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Research Article

Comparison of thewound healing effects of vascular endothelial growth factor a (VEGF-A) and transforming growth factor beta 3 (TGF-β3) on gingival cells

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ABSTRACT

Each tissue has its own anatomy and physiology. The deterioration of these structures by various effects (crushing, puncture, pathogens, etc.) is called as wound. The tissue exposed to these effects wants to gain the same function and anatomy. This phenomenon is called wound healing. Wound healing is a process that involves many reactions and cytokines. Since it is known that TGF-β3 and VEGF-A proteins which are among these cytokines, play an active role in wound healing stages. In the present study, it was decided to examine the wound healing effects of the mon human gingival fibroblast cells comperatively. Wound healing process under the influence of these cytokines was performed by *in vitro* scratch analysis which is known as easy, reliable and cheap technique. In this context, firstly cell viability analysis was performed to determine the most effective dose for inducing cell proliferation. After this experimental step, gingival cells were incubated for 72 hours with suitable TGF-β3 and VEGF-A doses. As a result of the study, it was concluded that TGF-β3 and VEGF-A proteins are not cytotoxic and have a wound-healing effect on gingival fibroblast cells.

Keywords: TGF-β3, VEGF-A, Gingival fibroblast cells, wound healing.

1. INTRODUCTION

Every living tissue has a normal anatomy and functional integrity. The disruption of this tissue integrity after any trauma (crushing, puncture, chemical solutions, bacteria, etc.) is called wound. All cellular and biochemical events that take place after the effect of this trauma is over and the tissue can regenerate is called wound healing.[1] The wound healing process is a regular yet complex set of events[2]. The stages of wound healing are listed as haemoastasis, inflammation, proliferation and remodeling, respectively.[2-4].Wound healing process involves a complex set of reactions which include inflammation, granular tissue formation, epithelium regeneration and matrix remodeling.[4-6] In addition to these stages, the release of mediators, which are various growth factors, cytokines and various small molecule-weighted compounds, begins immediately after injury.[1,6]Systemic mediators in wound healing stages may differ according to factors such as injury type, sequential molecular and cellular events, local wound factors, inflammation, angiogenesis, fibroplasia, wound epithelialization and matrix remodeling. These factors are known as markers that indicate the presence of acute or physiological wound healing and whether there is an abnormality in healing. Acute wounds are clean wounds that are easy to heal. Chronic wounds heal with difficulty and even healing may not be complete due to various diseases like diabetes mellitus. Whether the wound formed in thetissue is chronicor acute, the healing process generally consists of four basic steps. The first of these is the homeostasis step and this process starts right after the wound formation.[7,8] The most important requirement in this stage is to stop the bleeding and alpha granules are formed as the platelets. These alpha granules also stimulate the release of various cytokines in

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platelets. The main cytokines are platelet derived growth factor (PDGF), transforming growth factor (TGF-β), vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) [9] These cond phase of wound healing is called as inflammation phase.[9]

In the early stage of inflammation, neutrophil cells predominate. Neutrophils are cells that come to the wound site and initiate acute inflammation. Neuneutrophils eliminate impaired matrix and dead tissues by secreting elastase, collagenase and protease.[10] In the late inflammation stage, macrophage cells are predominate and they proliferate in the wound area quickly. They are responsible for antimicrobial defense and phagocytosis at the wound site. Activation of macrophage cells leads to there lease of cytokines (VEGF, TGF-β, TNF-α, Interleukin-, etc.) that cause angiogenesis and fibroplasia.[9,11] After the completion of this step, during the proliferation process, fibroblast and endothelial cells become active and begin to migrate to close the wound are while proliferating on the one hand. In this recovery phase, as in the previous step, VEGF and TGF-β cytokines play an important role. Towards the end of the proliferation phase, keratinocytes multiply and migrate to the wound site and connect the wound edges to each other by building bridges. After this phase, the maturation/remodelling phase begins. [10-12]

As mentioned above, one of the most important factors affecting a healthy wound closure process is VEGF. The VEGF family has multiple members. VEGF-A, VEGF-B, PIGF, VEGF-C, VEGF-D and VEGF-E are known in this family.[13,14,19] VEGF-A, an isoform of the VEGF protein, consists of 8 exons, and homo dimeric, which is highly expressed in the heart, lung, kidneyand adrenal glands, is also a glycoprotein linked to disulfide. It is known that human VEGF-A protein plays an active role in wound healing, is a biomarker that occurs during the angiogenesis phase of wound healing.[15-18]

With the introduction of wound healing into abnormal processes, scar tissue, hypertrophic structure and kelloid tissue formation are observed. These fibrosis disorders are undesirable. There are many cytokines as regulators of these unwanted abnormal stages, and Transformative Growth Factor (TGF-β) is one of them. The upper family of TGF-β is one of the important strategies in tissue repair.[23] The majority of the members of the TGF-β super family are dimergic and consist of about 33 members. The most important members are the TGF-β1, TGF-β2 and TGF-β3 species.[24] TGF-β a cytokine that is synthesized in almost all cells. It is known that platelet cells have a large amount in alpha granules. For this reason, it is secreted by degranulation to the damaged area.[25] The most important feature of TGF-β is to stimulate the transmission of fibroblasts to the wound area, their proliferation and collagen production. Also suppression of inflammation, angiogenesis. They also play a role in steps such as reepithelization and connective tissue regeneration.[22]

A submember of the TGF-β family, TGF-β3 is a cytokine necessary for epithelial hyperplasia, extra cellular matrix synthesis, and immunity. TGF-β3 stimulates macrophages, increases collagen synthesis. It does this by stimulating fibroblast cells.[20] It is stated that TGF-β3 protein collect sand directs keratinocytes in wound healing, inhibits the epithelial and prevents scar tissue formation, that is, plays an anti-fibrotic role.[21]

The gingiva is an armor that completely covers the oral cavity, surrounds theteeth, prevents pathogens and irritants from entering the body and protects the underlying tissues. The gingiva is part of the chewing mucosa that surrounds the cervical part of the teet hand coversthe alveolar region. This mucosa consists of two layers, the lamina propria epithelial layer and the connective tissue underneath it.[32] Wounds of various sizes may occur on the gingiva for different reasons such as infection, trauma, invasive intervention for treatment (tooth extraction, tartar cleaning, root canal treatment, etc.). These wounds should be treated promptly before they reach larger irritating dimensions.[33,34] In the present study, it was tried to evaluate the possible effects of VEGF-A and TGF-β cytokines, which are thought to be effective in gingival wounds that negatively affect people's oral health and impair their quality of life, in vitro conditions. Therefore, it was thought that the data to be obtained would constitute a valuable the rapeuti capproach in dentis try and there search was designed in this direction.

2.MATERIALS AND METHODS

2.1.Materials

Human Gingival Cells were used from liquid nitrogen stocks of Tokat Gaziosmanpaşa University Faculty of Pharmacy, Animal Cell Culture Laboratory The VEGF-A and TGF-β3 proteins were purchased from Sigma.

2.2.Gingival cell culture and cell viability analysis

Human Gingival fibroblast (GF) cells were cultured with DMEM HG (Dulbecco'smodifiedEagle'smedium, 4.5 g/L glucose), 10% (v/v) inactive fetal bovine serum (FBS) at 37 ^{0}C in an environment consisting of 95% moistureand 5% CO_2. When GF cells that reached 80-90% confluency they were removed from the cell culture flasks and the number of cells were determined by Neubauer hemocytometer. GF cells were plated into 96 well culture plates with four replicate (n=4) with a concentration of 5 x 10^4 cells/mL. After 24 hours of incubation, TGF-β3 and VEGF-A proteins were added to the GF cells in various dilutions (20-0.625 ng/ml). Cells were incubated with these proteins for 72 hours and MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium

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bromide) analysis was performed every 24 hours and the effects of the relevant proteins on cell viability were determined.[36] During this analysis, the formazan crystals formed on the cells incubated for 3 hours with MTT solution (0.5 mg/mL) in a dark environment were dissolved by adding DMSO and the absorbance values were recorded by reading the relevant plate on the UV plate reader at a wave length of 570 nm. Using these values, percentage cell viability was calculated.

2.3. *In vitro* wound healing analysis

In vitro scratch analysis, which is often preferred as a wound healing analysis and gives very good results, was performed.[35] GF cells were transplanted into 6 well culture plates and when the cells reached 80-90% confluence a scratch was formed with the P200 sterile pipette tip. Upon scratch, TGF-β3 and VEGF-A proteins were administered at the dose (10 ng/mL) determined by MTT test and most effectively inducing cell proliferation. The morphological changes of the wound models exposed to the relevant proteins for 72 hours were photographed under inverted light microscope every 24 hours.[37] The micrographs were analyzed with Image J Software and % migration rates were calculated and graphed with Graph Pad Prism 9.0 program and statistically evaluated.

3.RESULTS AND DISCUSSION

In this study, in which the possible effects of VEGF-A and TGF-β3 proteins on the healing of gingival wounds were examined comparatively, in vitro scratch analysis was performed and the obtained data were presented qualitatively and quantitatively. In the first step of the study, MTT analysis was performed to determine the most effective cytokine doses to support wound healing in the gingival tissue. As a result of this analysis, it was determined that both proteins did not show cytotoxic effects on gingival fibroblasts and increased cell proliferation compared to the negative control. Among the studied protein concentrations, it was determined that the most effective result was achieved at 20 ng/mL in the acuteperiod (24 hours) and at 10 ng/mL in the chronic period (48 hours). This result was true for both VEGF-A and TGF-β3 (Figure 1). In addition, it was determined that the effect on the hand at the end of 48 hours continued until the end of the 72 hours period.

As mentioned above, 10 ng/mL is the the most effective protein concentration of the chronic period for both VEGF-A and TGF-β3. This result is consistent with previous studies in the relevant field.[26] In 2018, while Wongkhum et al. İnvestigated the effect of VEGF protein on angiogenesis, cell viability tests again gave the best result with a concentration of 10 ng/mL.[27] In the study in which Zhu et al. İnvestigated the effect of TGF-B protein on amylase secretion of rat submandibular gland cells, they applied MTT colorimetric method at 0.5, 1.0, 5.0, 10.0 and 25.0 ng/mL dosages at 0.5, 1.0, 72 and 96 hours and found that amylase secretion was significantly stimulated at 0.5-10 ng/mL concentrations, but not at high doses.[38]

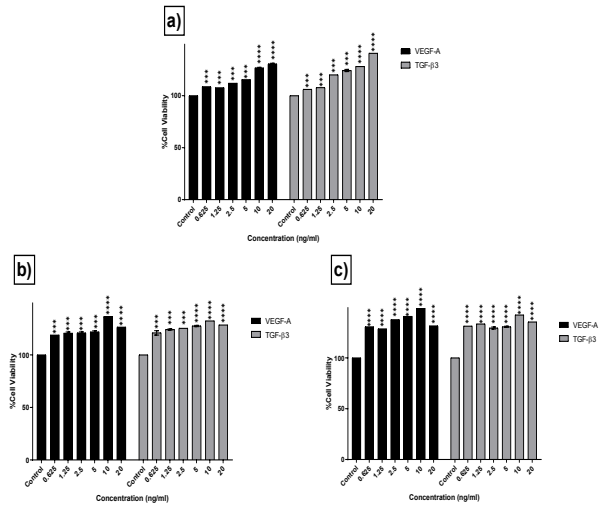

Figure 1. The effect of VEGF-A and TGF-β3 on the viability of GF cell line. Viability was measured by the MTT assay after 24h (a), 48h (b) and 72h (c). These results were analyzed using the Two-way ANOVA test and the differences between the groups were statistically significant (****P≤0.0001, ***P≤0.0005, **P≤0.01, *P≤0.05 vs Control)

In the study in which Ling et al. Looked at the effects of TGF-β isoforms on renal fibrogenesis, the MTT test was used and three TGF-β isoforms were used at concentrations of 0.001 to 10 ng/mL, and the most effective concentration of TGF-β3 protein was reported as 10 ng/mL,[39] and it was shown that it had fibronetic effects in kidney fibroblasts. The most effective concentration used in this study is the same as in our experiment.

To investigate the effects of VEGF-A and TGF-β3 proteins on gingival wound healing *in vitro,*the most effective dose of 10 ng/mL was used which was determined by MTT analysis. The responses of the cells to the both of proteins at the relevant dose were photographed under inverted microscope. Micrographs were analyzed by Image j softw are and obtained quantitative data were graphically presented.

At the end of the first 24 hours, it was observed that the closure rate of the wounds formed in vitro conditions was low, however, regeneration was realized at a higher rate compared to the negative control. When the results after 48 hours of incubation with VEGF-A and TGF-β3 were examined, it was observed that the wound are as were completely closed and the gingival healing was fully realized (Figure 2).

In the wound healing test, the closure rate of the negative control after the first 24 hours was found to be lower than the closure rate of the experimental groups interacting

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with VEGF-A and TGF-β3. In addition, when the effects of the two proteins were compared, it was found that GF cells treated with TGF-β3 protein closed by 93% and VEGF-A protein by 95%. Therefore, the effect of VEGF-A was found to be more positive, albeit at a very mild level, than TGF-β3. After 48 hours, it is seen that the wound areas are completely closed in all three groups. When the photographs taken are examined, it is seen that the cell densities of the treatment groups were more intense than the negative control group from the areas closed after 48 hours. After 72 hours, it was observed that the wound areas were completely closed (Figure 3).
In the study conducted by Prapulla et al. using GF cells, it was investigated whether VEGF proteins play a role in the treatment of periodontal diseases and the results obtained showed that VEGF protein has high bioactivity in wound healing of GF cells.[40]

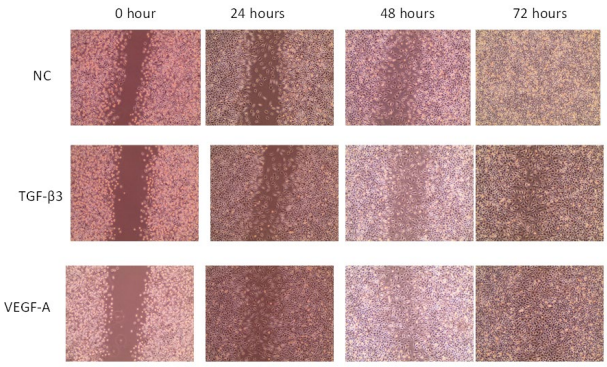

Figure 2. Wound healing situations according to time intervals

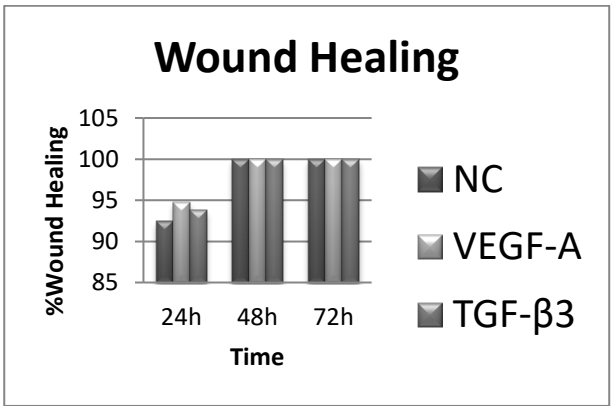

Figure 3. Graphical representation of wound healing.

The development of the palate in the embryonic period is called palatogenesis. The most common deformity in palatogenesis is the cleft palate. The palate midline epithelium suture (MES) cells, which are necessary for the union of the mesenchymal cells of the palate, must be broken down by apoptosis.[30,31] TGF-β3 is also responsible for this breakdown and cell migrations as a result of disintegration. In the study conducted by Ahmed et al. On the mechanisms of disintegration of epithelium sutures with TGF-β3; Single palate racks collected from CF1 mouse embryos were placed with the opposite palate rack and incubated with 5 ng/mL TGF-β3 protein for up to 72 hours. MES cells were checked by phase contrast microscopy every 24 hours. As a result of the study, it was observed that the MES cells were mobile and closed the gaps compared to the control groups.[29]A similar study was conducted by Jalali et al. and used the same amount, namely 5 ng/mL TGF-β3 protein. In this study, they proved that TGF-β3 protein plays a major role in MES breakdown and mesenchymal fusion for palate development.[28] In the study conducted by Nawshad et al., they applied 2, 5 and 10 ng/mL TGF-β3 protein in the scratched wound model in palate wound healing. MES cells started to migrate after 12 hours. In the study, the increase in protein concentration increased cell migration.[41] These studies show that TGF-β3 protein plays an important role in wound healing and the concentration of 10 ng/mL we use supports it.

4.CONCLUSION

In our wound healing experiment with VEGF-A and TGF-β3 proteins, it was determined that both proteins were not cytotoxic, although their bioactivity did not differ significantly from the negative control. It is planned that this result, which was obtained as a preliminary, will be transferred to in vivo wound healing processes and supported by further analysis.

Conflict of interests

I declares that there is no a conflict of interest with any person, institute, company, etc.

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