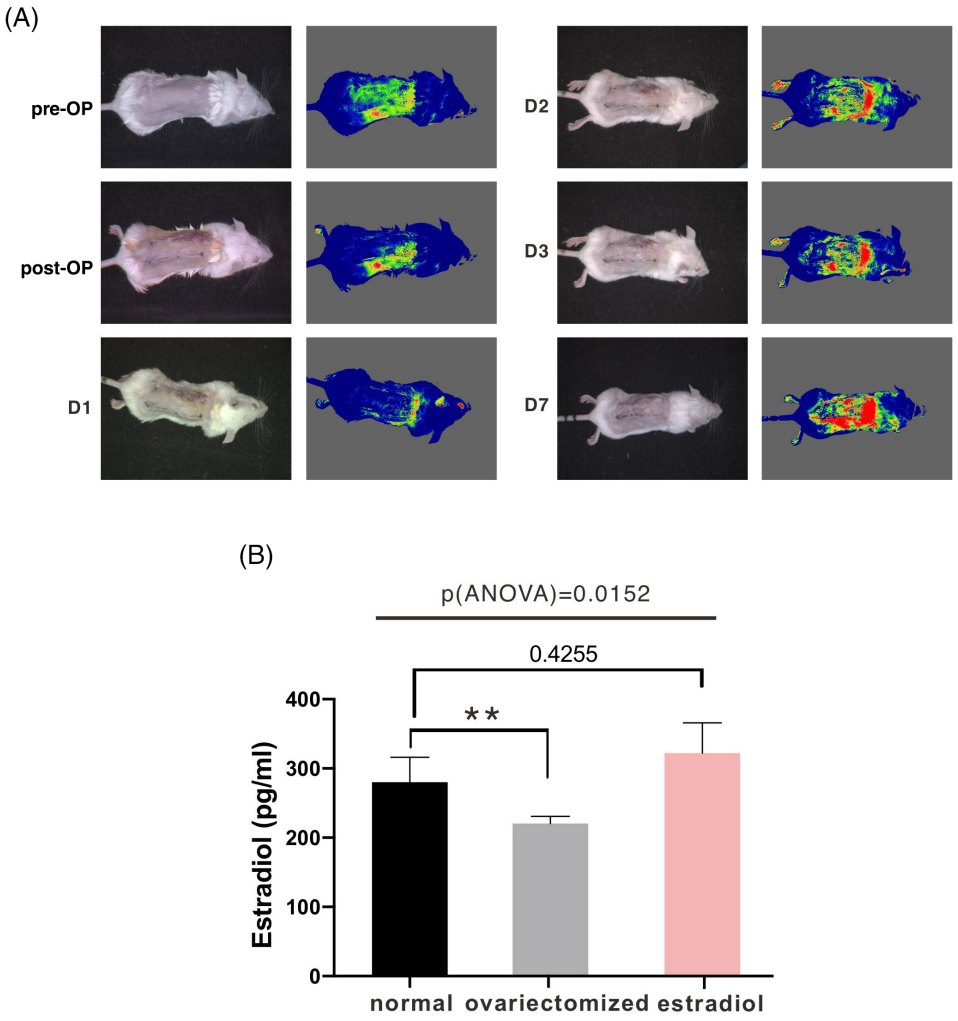

FIGURE 1 Superficial blood perfusion in murine skin and serum estradiol level after oophorectomy. (A) LSCI images of superficial blood perfusion in murine skin after oophorectomy. Pre-OP—preoperative day; post-OP—D1–D7 postoperative days 1–7. (B) Estradiol level in different groups. Data are presented as the mean ± standard deviation, $n = 6$ mice per group, $^{**}p < 0.01$.

that received estradiol capsules for 7 days were 280 ± 30 pg./mL, 220 ± 10 pg./mL and 320 ± 44 pg./mL, respectively. The estradiol level in the OVX group was significantly lower than that in the normal group ($p = 0.0030$). Conversely, OVX mice receiving estradiol capsules had significantly higher estradiol levels than the OVX groups ($p = 0.0098$). Additionally, no significant difference was observed between estradiol levels in the control and estradiol groups ($p = 0.4255$) (Figure 1B).

3.2 | Estradiol capsules reduced necrosis of random skin flap tissue in OVX mice

Failure to promptly restore microvascular circulation can lead to ischemic necrosis originating from the marginal regions of the flap tissue.[1] Three days after random skin flap surgery, the flap was discoloured in some areas (control: 40.5 ± 5.6%, SC: 38.2 ± 7.6% and E2: 32.8 ± 5.8%), no significant difference was observed between groups ($p = 0.1077$). On days 7 and 9, the black (necrotic) and reddish brown (cyanotic) discoloured areas in the control, SC and E2 groups were 41.714.5%, 52.7 ± 7.5% and 40.6 ± 8.6% on days 7, and 56.0 ± 8.8%, 43.2 ± 10.5% and 38.0 ± 8.7% on days 9, respectively. The discoloured area in the E2 group was significantly smaller than that in the control ($p = 0.0095$ on day 7 and $p = 0.0032$ on day 9) and SC ($p = 0.0344$ on day 7) groups (Figure 2A,B). The H&E staining of random skin flaps on D9 revealed that the E2 group had fewer necrotic areas than the other groups (control: 71.5 ± 19.3%, SC: 55.0 ± 9.6% and E2: 32.9 ± 8.6%). Significant differences were observed between the groups ($p = 0.0011$ compared with the control group and $p = 0.0310$ compared with the SC group) (Figure 2C,D). These findings indicate that E2 reduced necrosis in random skin flaps.

3.3 | Estradiol capsules improved the blood perfusion of cutaneous flap tissue in OVX mice

According to the LSCI images, the E2 groups had increased blood perfusion in the random skin flaps on

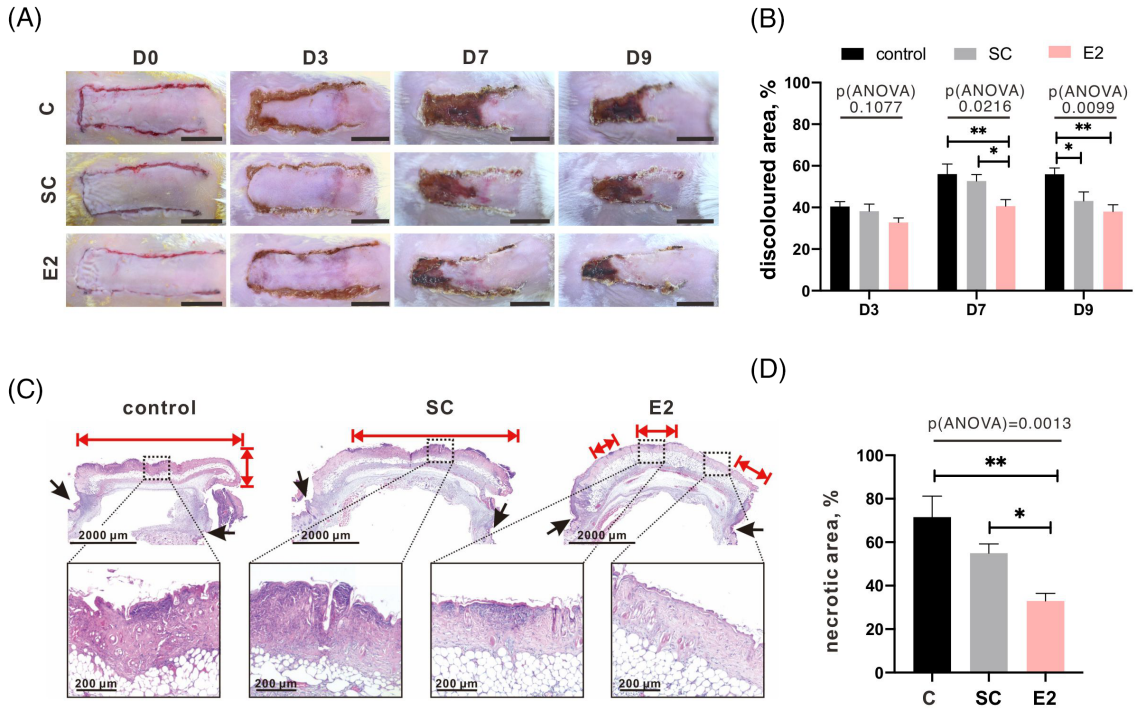

FIGURE 2 The effect of estrogen on the wound area in random skin flap wound healing. (A) Photographic images of random skin flap in OVX mice. D0: Random skin flap surgery day, D3/D7/D9: 3/7/9 days postoperatively (scale bar = 1 cm). Control (C), sesame oil capsule (SC) and estradiol (E2) (B) Quantitative data of the discoloured area, data are presented as the mean ± standard deviation, $n = 6$ mice per group, $^{*}p < 0.05$, $^{**}p < 0.01$. (C) Haematoxylin and eosin staining of random skin flaps on D9. Red double arrows indicate the necrotic area. (D) Quantitative data of the necrotic areas in haematoxylin and eosin staining data are presented as the mean ± standard deviation, $n = 6$ mice per group, $^{*}p < 0.05$, $^{**}p < 0.01$.

day 7 compared with the control or SC groups (Figure 3A). Quantitative analysis revealed that the ischemic area of the E2 group was significantly smaller than that of the SC group (control: 7143 ± 2228 pixel, SC: 7046 ± 3351 pixel and E2: 3226 ± 855 pixel, $p = 0.0308$ and 0.00354 compared with the control and SC groups, respectively) (Figure 3B). Superficial blood perfusion was increased in the E2 group than in the SC group (control: 237 ± 75, SC: 241 ± 69 and E2: 348 ± 71, $p = 0.0393$ and 0.0471 compared with the control and SC groups, respectively) (Figure 3C).

3.4 | The effect of estradiol capsules on tissue hypoxia, angiogenesis and fibroblast differentiation in cutaneous flap tissue

We further investigated whether estradiol regulated blood flow by increasing angiogenesis because of the upregulated blood flow in the E2 group. Hypoxia is accompanied by HIF-1α expression and is closely associated with angiogenesis. It is also an upstream regulator of *vascular endothelial growth factor (VEGF) mRNA*.[36] As an angiogenic biomarker, CD31 detects new capillaries or vessels.[37] We detected the protein expression of HIF-1α using IHC staining and quantified it to a related fold. On day 9, the E2 group displayed less HIF-1α protein expression in the dermis (control: 1 ± 0.06, SC: 0.97 ± 0.05 and E2: 0.89 ± 0.05) and subcutaneous fascia (control: 1 ± 0.18, SC: 0.92 ± 0.30 and E2: 0.96 ± 0.05) of the flap. Furthermore, the dermis of the E2 and control groups significantly differed ($p = 0.0088$ compared with the control group, $p = 0.0491$ compared with the SC group). However, HIF-1α in the subcutaneous fascia did not significantly differ among the control, SC and E2 groups ($p = 0.7979$) (Figure 4A,B). We determined the number of blood vessels in the dermis via CD31 IHC staining (control: 33.2 ± 14.4, SC: 26.0 ± 10.0 and E2: 27.3 ± 11.3). However, no significant differences existed among the control, SC and E2 groups ($p = 0.5869$) (Figure 4C,D).

Fibroblast proliferation and extracellular matrix are crucial for wound healing. Estrogen can increase the proliferation of fibroblasts and the synthesis of collagen, TGF-β and IGF-1.[12] Estradiol capsules accelerated wound healing in the skin flap model. We chose alpha–smooth muscle actin (α-SMA) to further understand the mechanism of estradiol in wound healing, which is a kind of fibroblast differentiation biomarker, to target myofibroblasts, which

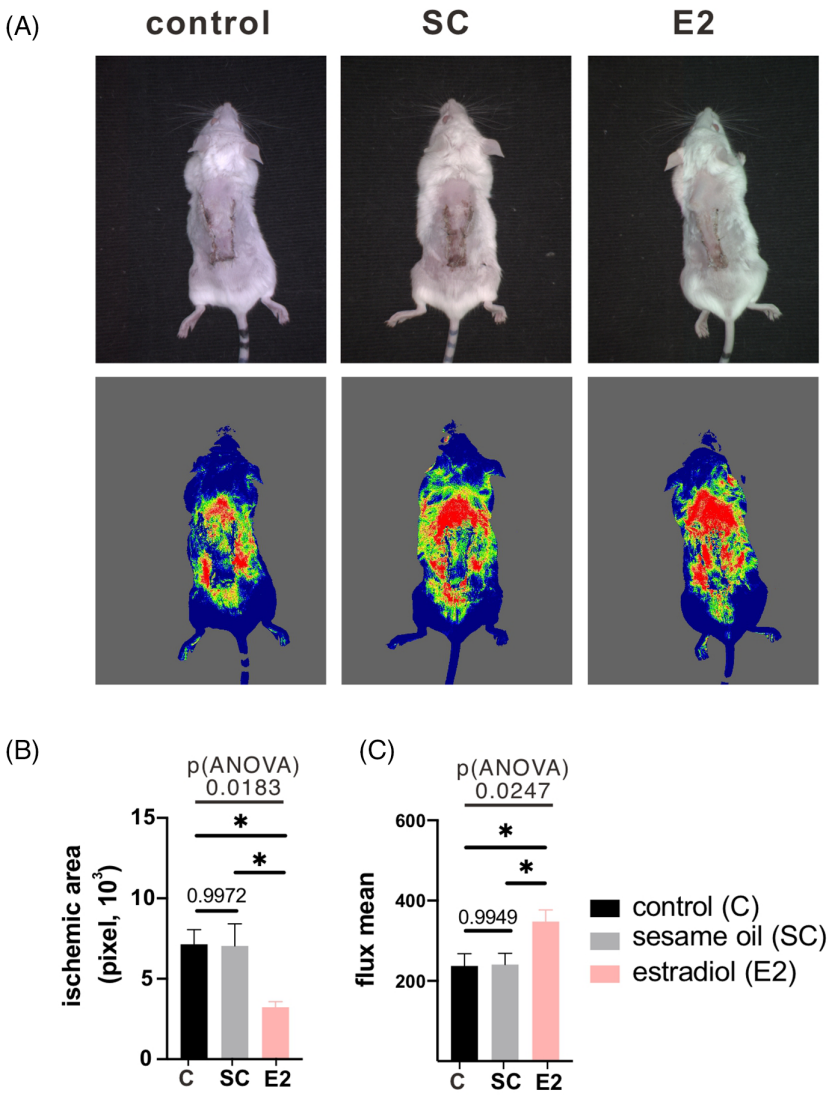

FIGURE 3 The effects of estrogen on the ischemic area and superficial blood perfusion in random skin flap wound healing. (A) LSCI images of the control, sesame oil (SC) and E2 groups on day 7. (B) Quantitative data of the ischemic area in the control, SC and E2 groups. Data are presented as the mean ± standard deviation, $n = 6$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$. (C) The superficial blood perfusion in the control, SC and E2 groups. Data are presented as the mean ± standard deviation, $n = 6$ mice per group, $^{*}p < 0.05$.

often appear in healing wounds or scar tissue. On day 9, the E2 group had less α-SMA protein expression related fold in the centre (control: 1 ± 0.37, SC: 0.80 ± 0.24 and E2: 0.71 ± 0.14) and edge (control: 1 ± 0.34, SC: 0.84 ± 0.43 and E2: 0.61 ± 0.13) of the flaps. However, there were not significant different among groups (centre: $p = 0.1841$, edge: $p = 0.1539$) (Figure 4E,F).

3.5 | Estradiol capsules reduced apoptosis in the random skin flap tissue

Cell death involves apoptosis and necrosis. In laboratory, TUNEL assay detects DNA breaks during DNA fragmentation in the last apoptosis phase. Hence, we used a TUNEL assay to monitor apoptosis in the skin flap model. On day 9, the E2 group had fewer TUNEL-positive cells than the other groups in the dermis and subcutaneous fascia (Figure 5A). We measured the expression of cleaved caspase-3 using IHC staining to further investigate the effect of estradiol on the cleaved caspase-3 protein that is activated during apoptosis induction. The E2 groups had less expression of cleaved caspase-3 (clv-caspase-3) protein in the subcutaneous fascia than the other groups (control: 1 ± 0.03, SC: 0.98 ± 0.03 and E2: 0.86 ± 0.04), and the control, SC and E2 groups significantly differed ($p < 0.0001$) (Figure 5B,C). These data support the notion that estradiol can reduce apoptosis in skin flaps.

4 | DISCUSSION

In this study, we demonstrated that the estrogen silastic capsule elevated estrogen to physiological levels in OVX mice, which increased superficial blood perfusion, reduced tissue ischemic area and resulted in decreased tissue necrosis of the random pattern skin flap.

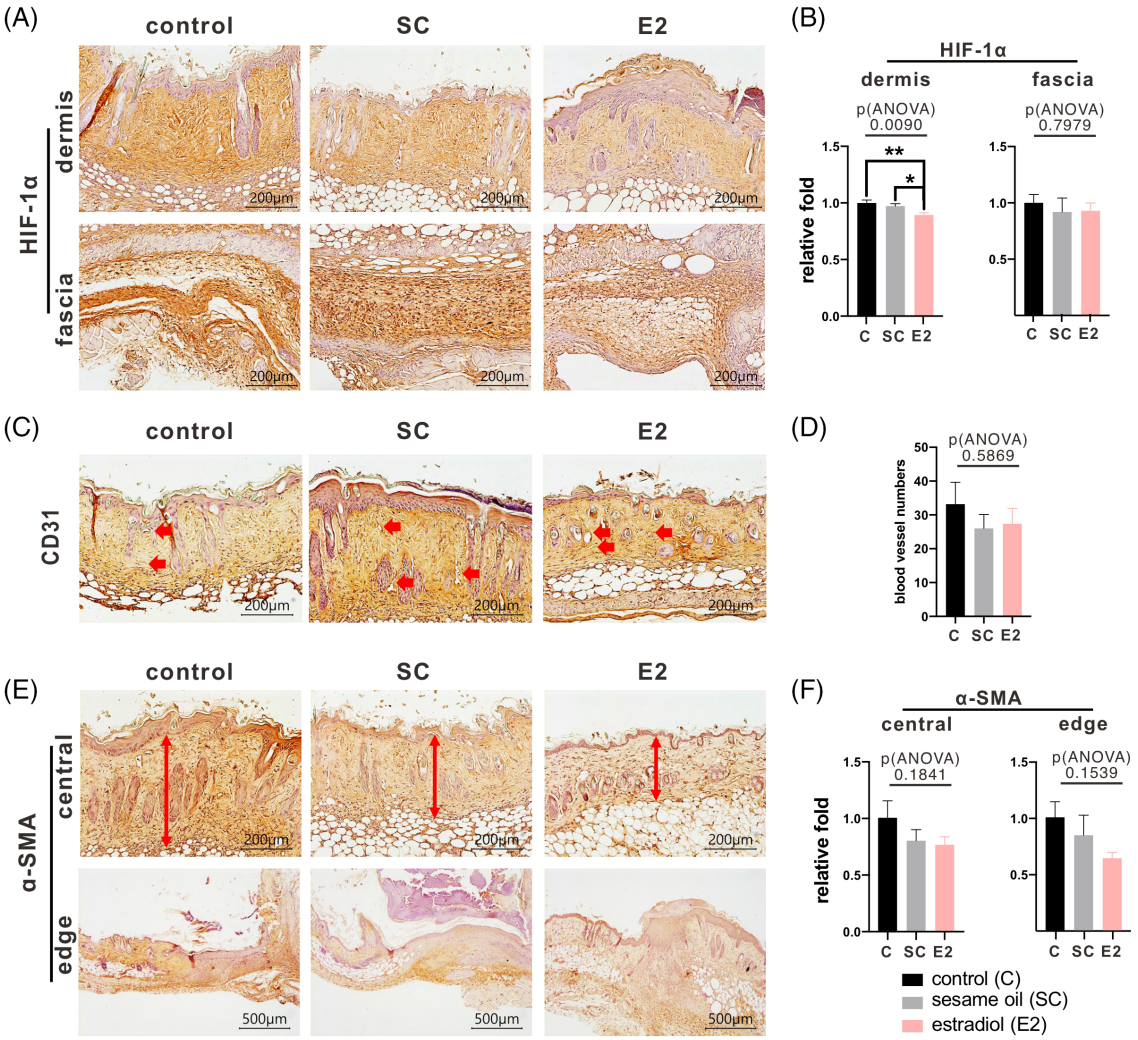

FIGURE 4 The effects of estrogen on the distribution of HIF-1α, CD31 and α-SMA in random skin flap wound healing. The photomicrograph of immunohistochemical staining and protein expression quantitative data for (A, B) HIF-1α, (C, D) CD31 (red arrow indicates the blood vessel) and (E, F) α-SMA (red double arrowhead represents the dermal thickness) revealed in the control, sesame oil (SC) and E2 groups. Data are presented as the mean ± standard deviation, $n = 6$ mice per group, $^{*}p < 0.05$, $^{**}p < 0.01$.

Our results revealed that the positive effect of estrogen silastic capsules on superficial blood perfusion and decreased tissue necrosis were consistent with findings from studies involving estrogen pellets in ischemic skin flaps,[15] intraperitoneal injection of estrogen following flap ischemia–reperfusion injury in male Wistar rats,[16,17] and research examining factors associated with necrosis after single-digit replantation in 946 patients.[38] In female patients, estrogen levels are lower during the menstrual period and menopause, which markedly influenced the digital necrosis rate. This was because the blood was in a state of hypercoagulability during the menstrual period.[38] Furthermore, estrogen induces the production of nitric oxide, which facilitates vasodilation and enhances capillary perfusion.[39–41] This may explain the increased flap perfusion in the presence of estrogen.

Our data showed that estrogen decreased HIF-1α protein expression in a mice random pattern skin flap model, consistent with previous research indicating estrogen's potential to downregulate HIF-1α expression during tissue regeneration.[42] HIF-1α could induce DNA damage and cell death,[43] which is caused by cleaved caspase-3 activation.[44] Consistently, our data revealed that estrogen silastic capsules reduced cell death via the analysis of TUNEL-positive cells and cleaved *caspase-3* protein, consistent with the status of HIF-1α in our model.

Estrogen enhances angiogenesis by promoting endothelial cell proliferation and migration.[39] Mice with skin flap necrosis treated with higher concentrations of estrogen exhibited increased expression of growth factors such as FGF-2 and VEGF, which promote vascular endothelial cell proliferation.[15] Sex hormones are crucial in sexuality

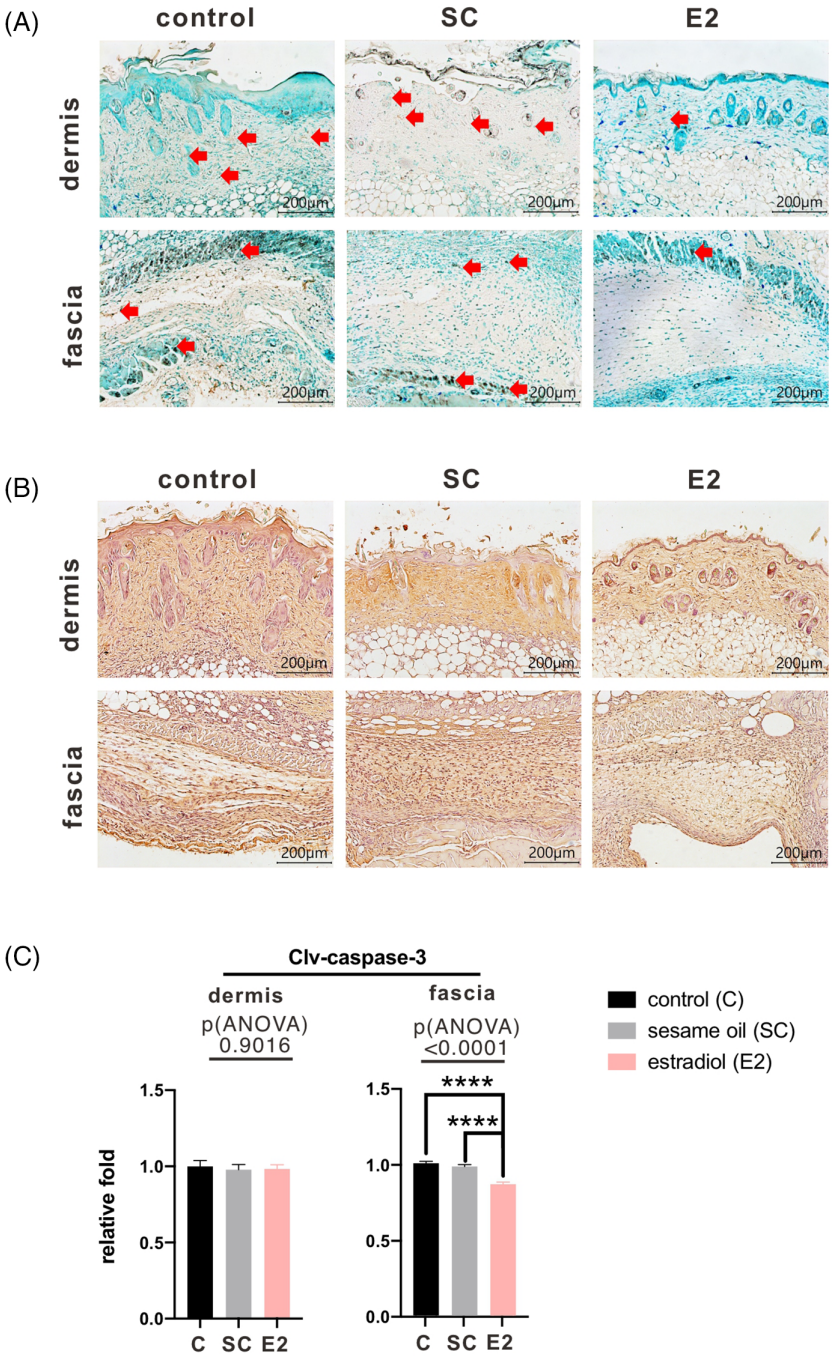

FIGURE 5 The effects of estrogen on apoptosis and clv-caspase-3 in random skin flap wound healing. (A) TUNEL stain of the random skin flaps on D9. Red arrows indicate the TUNEL expression in the dermis or fascia of the flap of the control, sesame oil (SC) and E2 groups. (B) The photomicrograph of immunohistochemical staining for clv-caspase-3. (C) Quantitative data of clv-caspase-3 protein expression in dermis and fascia. Data are presented as the mean ± standard deviation, $n = 6$ mice per group, $^{****}p < 0.0001$.

and fertility and influence wound healing and finger replantation. Estrogen exerts a protective effect on blood vessel lining.[45,46] Numerous studies have demonstrated that estrogen is essential in wound healing by promoting angiogenesis, fibroblast differentiation and collagen synthesis.[47–49] However, in this study, the number of CD31-positive cells in the dermis and superficial fascia did not increase following estradiol treatment. Additionally, the protein expression of α-SMA, a marker of fibroblast activation, did not show significant differences among the groups. It is possible that the physiological concentration of estrogen cannot induce angiogenesis to the same extent as higher concentrations. In the future, different concentrations of estrogen may be administered to mice ischemic flap models to find an effective and safe dosage to promote angiogenesis in the ischemia wound.

Recent studies suggest that estrogen may act according to hormetic principles in conditions such as cerebral ischemia, inflammation, cardiovascular diseases and anxiety. At physiological levels, estrogen offers protection, but when present in higher concentrations or for extended periods, it can have detrimental effects.[19] Previous studies primarily utilized different estrogen doses (one dose and three doses) and delivery pathways

(estradiol pellets releasing estradiol 80 and 200 μg/kg/day), mediated through the p38 mitogen-activated protein kinase signalling pathway and the vascular network for preventing skin flap necrosis in rat and mouse models.[15–17,21,22] However, these studies resulted in elevated serum hormone levels beyond projections and inconsistent hormone delivery. Estrogen can downregulate its receptors at very high doses and activate other nuclear receptors, activating a completely different set of genes compared with the physiological concentration range.[50,51] The classical pathway of estrogen is the nuclear estrogen receptors α and β (ERα and ERβ) or potentially mediated by membrane-bound receptors, such as GPR30.[52–54] Different subsets of membrane receptors can result in non-monotonic dose–response relationships.[19,20]

Different estrogen delivery systems for animals include routine injections, oral gavage, commercially prepared pellets and implantation of silicone capsules. Silicone capsules used for hormone delivery are acknowledged for their stability and are commonly utilized in various research applications.[24,25] This study explores the use of silicone capsules for estrogen delivery to stabilize estrogen levels, bringing them closer to physiological levels, which may have a beneficial impact in reducing the aforementioned negative systemic effects. Our findings support that the estrogen silicone capsule could stably elevate estrogen levels in OVX mice and benefit wound healing of skin flaps. However, the complexity of its target receptors is mediated by genomic and non-genomic effects on the regulation of target genes involved in wound healing. A more direct analysis, such as RNA-sequencing and chromatin-immunoprecipitation strategies, might reveal the working mechanism of estrogen at each stage of the wound healing process in our skin flap model.

5 | CONCLUSION

This study showed that the estrogen silastic capsule increased superficial blood perfusion, leading to improved tissue hypoxia and reduced cell necrosis in a mouse random pattern skin flap model. Administering estrogen at a lower concentration to maintain physiological levels might enhance flap survival and reduce side effects compared with the higher concentrations of estrogen supplements.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

Data associated with the publication are available upon request by the corresponding author.

ETHICAL STATEMENT

All animal experimental procedures were reviewed and approved by the Experimental Animal Committee of the National Defense Medical Center (Application Form No. IACUC-20-215).

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