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Leptin Accelerates Endothelial Wound Healing: Role of Endothelial Nitric Oxide Synthase Expression

ORIGINAL INVESTIGATION

ABSTRACT

Background: The endothelium is crucial for the control of vascular homeostasis and plays a role in angiogenesis. Leptin, a protein released mainly by adipose tissue, plays a key role in the regulation of energy balance and angiogenesis. We aimed to investigate the changes of endothelial nitric oxide synthetase expression on human umbilical vein endothelial cells wound healing model after leptin treatment.

Methods: In this study, 5 groups were planned as Group 1: control (untreated), Group 2: treated with 0.1 ng/mL leptin, Group 3: treated with 1 ng/mL leptin, Group 4: treated with 10 ng/mL leptin, and Group 5: treated with 100 ng/mL leptin. Closure rates of wound areas were calculated by the Image J program after 24 hours of leptin treatment. The WST-1 assay was used to calculate the cell viability. Immunocytochemical analysis was performed for endothelial nitric oxide synthase expression and H-Score was calculated.

Results: The closure rates of wound areas were calculated as 80.24%, 89.73%, 87.40%, 90.73%, and 93.70%, respectively. When all groups treated with leptin were compared with the control group, there was a statistically significant difference ($P < .05$). The WST-1 results showed that the most increasing levels of viable cells were found in the groups treated with 0.1 ng/mL leptin and 100 ng/mL leptin when compared to the control group. H-Score values of each group were calculated as 284.8 ± 15.22, 288.6 ± 8.41, 291 ± 8.16, 295.2 ± 11.60, and 308.8 ± 4.32, respectively. The difference between the control group and the group treated with 100 ng/mL leptin was statistically significant ($P < .05$).

Conclusions: Endothelial nitric oxide synthase expression in human umbilical vein endothelial cells increased depending on the leptin dose and the highest increase was in the group treated with 100 ng/mL leptin.

Keywords: Leptin, eNOS, endothelial wound healing

INTRODUCTION

Cardiovascular disease (CVD) is very common in the adult population and is the leading cause of morbidity and mortality globally. Numerous studies have shown that endothelial dysfunction plays a vital role in CVD.[1] The endothelium is essential for the control of vascular homeostasis and plays a role in the regulation of intracellular signal, vascular tonus, permeability, coagulation cascade, and angiogenesis. Endothelial injuries trigger the inflammatory response, causing endothelial cell dysfunction, stiffness of the vascular wall, and atherosclerotic plaque formation.[2] Endothelial dysfunction is caused by an imbalance in the production of vasodilatory agents, such as nitric oxide (NO).[3]

Nitric oxide is produced and released by endothelial cells. It is synthesized endogenously from L-arginine by the NO synthase (NOS).[4] Three isoforms of NOS are described; neuronal NOS (nNOS or NOS-1) and endothelial nitric oxide synthase (eNOS or NOS-3), which are the major contributors to the circulating NO together with the inducible NOS (iNOS or NOS-2).[3,5] Both nNOS and eNOS are constitutively synthesized under physiological conditions and are mainly expressed in neurons and endothelial cells, respectively.[5] Endothelial nitric oxide synthase plays an important role in vasodilation, blood pressure, platelet aggregation, and leukocyte-endothelial interactions. Nitric oxide is required to maintain vascular

Gürkan Yiğittürk[1]
Melike Özgül Önal[1]
Ekin Subaşı[2]
Beyza Doğan[2]
Melih Türkmenoğlu[2]
Merve Kaplan[2]
Volkan Yaşar[1]
Hülya Elbe[1]
Feral Öztürk[1]

[1]*Department of Histology and Embryology, Faculty of Medicine, Muğla Sıtkı Koçman University, Muğla, Turkey*
[2]*Phase 6, Faculty of Medicine, Muğla Sıtkı Koçman University, Muğla, Turkey*

Corresponding author:
Hülya Elbe
✉ hulya.elbe@mu.edu.tr

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function in the endothelium. Therefore, regulation of NO production by activating eNOS phosphorylation in endothelial cells is essential for the treatment of CVDs.[4]

Leptin, a protein released mainly by adipose tissue, plays a key role in regulating food intake, energy balance, and glucose homeostasis.[6,7] Leptin is produced by some tissues other than adipose tissue such as the placenta, stomach, skeletal muscles, tooth germ, brain, and pituitary gland.[8] Under normal conditions, it circulates in the range of 1-15 ng/mL.[7,9] Plasma leptin levels of obese individuals have increased.[9] Leptin is a crucial cytokine for certain physiological processes such as inflammation, angiogenesis, hematopoiesis, reproductive and immune function through its effects on peripheral tissues.[5]

Recent studies have shown that leptin has potential beneficial effects on wound healing. However, the effect of leptin on endothelial cells has not been fully elucidated. Therefore, in this study, we aimed to investigate the dose-dependent effects of leptin on human umbilical vein endothelial cells (HUVECs) wound healing model and eNOS production.

METHODS

Studies on HUVECs have been recognized as a useful model for research on human endothelium. Although this model does not represent all endothelial cell types found in an organism, HUVECs are an excellent model for studying vascular endothelium and major biological pathways involved in endothelium function. More recently, this model has been used to study pathophysiological mechanisms involved in the development of CVD (Medina-Leyte et al[1] 2020). The HUVECs (ATCC® CRL-1730™) were purchased from American Type Culture Collection (ATCC) and grown in monolayer cell culture in Roswell Park Memorial Institute-1640 culture medium containing 10% heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin. Cells were cultured in 75 cm^2 polystyrene flasks and maintained in an incubator at 37°C in a humidified atmosphere in the presence of 5% CO_2. Growth and morphology were checked microscopically daily to ensure cell health. Cells were passaged once or twice a week, depending on their confluency. Cells were harvested using 0.25% trypsin-EDTA and centrifuged at 300 g. Cells were resuspended in a culture medium after centrifugation. Leptin (Sigma-Aldrich L4146, St. Louis, Mo, USA) was prepared as a stock solution in deionized water (1 mL/mg) and stored at –30°C. In this study, 5 groups were planned as Group 1: control (untreated), Group 2: treated with 0.1 ng/mL leptin, Group 3: treated with 1 ng/mL leptin, Group 4: treated with 10 ng/mL leptin, and Group 5: treated with 100 ng/mL leptin. Under normal conditions, leptin circulates in the range of 1-15 ng/mL.[7]

In Vitro Wound-Healing Assay

In the wound-healing assay, after starvation for 24 hours in a serum-free medium, HUVECs were seeded at a density of 1×106 cells/well per 6-well plate and grown to confluence. Cell monolayers were wounded by scratching with a pipette tip along the diameter of the well, and they were washed twice with a serum-free medium before their incubation with diverse concentrations of leptin. Wound areas were measured at the same positions at times 0 and 24 hours after leptin treatment.[10] To monitor cell movement into the wounded area, 5 fields of each wound were photographed immediately after the scratch time 0 and after 24 hours. The endpoint of the assay was measured by calculating the reduction in the width of the wound after 24 hours and compared to time 0, which is set at 100%. The area of wound healing was calculated by using the ImageJ software for Windows 10 (Microsoft Corporation, Redmond, Washington, DC, USA).

Cell Viability

The WST-1 analysis was used to determine the effect of leptin on the viability of HUVECs. Cells were seeded in 96-well plates at a density that provided ~70% confluency before culturing in leptin-containing media. Cells were treated with an increasing dose of leptin. After incubation, the WST reagent was added to the cells and the absorbance was determined after 2 hours at 450 nm wavelength using a microplate reader. The percentage of cell viability was determined using Microsoft Excel 2010 software for Windows 10 (Microsoft Corporation, Redmond, Washington, DC, USA).

Endothelial Nitric Oxide Synthase Expression

Endothelial nitric oxide synthase expression was determined by using the avidin-biotin complex immunocytochemistry (ICC) method. Human umbilical vein endothelial cells were grown on 12 mm sterile glass coverslips in 24-well plates for overnight. Cells were treated with 0.1 ng/mL, 1 ng/mL, 10 ng/mL, and 100 ng/mL leptin for 24 hours. After the cells were fixed with 4% paraformaldehyde for 30 minutes, the cells were treated with 0.1% Triton-X 100 on ice for 15 minutes and washed with phosphate-buffered saline (PBS). The cells were incubated in 3% H_2O_2 solution. They then washed the PBS 3 times and the blocking solution was treated for 30 minutes at room temperature. The cells were then incubated with the anti-eNOS (E-AB-32267, Elabscience, China) primary antibody (1:150) overnight in a humidified chamber at +4°C. The cells were then incubated with a biotinylated antibody (Cat No: AEN080, Lot#38705, ScyTek, USA) for 30 min, followed by three washes in PBS and then with strep tavidin-peroxidase conjugate for 30 minutes. For the visualization of eNOS immunostaining each well were treated with 3,3'-Diaminobenzidine. Mayer's hematoxylin was used for counterstaining. The wells were finally washed with distilled water and covered with mounting medium. The whole process was repeated 3 times. Antibody staining intensities were evaluated by H-Score analysis as follows: no staining (0), mean (1), moderate (2), strong (3), and very strong (4).

HIGHLIGHTS

- Leptin may play an important role in the wound healing process.
- Leptin significantly induces endothelial nitric oxide synthase (eNOS) expression from human endothelial cells.
- The most effective dose for both wound closure and induction of eNOS expression was 100 ng/mL leptin.

Semiquantitative H-Score values were calculated by counting positive staining cells in 5 randomly selected fields for each group [H-Score: ∑Pi (i + 1) (Pi: % positively stained cells; i: intensity of staining)]. The mounted slides were visually evaluated with a Nikon Eclipse 80*i* image analysis system (Nikon Instruments, Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed using the SPSS 17.0 statistical program (SPSS Inc., ver. 17, Chicago, Ill, USA). All experiments were carried out in triplicate, and presented as mean ± SD. Statistical analysis was performed by using one-way analysis of variance, followed by Tukey's or Dunnett's post hoc test. $P < .05$ was considered to indicate a statistically significant difference.

RESULTS

Effects of Leptin on Cell Viability

The viability of control cells was accepted as 100%. Cell viability of each group was determined as 106.463% (0.1 ng/mL leptin), 104.765% (1 ng/mL leptin), 104.610% (10 ng/mL leptin), and 105.730% (100 ng/mL leptin). It was shown that the highest increase in the number of viable cells was in the group treated with 0.1 ng/mL leptin and the group treated with 100 ng/mL leptin compared to the control group. There was no significant difference between the groups ($P > .05$) (Figure 1).

Effects of Leptin on Endothelial Wound Healing

Wound closure rates were 80.24% (control), 89.73% (0.1 ng/mL leptin), 87.40% (1 ng/mL leptin), 90.73% (10 ng/mL leptin), and 93.70% (100 ng/mL leptin). When all groups treated with leptin were compared with the control group, there was a statistically significant difference ($P < .05$) (Figures 2 and 3).

Effects of Leptin on Endothelial Nitric Oxide Synthase Expression

The highest eNOS expression was found in the group treated with 100 ng/mL leptin and the lowest eNOS expression was found in the group treated with 0.1 ng/mL leptin (Figure 4). The H-Score of eNOS expression was calculated as 284.8 ± 15.22 (control), 288.6 ± 8.41 (0.1 ng/mL leptin), 291 ± 8.16 (1 ng/mL leptin), 295.2 ± 11.606 (10 ng/mL leptin), and 308.8 ± 4.3243 (100 ng/mL leptin). The difference between the control group and the group treated with 100 ng/mL leptin was statistically significant ($P < .05$). There was no significant difference for the groups treated with 0.1 ng/mL, 1 ng/mL, and 10 ng/mL leptin compared to the control group ($P > .05$) (Figure 5).

DISCUSSION

Wound healing is a highly dynamic process in which cellular and biochemical events are regulated, and tissues are successfully repaired. The microvascular system plays an essential role in tissue repair. Endothelial cells rapidly respond to angiogenic factors by forming a complex capillary network.[11] The main feature of wound repair is the formation of granulation tissue, that is, fibrovascular tissue including fibroblasts, collagen, and blood vessels.[12] Wound healing is a complex event in which various types of cells interact with various functions. Coagulation and hemostasis are triggered in the wound immediately after injury. The dynamic balance between endothelial cells, platelets, coagulation, and fibrinolysis regulates hemostasis. Endothelial cells have a special role. It provides growth and survival of newly formed tissue.[13] Although most of the essential biochemical steps in wound healing have been characterized, some key regulatory molecules have yet to be identified. To better understand leptin's role in wound healing, we analyzed leptin for its effects on endothelial cell migration and proliferation.

Leptin, a protein released mainly by adipose tissue, plays a key role in regulating food intake, energy balance, and glucose homeostasis.[6,7] Under normal conditions, it circulates in the range of 1-15 ng/mL. Since hyperleptinemia is associated with altered NO activation, oxidative stress, inflammation, and changes in vascular smooth muscle cell activity, high leptin levels may have deleterious effects on vascular function.[7] In our study, we observed that leptin plays a neutral role at physiological levels that it can change neither proliferation nor migration. Williams et al[14] reported that leptin selectively increases the expression and secretion of matrix metalloproteinases in human gingival fibroblasts. In another study, it was reported that leptin increased production of IL-6 and IL-8 in dental pulp fibroblasts.[15] In an *in vitro* study, it was reported

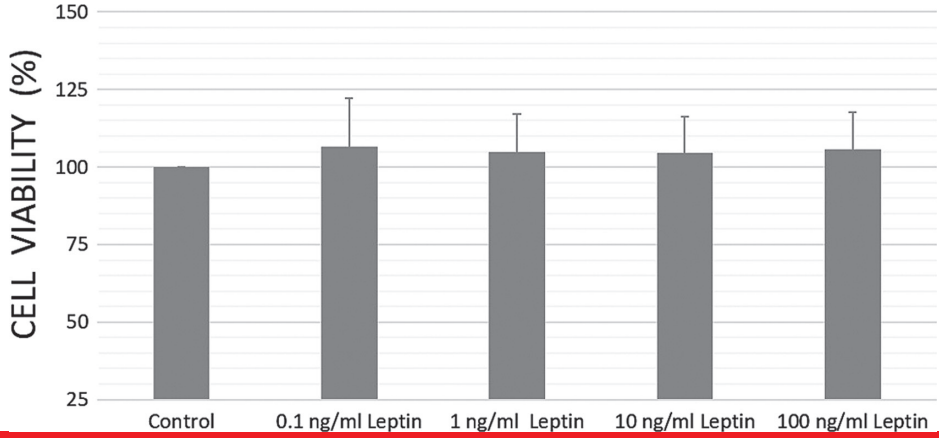

Figure 1. WST-1 cell viability analysis. Dose-dependent effects of leptin on HUVECs viability were analyzed. There was no significant difference between the groups ($P > .05$). HUVECs, human umbilical vein endothelial cells.

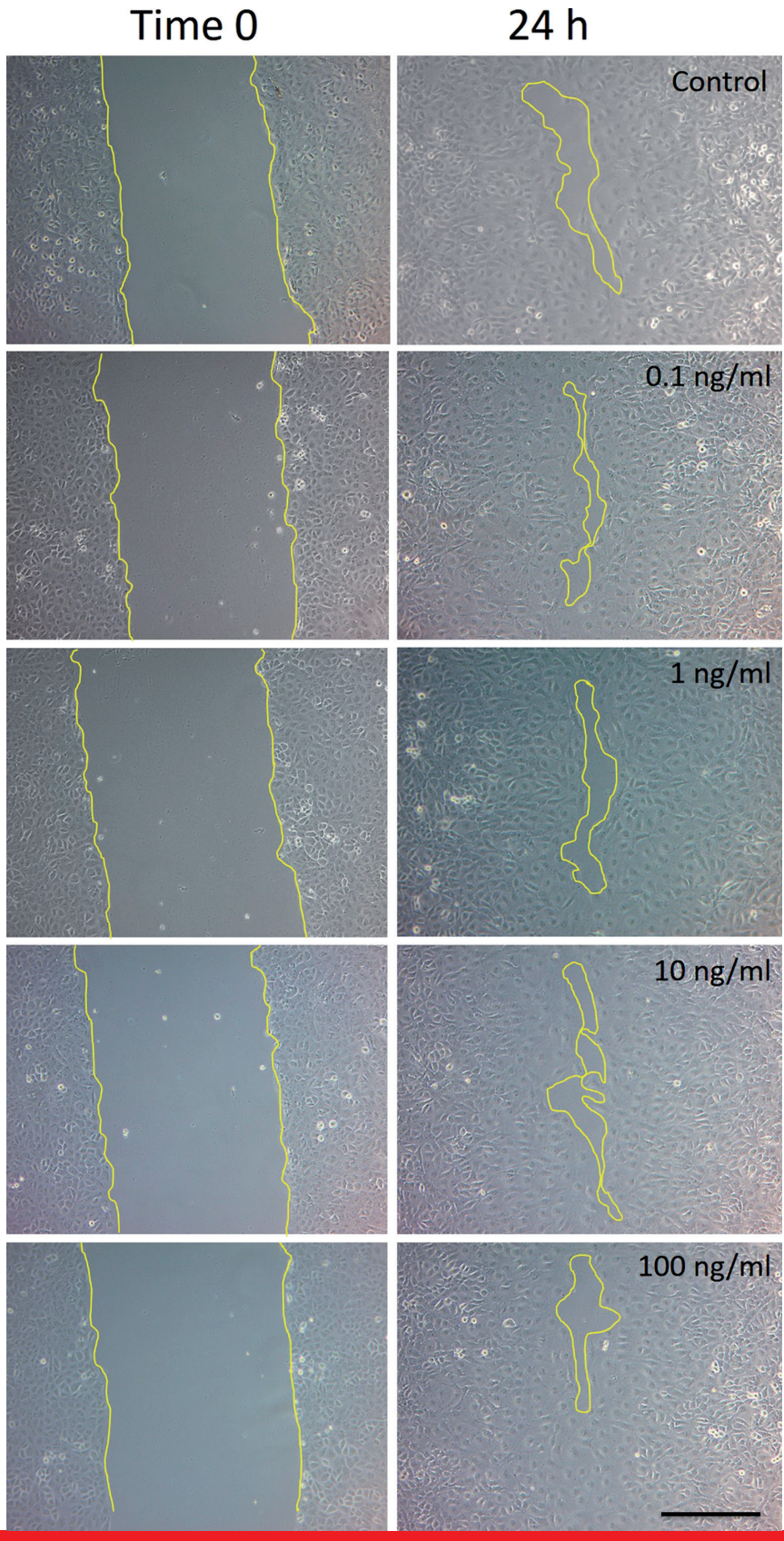

Figure 2. ***In vitro*** **wound-healing assay. Images were captured by a camera coupled to the inverted microscope, with 10× magnification, before (time-0) and after 24 hours of the leptin application, and compared with the control. The images were analyzed using the Image J software to evaluate the scratch by quantification of the areas occupied by the lesion. 10×. Scale bar: 100 µm.**

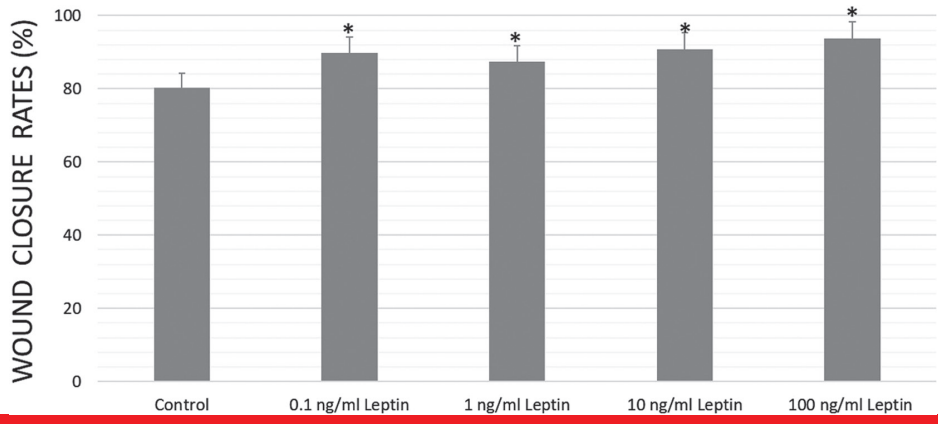

Figure 3. Closure rates of wound areas (%). *Statistically significant compared to the control group ($P < .05$).

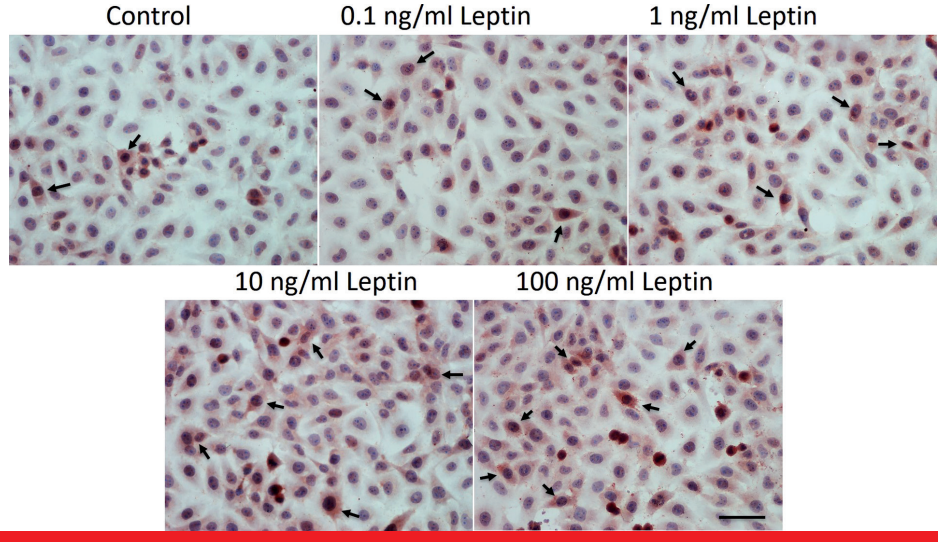

Figure 4. Expression of eNOS in HUVECs. Strong eNOS immunostaining in human umbilical vein endothelial cells were stained brown color (arrows). 20×. Scale bar: 100 µm. eNOS, endothelial nitric oxide synthase; HUVECs, human umbilical vein endothelial cells.

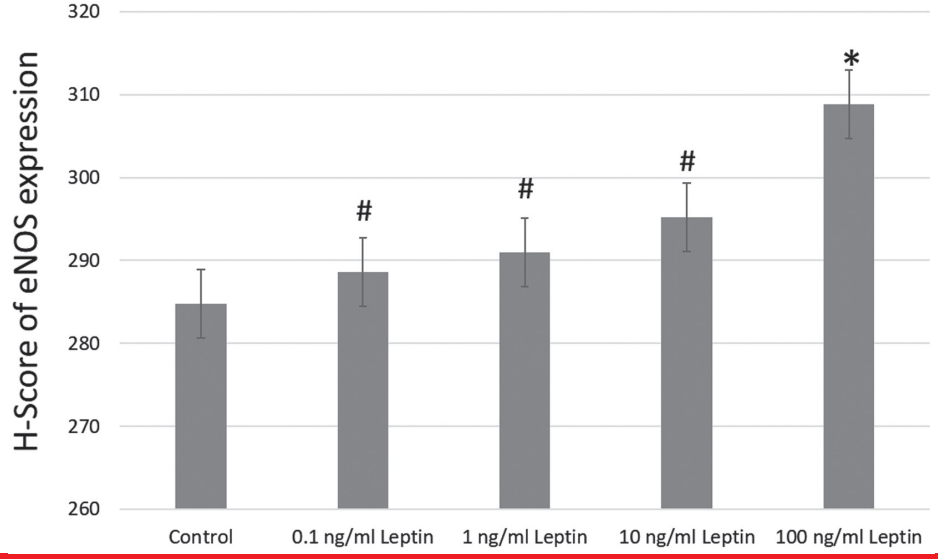

Figure 5. H-Score of eNOS expression. The positive cells were counted to record the expression and calculated H-Score. *Statistically significant compared to the control group ($P < .05$), #No significant difference compared to control group ($P > .05$). eNOS, endothelial nitric oxide synthase.

that leptin-administered rat cutaneous fibroblasts and collagen synthesis increased depending on the dose (0, 10, 50, 100, 200, and 400 ng/mL leptin), and leptin may play a role in the healing of skin wounds.[16] Recent studies have suggested that leptin may have direct effects on the endothelium and may lead to modulation of various physiological functions.[9] Leptin has been shown to promote the survival and proliferation of endothelial cells. The presence of leptin in a serum-free medium increases the number of viable endothelial cells. Moreover, when endothelial cells are exposed to leptin, they undergo migration, growth, and proliferation.[17] However, whether leptin's role in wound healing is mediated through angiogenesis or other mechanisms is controversial. More studies are needed to confirm these findings.

Nitric oxide is a free liposoluble molecule with a short half-life that functions as a messenger, found in a large number of tissues. Nitric oxide has multiple biological effects that acutely regulate vascular physiology or lead to long-term changes in cell function.[5] The vascular endothelium is an important component in the control of arterial pressure homeostasis. Endothelial cells secrete vasoactive factors such as NO, which have a potent vasodilator effect. Nitric oxide can spread to adjacent smooth muscle cells or be released into the bloodstream and cause vasodilation.[9] Endothelial nitric oxide synthase expression is predominantly found in endothelial cells.[4] Leptin induces endothelial NO synthesis, and leptin administration causes a dose-dependent increase in serum NO concentrations. Nitric oxide is considered to be the ultimate target of leptin activity on the endothelium.[9] Frühbeck[18] showed that bolus intravenous leptin administration increases NO production in a dose-dependent manner by activating NOS. Leibovich et al[19] showed that the production of angiogenic activity is dependent on NOS. Pu et al[20] demonstrated that endogenous NO production by endothelial cells is a prerequisite for the mitogenic and angiogenic effects of this factor. Noiri et al[21] (1997) reported that stimulated NO production serves a permissive role in the endothelin-induced acceleration of endothelial cell motility and wound healing. Leptin increases NO production in cultured bovine pulmonary artery endothelial cells, human aortic endothelial cells, and isolated rat aortic rings.[22] Under normal conditions, leptin circulates in the range of 1-15 ng/mL.[7] Manjunathan et al[23] reported that leptin induced changes in the localization and phosphorylation pattern of eNOS in cultured endothelium under various concentrations (1 nM, 5 nM, 10 nM, 25 nM, and 50 nM) for 6h. In our study, we applied 0.1 ng/mL, 1 ng/mL, 10 ng/mL, and 100 ng/mL leptin on HUVECs. We found that leptin increases eNOS expression in a dose-dependent manner. The lowest expression of eNOS was found in the control group and the highest expression was found in the group treated with 100 ng/mL leptin. Our data confirm that the nanomolar concentrations (ng/mL) of leptin accelerate eNOS expression in dose-dependent manner.

Recent studies have demonstrated that leptin acts as autocrine/paracrine regulator in wounded sites.[11] Umeki et al[24] demonstrated that local leptin administration can also promote wound healing in the oral mucosa cells. Previous studies reported that leptin induced the proliferation of various other cells such as keratinocytes,[25,26] lung epithelial cells,[27] hemopoietic cells,[28] pancreatic beta cells,[29,30] and endothelial cells.[31] Murad et al[11] reported that leptin directly causes angiogenesis on endothelial cells. Similarly, we have recently demonstrated that epithelial cell migration and wound healing are NO-dependent. From these findings, we conclude that leptin can promote wound healing on endothelial cells by stimulating both proliferation and migration by activating eNOS expression.

Study Limitations

This study has several limitations. First, we only evaluated the effect of leptin on HUVECs. The cells could be taken from experimental animals and a primary cell culture study could be performed. Second, the study included only eNOS expression by ICC staining. Other antibodies could also be used. Finally, this study, which was planned with the contributions of our medical faculty students, was financially limited.

CONCLUSION

Our data have shown that leptin may play an important role in the wound healing process and that the wound area treated with leptin is healed faster than the control group. However, we have shown that leptin significantly induces eNOS expression from human endothelial cells. We found that the most effective dose for both wound closure and induction of eNOS expression was 100 ng/mL leptin. These findings suggest that leptin may be a potential proliferative agent on wound healing, and further studies are required to examine the effects of leptin.

Ethics Committee Approval: Ethical approval is not applicable, because this article does not contain any studies with human or animal subjects.

Peer-review: Externally peer-reviewed.

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