

Hydrogen Sulfide Promotes Wound Healing by Attenuating Reactive Oxygen Species in Fibroblasts under Hyperglycemic Conditions

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SUMMARY

Chronic wounds are one of the common and serious diabetic complications that also impose a significant financial burden on society. Since comprehensive treatment for chronic wounds has not yet been found, new treatment recommendations are needed. The beneficial effects of hydrogen sulfide (H₂S) on wound healing have previously been demonstrated in healthy or diabetic animal models. H₂S has also been found to accelerate wound closure in cells and animal models. H₂S is beneficial in diabetic wound healing, but its effect on wound healing under diabetic conditions has not yet been elucidated. In this study, we investigated the effects of H₂S and reactive oxygen species (ROS) on wound healing in fibroblasts under high glucose conditions. We used 2,3-bis-(2-methoxy-5-nitrophenyl)-2H-tetrazolium-5-carboxanilide (XTT) and scratch migration assay to investigate fibroblast cell viability and wound healing migration. We showed that H₂S enhanced wound healing in fibroblasts incubated with high glucose by increasing cell viability, proliferation, migration, and attenuating ROS. According to our results, exogenous H₂S reduced oxidative stress during wound repair. In conclusion, H₂S accelerated wound healing, which may be related to inhibiting oxidative stress.

Key Words: Wound healing, fibroblast, high glucose, hydrogen sulfide

Hidrojen Sülfür Hiperglisemik Koşullar Altında Fibroblastlarda Reaktif Oksijen Türlerini Azaltarak Yara İyileşmesini Destekler

ÖZ

Kronik yaralar, topluma ciddi mali yük getiren, yaygın ve ciddi diyabet komplikasyonlarından biridir. Kronik yaraların kapsamlı bir tedavisi henüz bulunamamıştır ve yeni tedavi önerilerine ihtiyaç vardır. Hidrojen sülfürün (H₂S) yara iyileşmesi üzerindeki faydalı etkileri daba önce sağlıklı veya diyabetik hayvan modellerinde gösterilmiştir. H₂S'nin ayrıca hücrelerde ve hayvan modellerinde yara kapanmasını hızlandırdığı da bulunmuştur. H₂S'nin diyabetik yara iyileşmesinde faydalı olduğu gösterilmiştir ancak diyabetik koşullar altında yara iyileşmesi üzerindeki etkisi henüz açıklanmamıştır. Bu çalışmada; H₂S ve reaktif oksijen türlerinin (ROS) yüksek glukoz koşullarında fibroblasttaki yara iyileşmesi üzerindeki etkilerini araştırdık. Fibroblast hücre canlılığını ve yara iyileşmesi geçimini araştırmak için 2,3-bis-(2-metoksi-5-nitro-5-sülfofenil)-2H-tetrazolyum-5-karboksanilid (XTT) ve "scratch migration assay" kullandık. H₂S'nin yüksek glikozla inkübe edilmiş fibroblastlarda hücre canlılığını, çoğalmasını, göçünü artırarak ve ROS'u zayıflatarak yara iyileşmesini artırdığını gösterdik. Sonuçlarımıza göre eksojen H₂S yara onarımı sırasında oksidatif stresi azalttı. Sonuç olarak H₂S, oksidatif stresin inhibisyonu ile ilişkili olarak yara iyileşmesini hızlandırdı.

Anahtar Kelimeler: Yara iyileşmesi, fibroblast, yüksek glukoz, hidrojen sülfür

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INTRODUCTION

Wound healing is a complex pathophysiological process in diabetes. Impaired wound healing is one of the most serious diabetic complications, and effective treatment remains unknown. Several studies have reported that it benefits wound healing by improving angiogenesis, anti-inflammation and antioxidants in wound tissues (Zhang et al., 2014; Jeon et al., 2018; Yang et al., 2019). It was shown that diabetic rats with wounds showed inflammatory characteristics such as purulence and increased expression of TNF- α , reduced antioxidants and angiogenesis (Wang et al., 2015).

In recent years, research on wound healing has focused on gases that are formed endogenously. These gases are called gas transmitters because they can influence the functions of cells and tissues in picomolar quantities (Wang et al., 2004; Kolupaev et al., 2019). Hydrogen sulfide (H₂S) is one of these gas transmitters, which occurs endogenously in tissues and has a role as a regulatory molecule in wound healing. The first study showed an H₂S-mediated improvement of impaired wound healing in diabetic and obese *db/db* mouse models (Fang et al., 2014). Goren et al. showed that stimulation of wound tissue by exogenous application of H₂S-releasing drugs improved overall wound closure rates in the diseased mice. H₂S has been reported to accelerate gastric ulcer healing (Wallace et al., 2007) and healing of burn wounds in the skin (Cai et al., 2007; Papapetropoulos et al., 2009). Papapetropoulos et al. found that topical administration of H₂S improved the recovery from burn wounds in wild-type rats.

Oxidative stress is one of the main causes of wound healing impairment (Nathan et al., 1993). Reactive oxygen species (ROS) arising from inflammatory cells have vigorously participated in the pathogenesis of chronic ulcers (Singh et al., 2005). It was shown that ROS causing oxidative stress aggravates wound in diabetes, therefore; antioxidant productions may improve wound healing and healing of foot ul-

cers in diabetes (Park et al., 2011). Furthermore, ROS activates cellular molecular signals to disturb angiogenesis or cytokine secretion to delay wound healing (Zhang et al., 2014; Jeon et al., 2018). The ability of antioxidant effect of H₂S has been demonstrated in several systems by many studies (Meng et al., 2015; Xie et al., 2016; Lin et al., 2018; Meng et al., 2018; Wang et al., 2018). It has been reported that H₂S accelerated diabetic wound healing by inhibiting ROS production (Yang et al., 2019). Some studies indicated that H₂S itself is not a potent antioxidant compared with other antioxidants (Kimura et al., 2004; Jha et al., 2008; Hamar et al., 2012) but H₂S enhances the antioxidant effect via elevating endogenous antioxidantase such as superoxide dismutase (SOD) (Searcy et al., 1995; Sivarajah et al., 2009). Wang et al. reported that H₂S treatment increased the activity of SOD, decreased malondialdehyde (MDA) content (Wang et al., 2018).

Endogenous H₂S is produced from L-cysteine by catalysis of cystathionine beta-synthase (CBS) and cystathionine-gamma-lyase (CSE) (Wang, 2003). Inhibition of CSE promotes endothelial cell dysfunction induced by hyperglycemia (Degterev et al., 2005) and reduced H₂S levels in streptozotocin-induced diabetic rats (Jain et al., 2010).

Some studies on animal models and cell cultures, like L929 fibroblast cells, indicated that H₂S could promote wound healing. Fibroblasts play a crucial role in wound healing by producing collagen which is essential for tissue repair and remodeling. H₂S has been proposed to enhance the migration and proliferation of fibroblast cells, thereby potentially speeding up the wound healing process (Fang et al., 2014). Moreover, it has been shown that significant improvement in wound closure, an increase in the number of fibroblasts and inflammatory cells, and higher collagen I and collagen III levels in hyaluronic acid-treated rats (Taskan et al., 2021). Diabetes-induced impaired migration and proliferation of keratinocytes and fibroblasts has been reported in animal models (Patel et al., 2019). It is also known that high glucose impairs

the proliferation and migration of human gingival fibroblasts (Buranasin et al., 2018).

The aim of this study is to investigate the role of H₂S and ROS in wound healing under hyperglycemic (HG) conditions in fibroblasts.

MATERIAL AND METHODS

Cell culture

L929 mouse fibroblast cell line (Republic of Türkiye Ministry of Agriculture and Forestry, Institute of Şap; Mouse C3/An connective tissue, 92123004) was used in the experiments. Dulbecco's Modification Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2% L-glutamine and 1% penicillin-streptomycin was used as the medium in all groups. Fibroblasts were incubated in DMEM medium containing 10 mM and 25 mM glucose for 48 hours (Ueck et al., 2017). Control group was incubated with only DMEM medium which already contains 5 mM glucose which approximates normal blood sugar levels *in vivo*. Fibroblasts were incubated in DMEM containing 25 mM glucose for 48 hours, then H₂S donor sodium hydrogen sulfide (NaHS; 100 µM) and/or ROS scavenger SOD (50 U/ml) and CSE enzyme inhibitor propargylglycine (PAG; 30 mM) was added for 30 min, in line with a previous study (Fang et al., 2014). To investigate the effects of osmolarity, we used a mannitol treatment group with the same molarity as the HG group. In the mannitol group, fibroblasts were incubated for 48 hours in DMEM with 20 mM mannitol.

XTT assay

L929 cells were seeded into 96-well plates and incubated with medium for 24 hours (5 % CO₂, 37 °C) to form a confluent monolayer. After 24 hours of incubation, the medium was aspirated from the surface of the cells. Cells were incubated with 5, 10 and 25 mM glucose for 48 hours. After 48 hours of treatments, the medium was removed from wells. 100 µl of fresh medium and 50 µl of the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT)

/Phenazine methosulfate (PMS) solution was added to each well and the plates were incubated for 4 hours in the incubator (Nüve EN 400 CO₂ incubator; 5 % CO₂, 37 °C). Then, an aliquot of 100 µl was transferred from each well into the corresponding well of a new 96-well plate. The absorbances were measured with a microplate reader (Biotek Powerwave XS2) at 450 nm (reference wavelength 630 nm). The results were normalized to the control group. In another group of experiments, cells were seeded into 96-well plates and incubated with 5 and 25 mM glucose for 48 hours, then 30 min with NaHS and/or SOD and PAG. After incubation, the medium was aspirated and XTT assay was performed as described above.

Scratch migration assay

The migration of fibroblasts was analyzed by the scratch migration assay as previously described (Liang et al., 2007). L929 cells were seeded in 24-well plates and incubated with DMEM at 37 °C and 5 % CO₂ for 24 to 48 hours to permit cell adhesion and the formation of a confluent monolayer. After 90% confluence was achieved, an artificial gap, so called "scratch", approximately 0.2-0.4 mm in width, was created with a 200 µL sterile pipette tip. The medium, along with any detached cells and debris, was immediately removed. Cells were incubated with 5 and 25 mM glucose for 48 hours and then 30 min with NaHS and/or SOS and PAG. Images were taken by an inverted microscope (Leica DM IL) equipped with a digital camera to follow cell migration and morphological changes of cells. Images were taken just after the "scratch" was created which was accepted as the initial wound area, and images were taken at the 24th and 48th hours of incubation. The area of the scratch and wound closure were measured by ImageJ 1.53e software (National Institute of Health). Wound closure was quantified as wound area relative to the initial wound area.

Chemicals

DMEM was purchased from Wisent Bioproducts (Quebec, Canada). FBS and trypsin-EDTA were purchased from Cegrogen-Biotech (Germany). L-gluta-

mine and penicillin-streptomycin were purchased from Biochrom (Cambridge, UK). XTT was purchased from PanReac AppliChem (Germany). PMS, glucose, mannitol, NaHS, SOD and PAG were purchased from Sigma Aldrich (St Louis, USA).

Statistical analysis

Statistical analysis was performed with a one-way ANOVA *post hoc* Tukey test. Data were represented as mean \pm standard error mean (SEM). $P < 0.05$ was accepted as statistically significant. GraphPad Prism 5.0 software was used for statistical analysis (San Diego, USA).

RESULTS AND DISCUSSION

Firstly, we examined the cytotoxic effect of increasing concentration of glucose on L929 cells at 48 hours. 10 mM glucose did not affect cell viability compared to control group which was incubated with

5 mM glucose, but incubation with 25 mM glucose decreased cell viability after 48 hours compared to control group (Figure 1). 10 mM glucose is known as pre-diabetic as well as higher glucose concentration is considered as diabetic in *in vivo* studies (https://www.sigmaaldrich.com/IT/en/technical_documents/technical-article/cell-culture-and-cell-analysis/mammalian-cell-culture/glucose). The incubation with glucose at 25 mM for 48 hours was chosen as the HG condition for further experiments to observe the exaggerated responses. Control group was incubated with only DMEM medium which already contains 5 mM glucose which approximates normal blood sugar levels *in vivo*. The cell viability in mannitol group was not different from that of the control group, which showed that the difference in HG group was not related to hyperosmolarity.

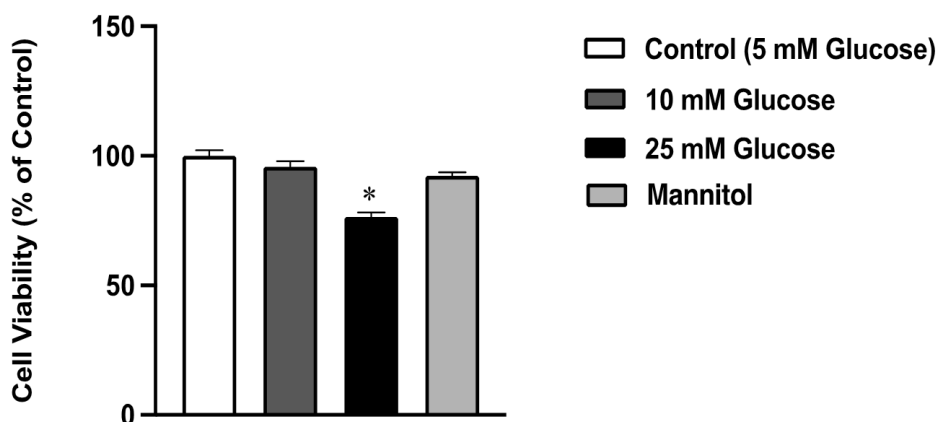


Figure 1. Effect of glucose incubation (10, 25 mM) and mannitol on L929 cell viability after 48 hours. Results were given as a percentage of control group (* $P < 0.05$ significant compared to control group; $n=5$).

In the second part of our study, we examined cell viability and proliferation were assessed with NaHS, SOD and PAG in control group (5 mM glucose) and HG (25 mM glucose) conditions. H_2S was administered in the form of NaHS, which is well established as a reliable H_2S donor. NaHS is a rapid-releasing H_2S donor widely used in recent *in vivo* and *in vitro* H_2S studies (Sivarajah et al., 2006; Cai et al., 2007; Papa-

petropoulos et al., 2009; Wang et al., 2010). In our results, NaHS, SOD and PAG did not change cell viability in control group (incubation with 5 mM glucose, for 48 hours). In HG conditions (incubation with 25 mM glucose, for 48 hours), PAG treatment did not alter the decrease in cell viability, whereas NaHS and SOD significantly increased cell viability (Figure 2). Liu et al. have demonstrated that exogenous

H₂S significantly prevented cell death, decreased the generation of apoptotic markers, and suppressed mitochondrial ROS production in rat aortic endothelial cells under HG conditions (Liu et al., 2016). Moreover, it was reported that NaHS treatment reduces HG-induced cytotoxicity, apoptosis, oxidative stress and inflammation in human umbilical vein endothelial cells (HUVECs) (Fengxia et al., 2020). Increasing studies indicate that H₂S executes various biological functions, such as reducing oxidative stress (Calvert et al., 2009; Vacek et al., 2010). Both clinical and ex-

perimental studies show that hyperglycemia results in reduced antioxidants and elevated oxidative stress involved in the development of diabetes and its complications (Grieve et al., 2004; Molavi et al., 2004). In parallel with previous studies, ROS scavenger SOD significantly increased cell viability under HG conditions in the present study. Furthermore, the fact that the H₂S synthesizing enzyme-CSE inhibitor PAG did not change the decreased cell viability confirms the effect of H₂S on oxidative stress.

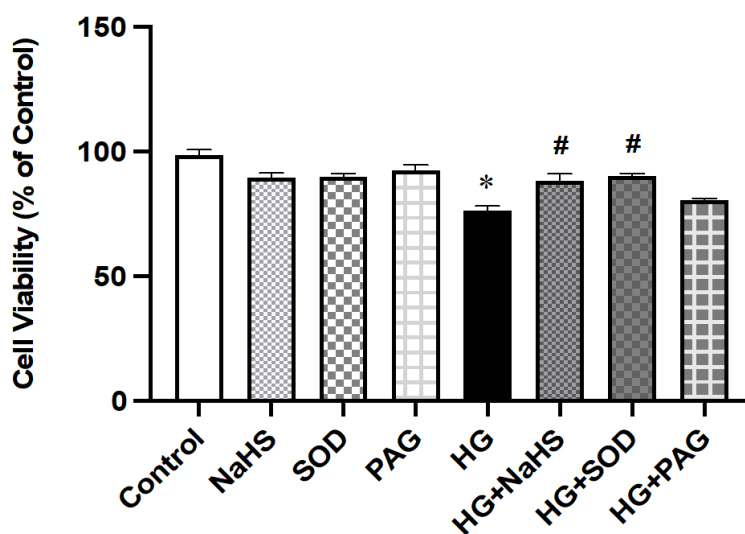


Figure 2. Effect of NaHS (100 μM), SOD (50 U/ml) and PAG (30 mM) on L929 cell viability in control group and HG conditions after 48 hours. Results were given as a percentage of control group (5 mM glucose). (**P*<0.05 significant compared to control group, #*P*<0.05 significant compared to HG; n=5).

Finally, we examined the effects of H₂S and/or SOD and PAG on wound closure in fibroblasts. In HG conditions (25 mM glucose), there is a significant decrease in wound closure compared to control group (5 mM glucose) after 24 and 48 hours. NaHS or SOD treatment significantly increased wound closure compared to HG after 24 and 48 hours (Figure 3A, B). In parallel with our study, it was reported that NaHS promoted cell migration in both skin fibroblasts and human keratinocytes around the wound healing area (Mengting et al., 2019). It was also shown that NaHS accelerated wound healing in ob/ob mice and this ef-

fect of NaHS was glucose-independent, as it did not affect the glucose levels (Zhao et al., 2017). When we investigated the effects of NaHS and SOD combination, there was a significant further increase in wound closure in NaHS and SOD combination treatment compared to NaHS and SOD alone under HG after 24 and 48 hours (Figure 3A, 3B). Over the past decade, H₂S has been discovered to improve skin wound healing in diabetic mice via antioxidants (Wang et al., 2015; Zhao et al., 2017). Treatment with H₂S in diabetic rats accelerated wound closure and increased the activity of SOD, decreased MDA content (Guoguang

et al., 2015). These findings suggest that H₂S improves wound healing in diabetes by enhancing antioxidants. It was also reported that H₂S increased SOD activity,

enhanced haem oxygenase-1 (HO-1) protein expression, and finally alleviated oxidative stress injury in the skin (Wang et al., 2015).

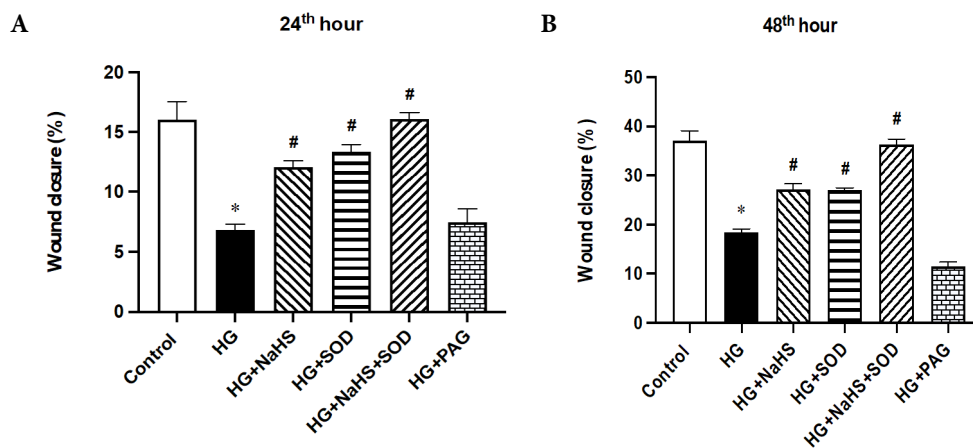


Figure 3. Effect of NaHS (100 μ M) and/or SOD (50 U/ml) and PAG (30 mM) treatments on wound healing of fibroblasts in HG for 24 (A) and 48 (B) hours after scratch assay. Wound closure was calculated as a percentage of the initial wound area. (* P <0.05 compared to control group; # P <0.05 significant compared to HG; n=6).

On the other hand, H₂S synthesizing enzyme-CSE inhibitor PAG did not change the decreased wound closure in HG conditions (Figure 3A, B). CSE appeared to be the most relevant H₂S-producing enzyme in wound tissue (Goren et al., 2019). There are many studies stating that impairment in wound healing is associated with enzymes involved in H₂S production (Zhao et al., 2017; Liu et al., 2014; Degterev et al., 2005; Jain et al., 2010). Zhao et al. were reported that impaired diabetic wound healing is associated with reduced CSE expression. H₂S improves diabetic wound healing by restoring endothelial progenitor cell (EPC) function in type 2 diabetes. It has been reported that EPC functions of the db/+ mice were significantly reduced after in vitro PAG treatment or CSE silencing (Liu et al., 2014). Moreover, inhibition of CSE promotes endothelial cell dysfunction induced by hyperglycemia (Degterev et al., 2005) and reduces H₂S levels in streptozotocin-induced diabetic rats (Jain et al., 2010).

CONCLUSION

H₂S was suggested as one of the agents that have been found beneficial and may increase the migration and proliferation of fibroblasts. In our study, we showed that H₂S increased wound healing by enhancing the viability, proliferation and migration of fibroblasts and attenuating oxidative stress under HG conditions. The present study suggested that exogenous H₂S attenuated oxidative stress during wound repair. In summary, H₂S accelerated wound healing, which might be related to oxidative stress inhibition. This protective effect indicates that H₂S may be a potential therapeutic compound for skin wound closure in the clinic.

CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

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