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Investigation of the Effects of Pregabalin On Wound Healing In L929 Fibroblast Cells

Pregabalinin L929 Fibroblast Hücrelerinde Yara İyileşmesi Üzerine Etkilerinin Araştırılması

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ABSTRACT

Objective: Wound healing is a multifaceted, complex process consisting of sequential and interrelated phases including hemostasis/inflammation phase, proliferation phase and remodeling phase. Pregabalin (PGB), a gabapentin derivative, is an anticonvulsant agent with anti-inflammatory and antioxidant properties. Therefore, in this study, we aimed to show the effect of pregabalin on cell viability in L929 fibroblast cells and its effects on fibroblast migration and wound closure during the wound healing process.
Methods: In this study, the effect of different concentrations of pregabalin on cell viability and proliferation in L929 skin fibroblast cells was investigated using MTT assay. In addition, a scratch wound healing model was established in L929 skin fibroblast cells and the effects of pregabalin concentrations that increase cell proliferation on wound healing in MTT assay were shown. At the end of the experiment, TGF-β1 levels of all groups were measured by ELISA method.
Results: In our studies, it was observed that 100, 50, 25, 10 µM concentrations of pregabalin increased cell proliferation. In the scratch wound healing model, pregabalin at concentrations of 100 and 50 µM showed a significant closure compared to control and other groups. TGF-β1 levels were decreased in groups with good healing scores (50, 25 µM).
Conclusion: Pregabalin has been shown to enhance wound healing in in vitro experiments. This effect needs to be evaluated holistically within the organ system in vivo. There is also a need for experimental and clinical studies to evaluate the wound healing effects and mechanism of pregabalin.

Keywords: Wound Healing, Pregabalin, Scratch wound assay, L929 cell line

ÖZ

Amaç: Yara iyileşmesi, canlılarda hemostaz/iltihaplanma fazı, proliferasyon fazı ve yeniden şekillenme fazı olmak üzere sıralı ve birbiriyle ilişkili aşamalardan oluşan çok yönlü, karmaşık bir süreçtir. Çok sayıda endojen ve eksojen olumsuz faktör, fizyolojik iyileşme süreçlerini bozabilir. Son zamanlarda yara iyileşmesi üzerine yapılan çalışmalara bakıldığında, kullanılan tedavilerin yetersiz kaldığı görülmektedir. Bu durum yara iyileşmesinde yeni farmakolojik ajanlara ihtiyaç duyulduğunu göstermektedir. Bir gabapentin türevi olan pregabalin (PGB), epilepsi tedavisinde kullanılan antikonvülzan bir ajandır. Ayrıca pregabalinin antiinflamatuvar ve antioksidan özellikleri çeşitli çalışmalarda gösterilmiştir. Pregabalin ile yapılan çalışmalar incelendiğinde, yara iyileşmesi üzerine olan etkisini hücresel düzeyde gösteren herhangi bir çalışma bulunmamaktadır. Bu nedenle bu çalışmada L929 fibroblast hücrelerinde pregabalinin, hücre canlılığına etkisi araştırılıp; yara iyileşmesi sürecinde, fibroblast göçü ve yara kapanmasına etkilerinin gösterilmesi amaçlanmıştır.
Yöntemler: Bu çalışmada L929 deri fibroblast hücrelerinde farklı konsantrasyonlardaki pregabalinin hücre canlılığı ve proliferasyonuna olan etkisi MTT testi kullanılarak incelenmiştir. Ayrıca L929 deri fibroblast hücrelerinde çizik yara iyileşmesi modeli oluşturulup, MTT testinde hücre proliferasyonunu artıran pregabalin konsantrasyonlarının yara iyileşmesine olan etkileri gösterilmiştir. Deneyin sonunda tüm grupların TGF-β1 seviyeleri ELISA yöntemiyle ölçülmüştür.
Bulgular: Yaptığımız çalışmalarda pregabalinin 100, 50, 25, 10, µM konsantrasyonlarının hücre proliferasyonunu artırdığı gözlemlenmiştir. Çizik yara iyileşmesi modelinde ise 100 ve 50 µM konsantrasyonlardaki pregabalin, kontrol ve diğer gruplarla karşılaştırıldığında anlamlı bir kapanma göstermiştir.
Sonuç: Pregabalinin yara iyileşmesini artırdığı in vitro deneylerle gösterilmiştir. Bu etkinin in vivo olarak organ sistemi içinde bütüncül olarak değerlendirilmesi gerekmektedir. Ayrıca pregabalinin yara iyileştirici etkilerinin ve mekanizmasının değerlendirileceği deneysel ve klinik çalışmalara ihtiyaç vardır.
Anahtar Kelimeler: Yara İyileşmesi, Pregabalin, Çizik Yara Testi, L929 Hücre Hattı

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Introduction

The skin is an organ consisting of layers of tissue that protect the muscles and organs underneath. As a protective shield of the body against the external environment, the skin is constantly exposed to injuries. Therefore, wound healing is of great importance for the survival of living organisms (Takeo et al., 2015). Wound healing is a multifaceted, complex process consisting of sequential and interrelated phases including hemostasis/inflammation phase, proliferation phase and remodeling phase. After a skin injury, exposed subendothelium, collagen and tissue factor activate platelet aggregation. Chemotactic factors and growth factors are released by degranulation (Gauglitz et al., 2011). Neutrophils traveling to the site of the injury remove debris and bacteria, providing a favorable environment for the wound to heal. Subsequently, macrophages accumulate and facilitate phagocytosis of bacteria (Berman et al., 2017). The proliferative phase is characterized by the accumulation of large numbers of cells and abundant connective tissue. The extracellular matrix (ECM), which includes proteoglycans, hyaluronic acid, collagen and elastin, forms a granulation tissue to replace the original clot formation. This step is mediated by the transforming growth factor-β family (including TGF-β, TGF-β1, TGF-β2 and TGF-β3), the interleukin (IL) family and angiogenesis factors (vascular epidermal growth factor) (Su et al., 2010). The final step of wound healing is the remodeling phase, which requires a delicate balance between apoptosis of existing cells and the production of new cells. Any deviation at this stage can lead to excessive wound healing or chronic wounding (Plikus et al., 2017; Tsai et al., 2018).

Numerous endogenous and exogenous negative factors can disrupt physiological healing processes. Of the phases in the wound healing process, the inflammatory phase is the most sensitive to these negative factors (Kasuya & Tokura, 2014). Moderate inflammation facilitates the removal of necrotic tissue, kills local bacteria and promotes wound healing. However, excessive inflammatory infiltration inhibits normal healing events such as collagen deposition, angiogenesis and granulation tissue formation. It is therefore imperative that inflammation in the wound is sensitively modulated at a level appropriate to promote wound healing, but prevented from reaching a level that would inhibit it (Huang et al., 2022).

When we look at the recent studies on wound healing, it is seen that the treatments used to accelerate wound healing, prevent chronic wound formation, and treat stubborn wounds in an acute injury are inadequate. This shows that new pharmacological agents are needed in wound healing (El Ayadi et al., 2020).

Pregabalin (PGB), a gabapentin derivative, is an anticonvulsant agent used to treat epilepsy (Eutamene et al., 2000). PGB has a similar mechanism of action to gabapentin, acting through GABAergic neurotransmission, voltage-dependent potassium channels and calcium channels (Moore et al., 2009). It is also used in the treatment of central and peripheral neuropathic pain (Ceyhan M. , 2008). PGB has been suggested to

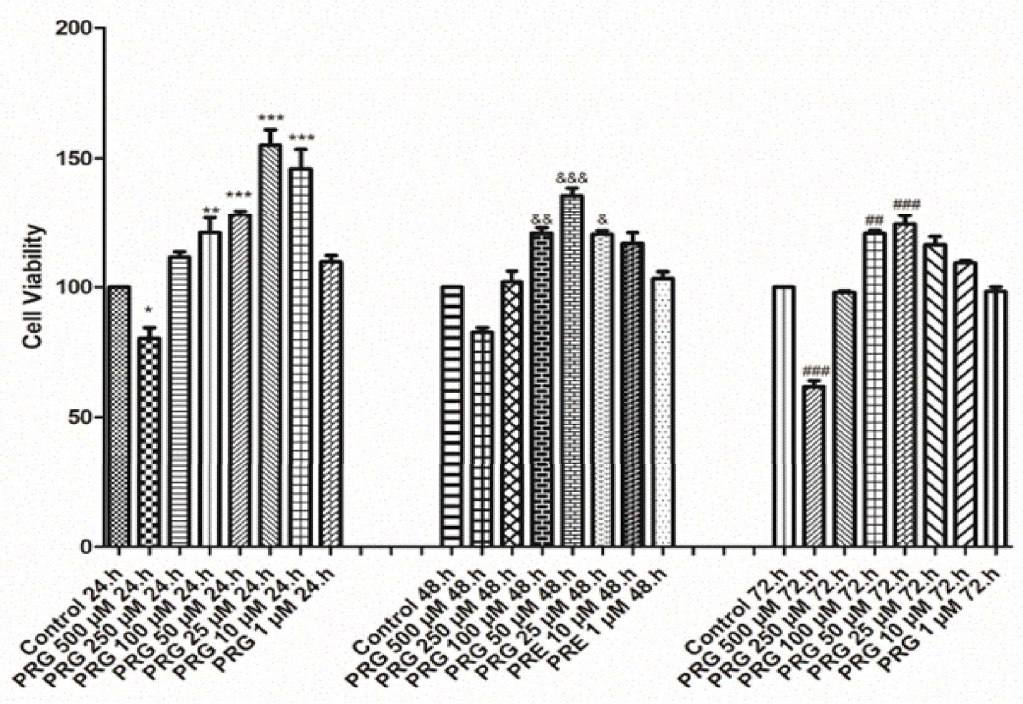

Figure 1: Cell viability results obtained form MTT test. *, #, & means p<0.05, **,##, && means p<0.01 and ***, ###, &&& means p<0.001 according to Tukey's post-hoc test.

exert antinociceptive effects in inflammatory pain by inhibiting the release of neuropeptides on sensory neurons (Fehrenbacher et al., 2003). PGB is reported to have antinociceptive effect in neuropathic pain as well as inflammatory pain (Abou-Khalil, 2016). It was also reported that gabapentin, which is structurally and functionally similar to pregabalin, showed anti-inflammatory effects in rats (Sinha et al., 2013). In addition, anti-inflammatory and antioxidant properties of pregabalin have been demonstrated in various studies (Abu-Rish et al., 2020; Salat et al., 2016). Therefore, in this study, we aimed to investigate the effect of pregabalin on cell viability in L929 fibroblast cells and to show its effects on fibroblast migration and wound closure during the wound healing process.

Methods

Evaluation of Cell Viability by MTT Method

L929 cell line obtained from American Type Culture Collection (ATCC, USA) and stored in Cryotube, were removed from the liquid nitrogen tank, and seeded in a T75 cm2 flask containing DMEM medium containing 10% FBS and incubated at 37°C, 90% humidity and 5% CO2. Cells were passaged successively and cell count was performed after the fourth passage and 5000 cells were seeded in each well of the 96-well plate. The cells were incubated for 24 hours to settle to the bottom of the well. At the end of this period, pregabalin was dissolved with PBS (phosphate buffered saline) and concentrations of pregabalin (500, 250, 100, 50, 50, 25, 10, 1 µM) were prepared and the drugs added to wells. At 24, 48, 72 hours, the absorbance at 570 nm was measured with a microplate reader spectrophotometer (Epoch Microplate Spectrophotometer, Bio Tek, USA) using the MTT method. Viability rates were analyzed in comparison with control wells.

Scratch Wound Healing Experiment

L929 cells in DMEM medium containing 10% FBS were seeded in each well (2×105 cells/well) of a 6-well plate and incubated at 37 0C, 5% CO2. After L929 cells completely covered the well bottom, a scratch was made vertically in each well with a sterile 200 µL pipette tip. Images before the addition of pregabalin were acquired using a Leica Inverted Microscope (Leica, DMIL LED). The wells were then exposed to pregabalin at the concentrations (100, 50, 25, 10 µM) that gave the best results in the cell viability assay. Images of all wells were taken at 0, 12, 24 and 36 hours. Wound closure rates were calculated using the formula below.

% wound closure=[(At=0h-At=Δh)/At=0h]*100

At=0h : Wound length measured at 0th hour (µm)

At=Δh : Wound length measured at Δth hour (µm)

Quantification of TGF-β1 Production in vitro

Supernatants of all experimental groups were taken at the 36th hour of the experiment.

TGF-β1 levels were measured with eBioscience ELISA kit (Lot No: 95303007) on Epoch Spectrophotometer System and Take3 Plate. Absorbances were read at 450 nm. The equation was obtained by plotting a curve from the absorbance of TGF-β1 standards. The ELISA procedure was performed according to the steps described in the kit protocol.

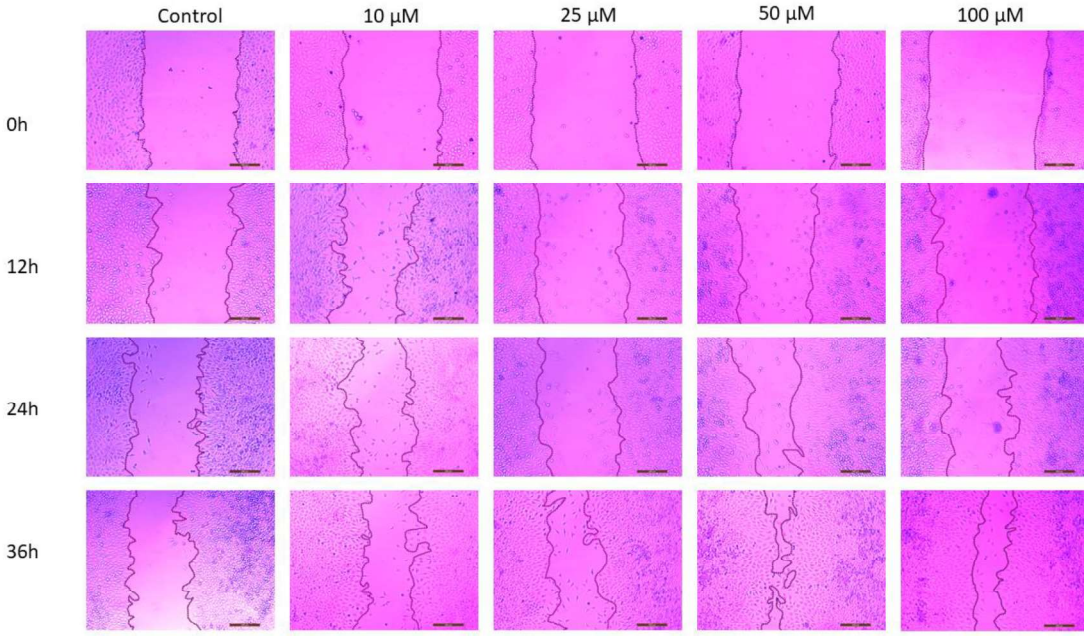

Figure 2: Photographs of scratch test after 0, 12, 24 and 36 hours.

Statistical Analysis

Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) followed by Tukey's test as a post test. Windows SPSS-20 (IBM Corp., NY, Armonk, USA) was used for statistical analyses. $p<0.05$ was considered significant.

Results

500, 250, 100, 50, 25, 10, 10, 1 µM concentrations of pregabalin were applied to L929 skin fibroblast cells and the effects on cell viability were measured by MTT assay at 24, 48, 72 hours and cell viability percentages were calculated. 24.hour MTT assay results showed that 500 µM concentration of pregabalin significantly decreased cell proliferation compared to the control group ($p<0.05$) (Figure 1). It was observed that 100 µM concentration of pregabalin significantly increased cell proliferation compared to the control group ($p<0.01$), and this increase was more significant at doses of 50, 25 and 10 µM ($p<0.001$). When the 48th hour MTT test results were analyzed, 100 µM ($p<0.001$), 50 µM ($p<0.001$) and 25 µM ($p<0.05$) concentrations of pregabalin significantly increased cell proliferation compared to the control group. In the 72nd hour MTT test results, 500 µM concentrations of pregabalin significantly decreased cell proliferation compared to the control group ($p<0.001$). At 100 µM ($p<0.01$) and 50 µM ($p<0.001$) doses, a significant increase was observed compared to the control group.

According to the MTT test results, the doses (100,50,25,10 µM) of pregabalin that increased the proliferation of L929 cells the most were selected and the scratch wound healing experiment was performed. Wound lengths in the images taken at 0, 12, 24, 36 hours were compared with the control group and

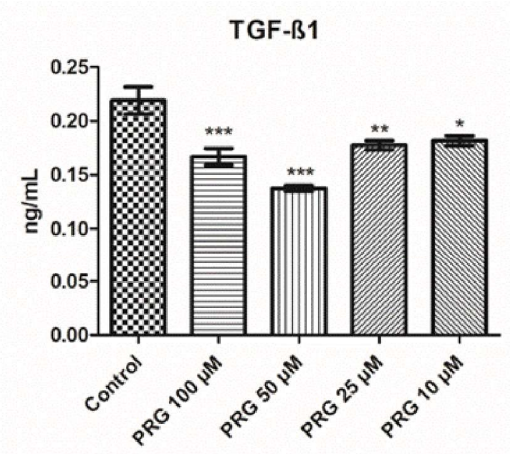

Figure 4: TGF-β1 ELISA results at 36th hour of scratch test. * means $p<0.05$, ** means $p<0.01$ and *** means $p<0.001$ according to Tukey's post-hoc test.

percent closure rates were calculated (Figure 2). At 12 hours, wound closure percentages were close in all control and drug groups. At 24 hours, the wound closure percentages of the drug groups increased compared to the control group. In particular, 100 and 50 µM concentrations of pregabalin increased facial wound closure more than 25 and 10 µM concentrations. At 36 hours, similarly, the wound closure percentages of the drug groups increased compared to the control group. Again, at 36 hours, the effect of 100 and 50 µM concentrations of pregabalin on wound closure percentage showed a clearer increase compared to 25 and 10 µM concentrations. In addition, 50 µM concentration of pregabalin increased percent wound closure more than 100 µM concentration at 24 and 36 hours (Figure 3).

At the 36th hour of the experiment, supernatants of all experimental groups were taken and TGF-ß1 levels were measured. Compared to the control group, 100 µM ($p<0.001$), 50 µM ($p<0.001$), 25 µM ($p<0.01$) and 10 µM ($p<0.05$) concentrations of pregabalin significantly decreased TGF-ß1 levels (Figure 4).

Discussion

In this study, the effect of pregabalin on cell viability in L929 skin fibroblast cells and cell migration in scratch wound healing assay was evaluated in vitro. TGF β1 levels, a biomarker of wound healing, were also examined to evaluate the wound healing effect and mechanism of pregabalin.

Fibroblast cells are the most frequently investigated cells for wound healing (Borges et al., 2017; Teplicki et al., 2018). In these cells, viability assays with the MTT assay are often preferred for both cell proliferation and other cytotoxic assays (Cangul et al., 2020; Ozdemir et al., 2009) . Wagener N. et al. showed that pregabalin increased both cell proliferation and cell viability in hOB, hMSC and MG63 cells (Wagener et al., 2022). Similarly, in a study by Şirin D.Y. and Karaarslan N., it was shown that pregabalin had no negative or toxic effect on cell viability and proliferation of chondrocytes in chondrocyte culture, and the number of viable cells was higher in the PGB-treated groups than in the control group (without PGB) (Sirin & Karaarslan, 2018). In our study, similar to the results in the existing studies, it was observed that pregabalin, especially at concentrations of 100, 50, 25, 10 µM, significantly increased cell viability and proliferation in skin fibroblast cells.

The scratch wound assay is a simple, reproducible assay widely used to measure cell migration parameters such as velocity, persistence and polarity. Cells are replicated until

confluent to the plate bottom. When the cells are confluent on the plate bottom, a thin "wound" is made by scratching with a pipette tip (Cory, 2011). Using this method, many studies have been conducted on wound healing, which is an important health problem all over the world (Jagiello et al., 2023; Zhang et al., 2018). Some studies on wound healing have shown that some substances with known anti-inflammatory and antioxidant properties such as resveratrol, curcumin, baicalin have positive effects (Hecker et al., 2022; Huang et al., 2019; Kant et al., 2015; Zhang et al., 2011). Salat K. et al. showed that pregabalin has anti-inflammatory and antioxidant properties in streptozocin-induced diabetic mice (Salat et al., 2016). Abu-Rish E.Y. et al. investigated the effect of pregabalin on cytokine secretion in a model of splenic inflammation in vivo and in vitro. The results of the study showed that pregabalin decreased cytokine secretion and showed anti-inflammatory properties both in vivo and in vitro (Abu-Rish et al., 2020). In our study, 50 and 100 µM concentrations of pregabalin significantly increased wound closure in the scratch wound healing test compared to the control group. We think that pregabalin at these doses prevents excessive or insufficient inflammation and increases the rate of wound closure by keeping inflammation in a certain balance during the inflammation phase of wound healing.

Cytokines and growth factors, especially TGF-β, have important roles in all phases of wound healing (Everts et al., 2006). Proinflammatory molecules IL-1, IL-2, IL-6, IL-17 and TNF have important roles in the inflammatory phase of wound healing and are especially responsible for the stimulation of adhesion molecules (Arango Duque & Descoteaux, 2014). In the early phase of the treatment process, TGF-β, PDGF and VEGF promote the division and differentiation of keratinocytes and fibroblasts and are the main responsible elements for collagen production (Barrientos et al., 2008; Seo et al., 2017). In the final phase of wound healing, the remodelling phase, collagen type 3 is converted to type 1 and TGF- β differentiates myofibroblasts and closes the wound (Desmouliere et al., 1993; Hosokawa et al., 2003; Ronnov-Jessen & Petersen, 1993). Of course, once these dynamic phases are over and the wound healing process is complete, these cytokines and growth factors decline. A decrease in these growth factors and cytokines indicates that the wound is healing (Nogueira et al., 2020). In our study, especially at doses of 50 and 100 µM at 36 hours, TGF decreased and the wound closed almost completely compared to the control in parallel with the microscopic findings, indicating that healing was supported by pregabalin.

Conclusion and Recommendations

In conclusion, pregabalin has been shown to increase wound healing. The fact that TGF-β is lower in the treatment groups than in the control group indicates that the healing process is close end to the end, accordingly, cytokine release has decreased. However, this effect needs to be evaluated holistically within the organ system in vivo. Additionally, experimental and clinical studies are needed to evaluate the wound healing effects and mechanism of pregabalin.

Etik Komite Onayı: Hücre kültürü çalışması olduğundan etik onaya ihtiyaç yoktur.
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