Investigation of biological and wound healing effects of Estrogen solution: An in vitro study

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Research Article

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Abstract

Background

Repairing dermal skin defects denotes a challenging obstacle in wound healing. Wound healing activities of estrogen have been noted in many experimental models proposing their beneficial role in wound closure and treatments of impaired wound healing. To study the most significant problem in dermal defect regeneration, namely collagen formation and insufficient blood supply, this study aimed to evaluate different concentrations of estrogen in the *co-culture* of fibroblast and endothelial cells.

Methods

The human fibroblast (C163) and Human umbilical vein endothelial cells (HUVEC) were *co-cultured* and treated with different concentrations of estrogen solution. The cytotoxic effect of estrogen solution was evaluated by MTT assay while expression of endothelial markers (CD31) and Vimentin in treated cells was examined using Real-time PCR and Immunofluorescence analysis. Wound healing capacity in human fibroblast cells was studied by a scratch test assay.

Results

Estrogen has a dose-dependent proliferation effect on C163 and HUVEC *co-culture* cells with a significant growth inhibition at concentrations higher than 75 ng/ml concentration. We demonstrated that estrogen increased the growth, proliferation, and migration of C163 and HUVEC *co-culture* cells, accordingly, cell viability and scratch tests. C163 and HUVEC *co-culture* cells were cultured by estrogen treatments, which also improved the expression of the CD31 and Vimentin markers.

Conclusions

These results provide further insight into the function of biological agents in the wound healing process and may have significant approaches for the use of estrogen in skin wound healing.

Introduction

Given the different deficiencies of present treatment choices, there is a crucial need to recognize novel targets and design new approaches to inhibit the issue of impaired skin wound regeneration [1]. The human skin plays a vital role in various processes such as protection from chemicals and pathogens, excretion, vitamin D synthesis initialization, and hydration [2]. In the skin, the dermis layer contains reticular, elastic, collagen fibers, mesenchymal stem cells, fibroblasts, endothelial cells and extracellular matrix proteins. The basement membrane isolates the dermis from the epidermis and modifies epithelial-mesenchymal interactions [3]. Wound healing is a complicated mechanism that applies a variety of

proliferation and migration of dermal and epidermal cells at the wound area [4]. Fibroblast cells are present in the wound healing area, from the inflammatory stage until full epithelialisation has emerged. They are migrated to the injury site, proliferate and lead to several key activities under the regulation of wound-mediated factors and the changing microenvironment of the healing wound, which is crucial to the end stage of the wound [5]. Concerning the skin wound process, the remodelling stage occurs from a few days after damage to several months [6]. Angiogenesis is an important component in many physiologic processes. Angiogenesis which resulted in wound regeneration supplies the nutritive and oxygen needs of the wounded tissue and plays an important role in clearing the waste materials of metabolism. Some agents associated with wound regeneration including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin, angiopoietin-1 and vascular permeability factor (VPF) could act as the inducer and/or regulators for wound healing [7]. Fibroblast and endothelial cells play a critical role in the precipitation of collagen and angiogenesis in the dermal wound regeneration process [8]. These cells are affected by their microenvironmental factors, such as morphogens, extracellular matrix composition, and circulating cytokines [9]. The wound-related impaired changes in the regenerations of dermal skin can be removed by topical estrogen application [10]. Estrogen is a steroid hormone which is comprehended to be useful in promoting the wound-healing process in different ways [11]. The effect of estrogen on dermal wound healing is studied in vivo models [12]. Many investigations reveal that estrogen is crucial in wound healing. It has been indicated that estrogen deficiency is associated with impaired wound healing or delayed wound repair and cutaneous ageing [13, 14]. Estrogen is a crucial agent for the proliferation and migration of fibroblasts, which is mediated by the Estrogen Receptor α. Estrogen causes a prompt re-organization of the myosin, actin and Vimentin cytoskeletons in dermal fibroblasts to the activation of non-classical receptor PI3 K/Akt, MAPK, ERK1/2, and GPR30 [15, 16]. Estrogen increases the circulating endothelial progenitor cells, and these cells support the creation and repair of damaged and/or injured endothelium layer. Estrogen also enhances proliferation, viability and migration in endothelial cells [17, 18]. The purpose of the current investigation was to evaluate in vitro endothelial and fibroblast cells *co-culture* in wound closure capacities by the response to various concentrations of estrogen.

Methods

Cell co-culture and estrogen treatment

Human fibroblast (C163) and Human umbilical vein endothelial (HUVEC) *co-culture* cells were cultured in the presence of different concentrations of estrogen solution (50 ng/mL, 75 ng/mL, and 100 ng/mL in cell media). C163 and HUVEC *co-culture* cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, sigma) containing 10% fetal bovine serum (FBS, GIBCO), 100 IU/ml penicillin, and 100 IU/ml streptomycin (Sigma).

Cytotoxicity Assay

Cell viability effects of estrogen solution were studied by MTT assay. C163 and HUVEC cells (1×10^4 cells/well) were cultured in a 96-well tissue culture plate and incubated with different concentrations of estrogen solution (50, 75, 100 ng/mL) for 1, 3, and 5 d. The absorbance of each sample was detected by a spectrophotometric plate reader (Rayeto) at 570 nm. DMSO was used as a negative control in all groups [19].

Real-time Pcr

C163 and HUVEC *co-culture* cells were grown in 12-well plates (5×10^4 cells/well). Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Japan), following the manufacturer's instructions. mRNA levels of CD31 and Vimentin were determined using the SYBR1 Premix Ex TaqTM II Kit (Tli RNaseH Plus, Chin). β-actin was used as an internal housekeeping gene in *co-cultured* cells, and the mRNA relative expression of the CD31 and Vimentin genes was quantified by the $2^{-\Delta\Delta Ct}$ method [20]. The primers used for Real-Time PCR analysis are listed in Table 1.

Scratch Test Assay

C163 and HUVEC *co-culture* cells were seeded in 6-well plates (5×10^5 cells/well) in a total volume of 2000 µL per well. The cells were treated with mitomycin C (10 µg/mL in cell media). After 2 h, a single scratch was created in the confluent monolayer of *co-cultured* cells, using a 10 µL pipette tip. The cells were washed with PBS, and different concentrations of estrogen solution were added to each well. Images were taken 48 h later using an inverted microscope (Olympus, Japan) equipped with a microscope camera (Olympus, Tokyo). The images were investigated, and the cell-free area was analyzed using the ImageJ software. Results are presented as a scratched area cell migration at 48 h compared with 0 h time point for the *co-cultured* corresponding wells [21].

Immunofluorescence Analysis

C163 and HUVEC *co-culture* cells were cultured in 12-well plates (2×10^4 cells/m). After 5 d post-treatment with different concentrations of estrogen, *co-culture* cells were fixed with 4% (w/v in PBS) paraformaldehyde (Sigma-Aldrich) for 20 min. All samples were then permeabilized by 0.2% Triton X-100 solution (Sigma-Aldrich) for 15 min at room temperature and washed with PBS (two times, 5 min each), each well was blocked with 0.1% bovine serum albumin (BSA, Sigma-Aldrich) for 30 min at room temperature. After washing two times with PBS, the *co-culture* cells were incubated with either the primary antibodies against CD31 (mouse monoclonal anti-human antibody; Abcam, 1:200) and Vimentin (mouse monoclonal anti-human antibody; sigma, 1:200) overnight at 4 ∘C. Then, they were incubated with Alexa Fluor 594 Goat Anti–Mouse IgG (1:750) secondary antibody for 1 h at room temperature. Nuclei were counterstained with 49, 6-diamidino-2- phenylindole (DAPI, Sigma-Aldrich). In the negative control group, only the Alexa Fluor 594 Goat Anti–Mouse IgG (secondary antibody) was used. The images were

obtained through a Nikon inverted-fluorescence microscope (Japan) and the intensity of the fluorescent signal was quantified using Image J software (Ver 1.8.0) [22].

Statistical Analysis

GraphPad Prism software ver. 7.0 (San Diego, CA, USA) was performed for statistical analysis. Results were revealed as the mean ± standard deviation (SD). The data were analyzed using one-way ANOVA test, followed by post hoc Tukey's multiple comparisons test to analyse the significance of the differences between co-culture groups. All the experiments were performed in triplicate. The P values were considered significant at $P < 0.05$, $P < 0.01$, and $P < 0.001$.

Results

Cell co-culture and estrogen treatment

After 24 h, the *co-culture* of C163 and HUVEC cells adhered and proliferated in the bottom of the flask. Then, these cells were incubated with different concentrations of estrogen solution. After 5 d post-treatment, C163 and HUVEC *co-culture* cells maintained their spindle-shaped appearance and flattened (endothelial cell appearance) morphology, respectively (Fig. 1).

Cytotoxicity Assay

Since the proliferation of C163 and HUVEC cells has been correlated with a strong prognosis in the repair and regeneration of wound process, we first investigated the effect of different concentrations of estrogen on the viability of *co-cultured* C163 and HUVEC cells using the cell Cytotoxicity (MTT) test. To study cell survival, we treated the *co-culture* cells for 1, 3, and 7 d with different concentrations of estrogen. After 24 h, MTT results showed that C163 and HUVEC *co-culture* proliferated in the presence of estrogen solution. On days 3 and 5 of *co-culture*, treated samples with 50 ng/mL estrogen solution also showed increased cell proliferation but treated samples with 75 ng/mL estrogen solution indicated a higher cell viability rate and proliferation compared with other cell specimens (control and estrogen-treated sample). As shown in Fig. 2, the treatment group with estrogen solution increased cell proliferation in a dose-dependent manner.

Real-time Pcr

mRNA levels of the CD31 and Vimentin significantly increased when C163 and HUVEC *co-culture* cells were treated with different concentrations of estrogen solution. As shown in Fig. 3, mRNA expression of the CD31 and Vimentin increased significantly in estrogen-treated C163 and HUVEC *co-culture* cells with respect to control, whereas the treatment with 75 ng/mL estrogen solution greatly enhanced mRNA expression. These data indicate that *co-cultured* C163 and HUVEC cells are susceptible to estrogen treatment.

Scratch Test Assay

To perform the scratch test, using a scraping tool (10 µL pipette tip), a part of the monolayer of *co-culture* was cleared of cells. The application of different concentrations of estrogen promotes the division of *co-cultured* C163 and HUVEC cells that, together with cell migration, are the two mechanisms involved in wound healing repair. In the *co-culture* of C163 and HUVEC cells, mean percentage of closure of the scratch area was 65.67% when treated with 50 ng/mL estrogen solution and 70.03% when treated with 75 ng/mL estrogen solution and 54.64% when treated with 100 ng/mL estrogen solution, compared to 30.11% for non-treated group (control) after 48 h of culture was shown in Fig. 4A and B.

Immunofluorescence Analysis

C163 and HUVEC cells were *co-cultured*, and dose-dependent changes in CD biomarker expression profiles (CD31 and Vimentin) were examined. The increased expression of the markers CD31 and Vimentin confirmed the level of these biomarkers' expression. After 5 d post-treatment, immunofluorescence analysis indicated that fibroblast and endothelial biomarkers (Vimentin and CD31) were expressed in all samples. The increased protein expression of CD31 and Vimentin was revealed in treated *co-cultured* cells compared to the control cells (Fig. 5A and B).

Discussion

Considering the importance of fibroblast and endothelial cells in wound repair and regeneration, scientific investigation has revealed an increasing interest in regulating their influence on tissue wound closure [1]. In the current in vitro study, we evaluated the effects of different concentrations of estrogen solution on human fibroblast (C163) and Human umbilical vein endothelial cells (HUVEC) *co-culture* cells. Here we studied the effect of estrogen on viability, proliferation, migration and gene expression characteristics of *co-cultured* cells. Fibroblast cells exert a complementary function to endothelial cells, because the fibroblast itself synthesis the main fibers (collagen, elastic, and reticular fibers) of connective tissue, thereby actively participating in the precipitation of collagen, promotion of angiogenesis, and regeneration of wound process [23]. Therefore, enhanced gene expression in fibroblast and endothelial cells could be used as a therapeutic approach for increasing the wound healing rate, such as the dermal wound healing process [24]. This investigation is the first to study estrogen treatment in *co-culture* of C163 and HUVEC cells in vitro. The epithelial-mesenchymal transition (EMT) is a complicated process that affects various phenotypic modifications, leading to enhanced migration properties of cells [25, 26]. During the EMT, migrating cells are activated to substitute epithelial-related genes with mesenchymal-related ones, resulting in the loss of epithelial markers such as E-cadherin, apical-basal polarity and cell-cell adhesions [27]. Our results denoted that the EMT of migrating cells was enhanced by estrogen treatment of C163 and HUVEC *co-culture* cells (Figs. 1, 3 and 5). Vimentin has been comprehended to play a critical function in cell migration [28]. Vimentin influences healing by regulating fibroblast proliferation, TGF-β1 signaling, collagen accumulation, and epithelial-mesenchymal transition processing

[29]. The frequently used mechanism to study endothelial and fibroblast cells gene expression is the evaluation of expression of CD31 and Vimentin, which is performed with Real-time PCR technique for detection and quantification of mRNA upregulation in investigated cells [30]. CD31 acts as an angiogenesis biomarker that attributes to the endothelial cells during the wound remodelling phase. The increased predominance of CD31 was demonstrated in the CA and sham groups compared to that in the COL groups and control. The CD31 quantitative chart showed that the staining in the sham group was greatly higher than that in the collagen-treated and control groups [31]. In the presence of estrogen, *co-culture* cells (C163 and HUVEC cells) expressed Vimentin (intermediate filament, a fibroblast marker) and CD31 (endothelial marker) (Fig. 3). Fibroblasts present high levels of Vimentin, which is an important intermediate filament in cell migration and wound closure because it induced epithelial formation [32]. Many studies have shown that the communication between CD31, proliferation and angiogenesis generates a microenvironment suitable for wound healing [33]. Thus, we observed that estrogen treatment induced endothelial cell proliferation, indicating that estrogen contained factors able to induce cell proliferation. Therefore, our findings indicated that the presence of Vimentin was increased by estrogen, indicating that estrogen exerted a migration activity, which is associated with fibroblast, cell playing a wound closure function, thereby raising wound healing (Fig. 4). Wounded skin samples including transplanted hair follicles were studied for fluorescence microscopy. The images revealed that endothelial cells grow from the sample toward the wound. Vessels developing in the follicle reacted to angiogenic signals arising from the injury area. Immunohistochemical staining indicated that CD31 was expressed in the new vessels developed into the wound [34]. In this study, we indicated that estrogen remarkably increased the viability of C163 and HUVEC *co-culture* cells in a dose-dependent manner and enhanced the genetic expression of Vimentin and CD31 especially expressed by fibroblast and endothelial cells, respectively (Fig. 5). As far as the C163 and HUVEC cell-specific CD markers are concerned, our results indicated that the estrogen used in our investigation could improve the expression of biomarkers in vitro (Fig. 5). Consistent with these data, we revealed that fibroblast cells showed an increase in cell viability when *co-cultured* with HUVEC cells and this increase was improved by the treatment with estrogen. The data of MTT test showed that treated samples with 75 ng/mL estrogen solution are a more suitable treatment than other cell *co-culture* for cell attachment and proliferation (Fig. 2). Hence, the experimental conditions of our investigation could be responsible for the presence of the estrogen effect on Vimentin and CD31 expression (Figs. 2, 3 and 5). To acquire additional data about the effects of estrogen, we also showed that Vimentin increased fibroblast-induced cell migration (Fig. 4). Accordingly, on our results, revealing the raising of C163 and HUVEC *co-culture* cells associated CD markers induced by estrogen, we hypothesize that it denotes the observed stimulatory effect on enhancing the CD markers by increasing the production of factors applied in the communications between fibroblast and endothelial cells (Fig. 5).

Conclusion

Our results revealed for the first time that estrogen influence the *co-culture* in C163 and HUVEC cells, by increasing the viability, proliferation and gene expression. Estrogen upregulates the expression of wound

healing-related markers. So, estrogen can be suggested for proliferation and improved function of fibroblast and endothelial cells and would be a suitable material for dermal wound healing. However, further investigations addressing the detailed analysis of the signaling mechanisms of *co-culture*, as well as in animal models, will support the provision of a clarified picture of this material.

Declarations

Conflict of interest

The authors report no conflicts of interest.

Acknowledgment

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Compliance with Ethical Standards

Funding

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Authors' contribution

“All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Dr. Atefeh Shamosi and Dr. Alireza Shams. The first draft of the manuscript was written by Dr. Atefeh Shamosi, Dr. Alireza Shams and Dr. Negar Nejati. All authors read and approved the final manuscript.”

Statements & Declarations

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Table

Table 1 is available in the Supplementary Files section.

Figures

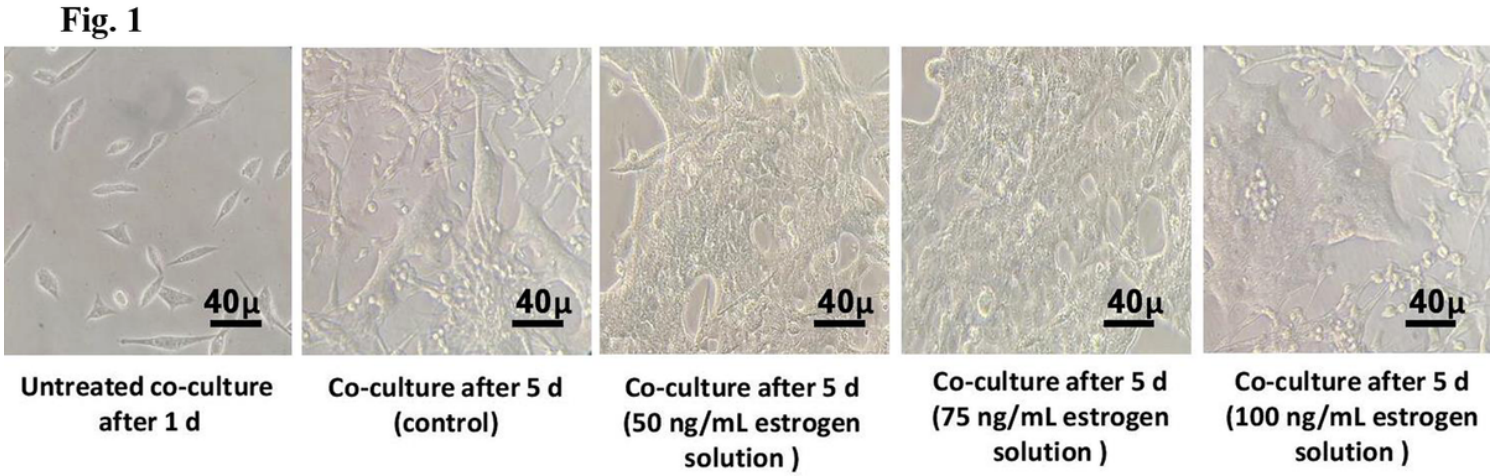

Figure 1

Morphological characterization of C163 and HUVEC co-culture cells. Phase-contrast images of co-culture cells before and after treatment with different concentrations of estrogen on day 5.

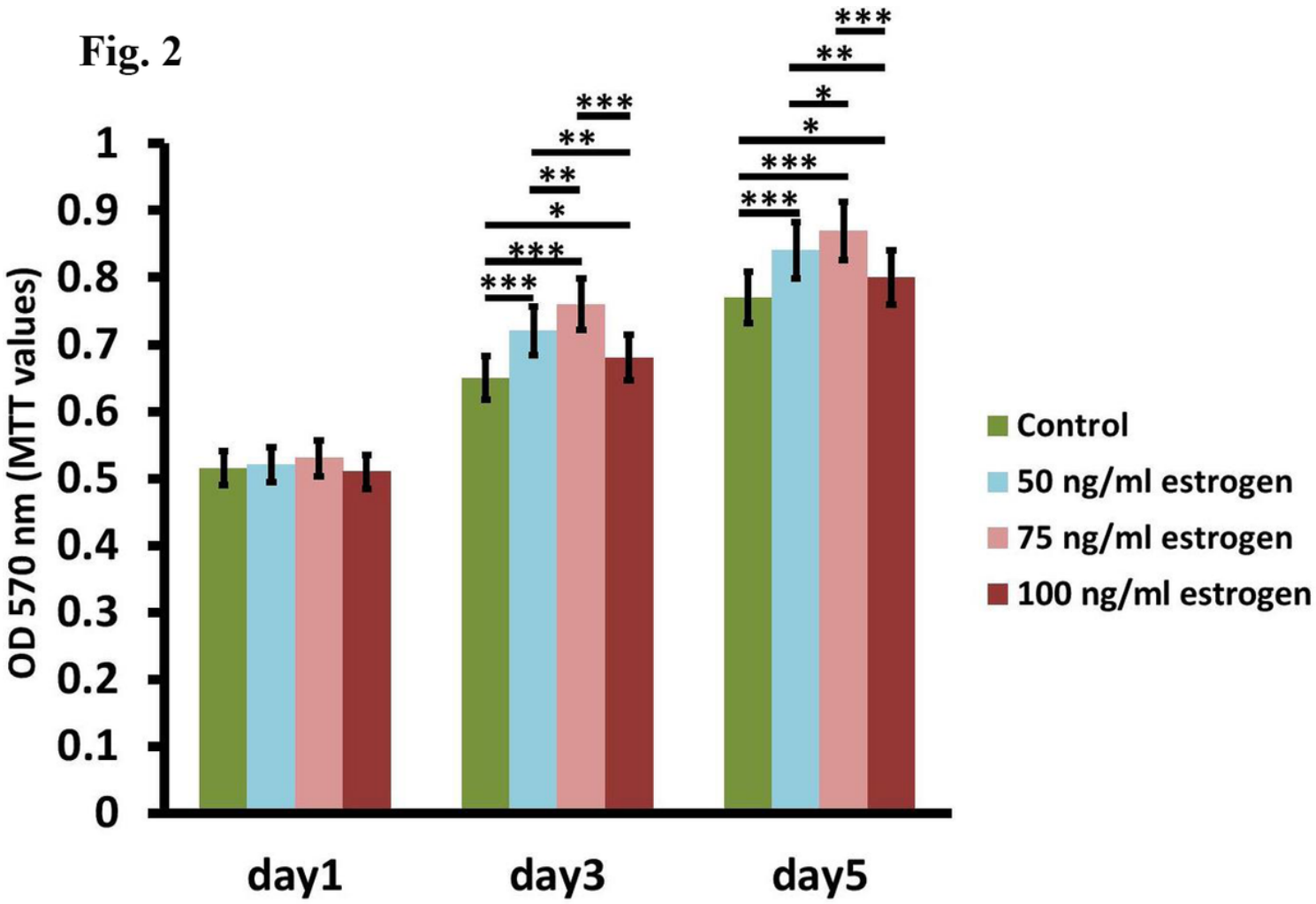

Figure 2

MTT test of C163 and HUVEC co-culture cells was cultured with different concentrations of estrogen after 1, 3, and 5 days. Results are presented as mean ± SD of three independent experiments (*P<0.05; **P<0.01, ***P<0.001), (n=3).

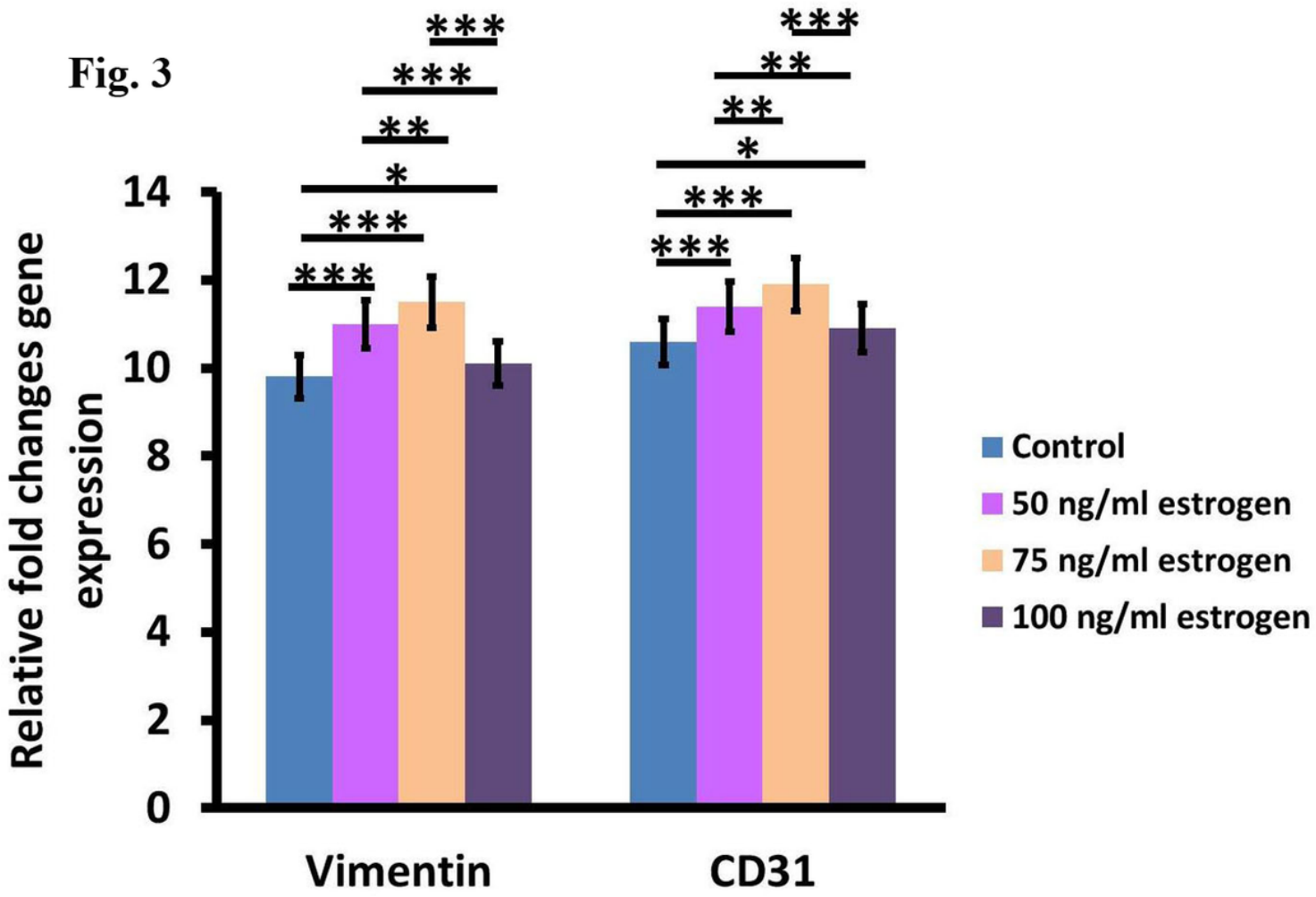

Figure 3

mRNA levels of CD31 and Vimentin. Results are presented as mean ± SD of three independent experiments performed in triplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

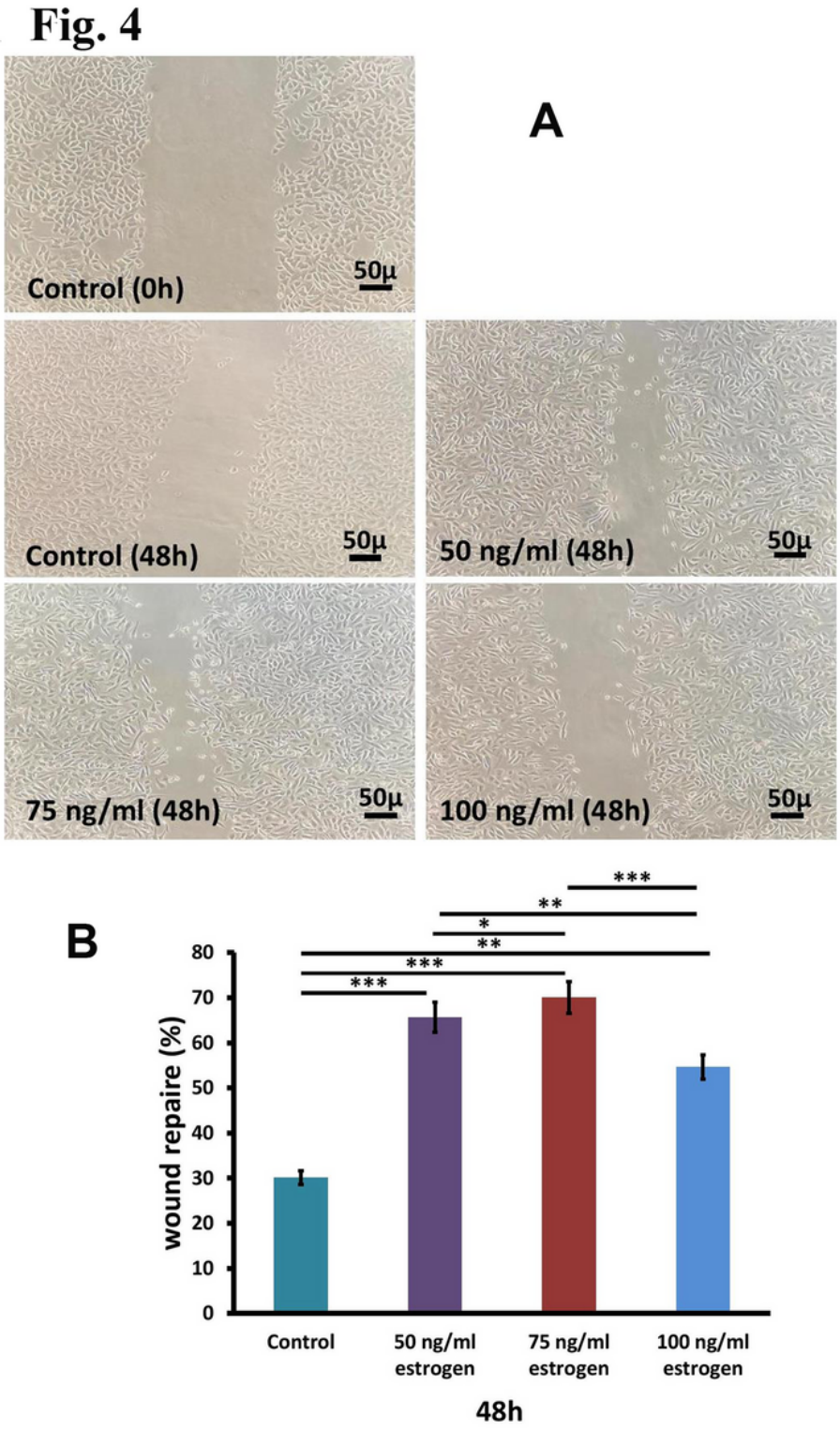

Figure 4

A Wound closure of co-culture of C163 and HUVEC is increased by estrogen solution. Microscopic photographs of scratch wounds at 48 h after scratch. **B** Quantification of the estrogen effect on wound closure was assessed by analyzing the decrement of the wound bed area over time employing Image J software. The data illustrate the mean ± SD from five independent experiments. $P < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) indicates significantly different from the control group.

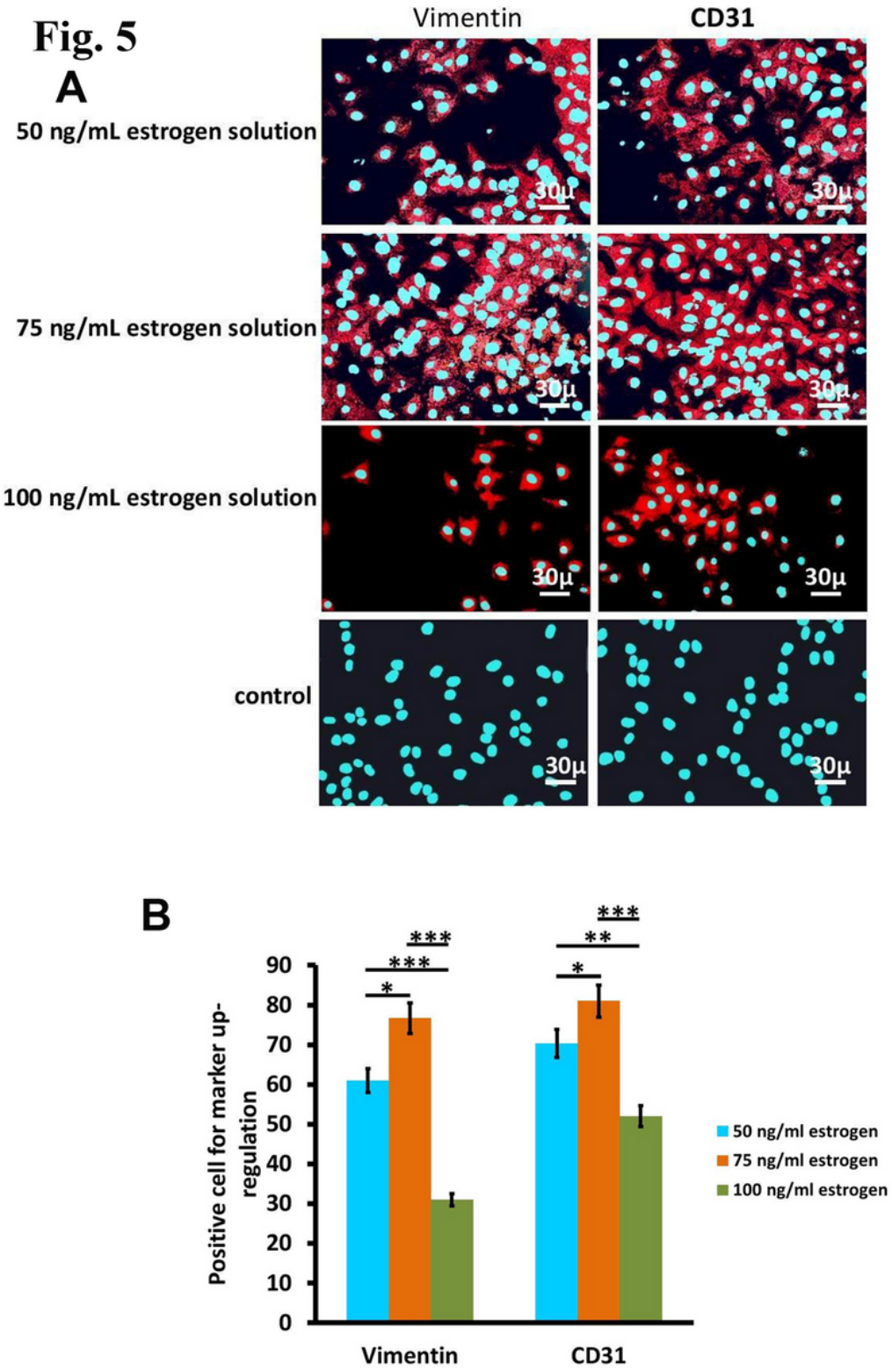

Figure 5

A Immunofluorescence images. CD31 and Vimentin were stained in red. Nuclei were stained with DAPI. **B** Percentages of co-cultured cells expressing biomarkers Vimentin and CD31 compared with 50 ng/ml, 75 ng/ml and 100 ng/ml estrogen treatments. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n=3, mean ± SD).

Supplementary Files

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- Table1.tif