FABAD J. Pharm. Sci., 48, 3, 539-548, 2023
Doi: 10.55262/fabadeczacilik.1354775
RESEARCH ARTICLE

Hydrogen Sulfide Promotes Wound Healing by Attenuating Reactive Oxygen Species in Fibroblasts under Hyperglisemic 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- -nitro-5-sulfophenyl)-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 Türevlerini 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 daha 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 türevlerinin (ROS) yüksek glukoz koşullarında fibroblasttaki yara iyileşmesi üzerindeki etkilerini araştırdık. Fibroblast hücre canlılığını ve yara iyileşmesi göçünü araştırmak için 2,3-bis-(2-metoksi--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 arttırdığını 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*

Received: 4.09.2023
Revised: 1.10.2023
Accepted: 2.10.2023

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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 (H_2S) 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 H_2S-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 H_2S-releasing drugs improved overall wound closure rates in the diseased mice. H_2S 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 H_2S 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 ulcers 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 H_2S 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 H_2S accelerated diabetic wound healing by inhibiting ROS production (Yang et al., 2019). Some studies indicated that H_2S itself is not a potent antioxidant compared with other antioxidants (Kimura et al., 2004; Jha et al., 2008; Hamar et al., 2012) but H_2S enhances the antioxidant effect via elevating endogenous antioxidase such as superoxide dismutase (SOD) (Searcy et al., 1995; Sivarajah et al., 2009). Wang et al. reported that H_2S treatment increased the activity of SOD, decreased malondialdehyde (MDA) content (Wang et al., 2018).

Endogenous H_2S 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 H_2S levels in streptozotocin-induced diabetic rats (Jain et al., 2010).

Some studies on animal models and cell cultures, like L929 fibroblast cells, indicated that H_2S could promote wound healing. Fibroblasts play a crucial role in wound healing by producing collagen which is essential for tissue repair and remodeling. H_2S 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 H_2S 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 H_2S 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 % CO_2, 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 CO_2 incubator; 5 % CO_2, 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 % CO_2 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 24^{th} and 48^{th} 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 ± 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.

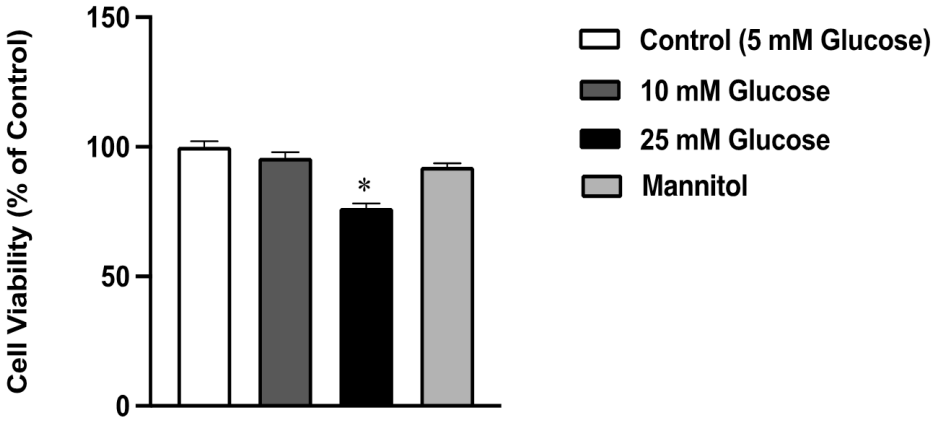

Figure 1. Effect of glucose incubation (10, 25 mM) and mannitol on L929 cell viability after 48 hours. Results were given as a percentage of control group (* $P<0.05$ significant compared to control group; n=5).

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; Papapetropoulos 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

H_2S 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 H_2S executes various biological functions, such as reducing oxidative stress (Calvert et al., 2009; Vacek et al., 2010). Both clinical and experimental 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 H_2S synthesizing enzyme-CSE inhibitor PAG did not change the decreased cell viability confirms the effect of H_2S on oxidative stress.

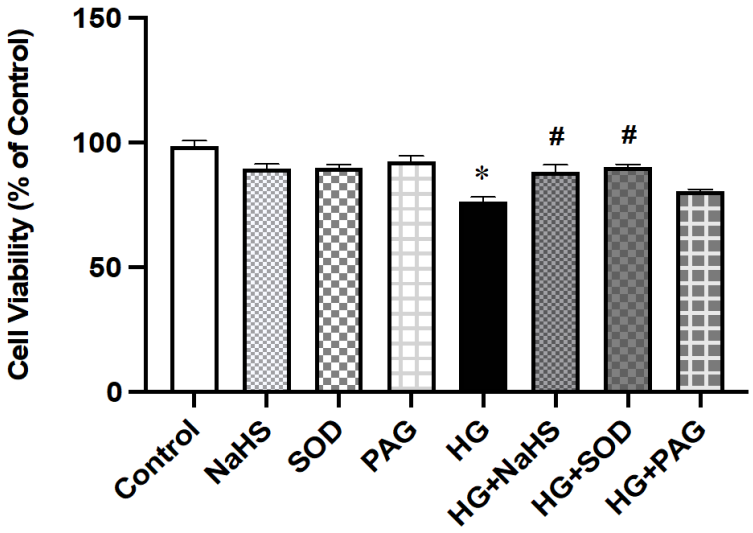

Figure 2. Effect of NaHS (100 μM), SOD (50 U/ml) and PAG (30 mM) on L929 cell viability in control group and HG conditions after 48 hours. Results were given as a percentage of control group (5 mM glucose). (*P<0.05 significant compared to control group, #P<0.05 significant compared to HG; n=5).

Finally, we examined the effects of H_2S 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 effect 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, H_2S has been discovered to improve skin wound healing in diabetic mice via antioxidants (Wang et al., 2015; Zhao et al., 2017). Treatment with H_2S in diabetic rats accelerated wound closure and increased the activity of SOD, decreased MDA content (Guoguang

et al., 2015). These findings suggest that H_2S improves wound healing in diabetes by enhancing antioxidants. It was also reported that H_2S increased SOD activity, enhanced haem oxygenase-1 (HO-1) protein expression, and finally alleviated oxidative stress injury in the skin (Wang et al., 2015).

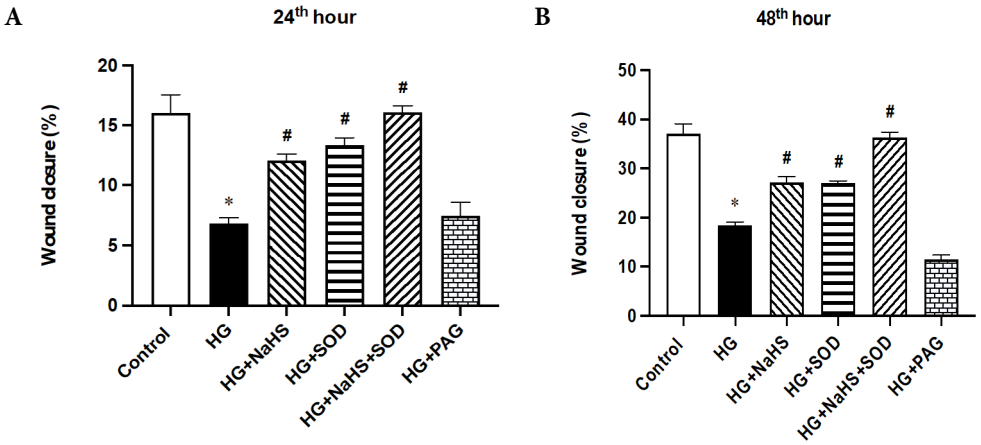

Figure 3. Effect of NaHS (100 μM) and/or SOD (50 U/ml) and PAG (30 mM) treatments on wound healing of fibroblasts in HG for 24 (A) and 48 (B) hours after scratch assay. Wound closure was calculated as a percentage of the initial wound area. (*P<0.05 compared to control group; #P<0.05 significant compared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

On the other hand, H_2S 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 H_2S-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 H_2S 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. H_2S 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 H_2S levels in streptozotocin-induced diabetic rats (Jain et al., 2010).

CONCLUSION

H_2S 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 H_2S 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 H_2S attenuated oxidative stress during wound repair. In summary, H_2S accelerated wound healing, which might be related to oxidative stress inhibition. This protective effect indicates that H_2S 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.

REFERENCES

Buranasin, P., Mizutani, K., Iwasaki K., Pawaputanon Na Mahasarakham, C., Kido, D., Takeda, K., Izumi, Y. (2018). High glucose-induced oxidative stress impairs proliferation and migration of human gingival fibroblasts. *PLoS One*, *13*(8). doi: 10.1371/journal.pone.0201855

Cai, W. J., Wang, M. J., Moore, P. K., Jin, H. M., Yao, T., Zhu, Y. C. (2007). The novel proangiogenic effect of hydrogen sulfide is dependent on Akt phosphorylation. *Cardiovasc Res, 76*, 29–40. doi: 10.1016/j.cardiores.2007.05.026

Calvert, J. W., Jha, S., Gundewar, S., Elrod, J. W., Ramachandran, A., Pattillo, C. B.,…Lefer, D. J. (2009). Hydrogen sulfide mediates cardioprotection through Nrf2 signaling. *Circ Res, 105,* 365-374. doi: 10.1161/CIRCRESAHA.109.199919

Cell culture and cell culture analysis. https://www.sigmaaldrich.com/IT/en/technical-documents/technical-article/cell-culture-and-cell-culture-analysis/mammalian-cell-culture/glucose (accessed on 27 March 2023).

Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N.,…Yuan, J. (2005). Chemical inhibitor of non- apoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol, 1*(2), 112–119. doi: 10.1038/nchembio711

Fengxia, L., Yiying, Y., Shanyin, W., Xiaojing, H., Zhijian, P., Xiao, K.,…Yinzhi, S. (2020). Hydrogen Sulfide Protects Against High Glucose-Induced Human Umbilical Vein Endothelial Cell Injury Through Activating PI3K/ Akt/eNOS Pathway. *Drug, Design, Development and Therapy, 14,* 621-633. doi: 10.2147/DDDT.S242521

Goren, I., Köhler, Y., Aglan, A., Pfeilschifter, J., Beck, K., Frank, S. (2019). Increase of cystathionine-γ-lyase (CSE) during late wound repair: Hydrogen sulfide triggers cytokeratin 10 expression in keratinocytes. *Nitric Oxide, 87,* 31–42. doi: 10.1016/j.niox.2019.03.004

Grieve, D. J., Byrne, J. A., Cave, A. C., Shah, A. M. (2004). Role of oxidative stress in cardiac remodeling after myocardial infarction. *Heart Lung Circ, 13,* 132-138. doi: 10.1016/j.hlc.2004.02.008

Hamar, J., Solymar, M., Tanai, E., Cseplo, P., Springo, Z., Berta, G.,…Koller, A. (2012). Bioassay comparison of the antioxidant efficacy of hydrogen sulfide and superoxide dismutase in isolated arteries and veins. *Acta Physiol Hung, 99,* 411-419. doi: 10.1556/Aphysiol.99.2012.4.5

Jain, S. K., Bull, R., Rains, J. L., Bass, P. F., Levine S. N., Reddy, S.,…Bocchini J. A. (2010). Low levels of hydrogen sulfide in the blood of diabetes patients and streptozotocin-treated rats cause vascular inflammation. *Antioxid Redox Signal, 12*(11), 1333–1337. doi: 10.1089/ars.2009.2956

Jeon, H. H., Yu, Q., Lu, Y., Spencer, E., Lu, C., Milovanova, T.,…Graves, D. T. (2018). FOXO1 regulates VEGFA expression and promotes angiogenesis in healing wounds. *J Pathol, 245,* 258- 264. doi: 10.1002/path.5075

Jha, S., Calvert, J. W., Duranski, M. R., Ramachandran, A., Lefer, D. J. (2008). Hydrogen sulfide attenuates hepatic ischemia-reperfusion injury: role of antioxidant and antiapoptotic signaling. *Am J Physiol Heart Circ Physiol, 295,* H801- 806. doi: 10.1152/ajpheart.00377.2008

Kimura, Y., and Kimura, H. (2004). Hydrogen sulfide protects neurons from oxidative stress. *FASEB J, 18,* 1165-1167. doi: 10.1096/fj.04-1815fje

Kolupaev, Y. E., Karpets, Y. V., Beschasniy, S. P., Dmitriev, A. P. (2019). Gasotransmitters and their role in adaptive reactions of plant cells. *Cytology and Genetics, 53*(5), 392-406. doi: 10.3103/S0095452719050098

Liang, C. C., Park, A. Y., Guan, J. L. (2007). In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc, 2*(2), 329-333. doi: 10.1038/nprot.2007.30

Lin, Z., Altaf, N., Li, C., Chen, M., Pan, L., Wang, D.,…Ji, Y. (2018). Hydrogen sulfide attenuates oxidative stress-induced NLRP3 inflammasome activation via S-sulfhydrating c-Jun at Cys269 in macrophages. *Biochim Biophys Acta Mol Basis Dis, 1864,* 2890-900. doi: 10.1016/j.bbadis.2018.05.023

Liu, F., Chen, D. D., Sun, X., Xie, H. H., Yuan, H., Jia, W., Chen, A. F. (2014). Hydrogen sulfide improves wound healing via restoration of endothelial progenitor cell functions and activation of angiopoietin-1 in type 2 diabetes. *Diabetes, 63,* 1763–1778. doi: 10.2337/db13-0483

Liu, J., Wu, J., Sun, A., Sun, Y., Yu, X., Liu, N.,…Zhang, W. (2016). Hydrogen sulfide decreases high glucose/ palmitate-induced autophagy in endothelial cells by the Nrf2-ROS- AMPK signaling pathway. *Cell Biosci, 6,* 33. doi: 10.1186/s13578-016-0099-1

Meng, G., Zhu, J., Xiao, Y., Huang, Z., Zhang, Y., Tang, X.,…Ji, Y. (2015). Hydrogen sulfide donor GYY4137 protects against myocardial fibrosis. *Oxid Med Cell Longev, 2015,* 691070. doi: 10.1105/2015/691070

Meng, G., Liu, J., Liu, S., Song, Q., Liu, L., Xie, L.,… Ji, Y. (2018). Hydrogen sulfide pretreatment improves mitochondrial function in myocardial hypertrophy via a SIRT3-dependent manner. *Br J Pharmacol, 175,* 1126–1145. doi: 10.1111/bph.13861

Mengting, X., Yuyun, H., Qi, Y., Meng, G., Yang, S. (2019). Exogenous hydrogen sulphide supplement accelerates skin wound healing via oxidative stress inhibition and vascular endothelial growth factor enhancement. *Experimental Dermatology, 28,* 776-785. doi: 10.1111/exd.13930

Molavi, B., and Mehta, J. L. (2004). Oxidative stress in cardiovascular disease: molecular basis of its deleterious effects, its detection, and therapeutic considerations. *Curr Opin Cardiol, 19,* 488-493. doi: 10.1097/01.hco.0000133657.77024.bd.

Nathan, D. M., Genuth, S., Lachin, J., Cleary, P., Crofford, O., Davis, M.,…Siebert, C. (1993). The effect of intensive treatment of diabetes on the development and progression of long- term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N Engl J Med, 329,* 977-986. doi: 10.1056/NEJM199309303291401

Papapetropoulos, A., Pyriochou, A., Altaany, Z., Yang, G., Marazioti A., Zhou, Z.,…Szabo, C. (2009). Hydrogen sulfide is an endogenous stimulator of angiogenesis. *Proc Nat Acad Sci, 106,* 21972–21977. doi: 10.1073/pnas.0908047106

Park, N. Y., and Lim, Y. (2011). Short term supplementation of dietary antioxidants selectively regulates the inflammatory responses during early cutaneous wound healing in diabetic mice. *Nutr Metab (Lond), 8,* 80. doi: 10.1186/1743-7075-8-80.

Patel, S., Srivastava, S., Singh, M. R., Singh, D. (2019). Mechanistic insight into diabetic wounds: Pathogenesis, molecular targets and treatment strategies to pace wound healing. *Biomed Pharmacother, 112,* 108615. doi: 10.1016/j.biopha.2019.108615

Searcy, D. G., Whitehead, J. P., Maroney, M. J. (1995). Interaction of Cu, Zn superoxide dismutase with hydrogen sulfide. *Arch Biochem Biophys, 318,* 251-263. doi: 10.1006/abbi.1995.1228

Singh, N., Armstrong, D. G., Lipsky, B. A. (2005). Preventing foot ulcers in patients with diabetes. *JAMA, 293,* 217-228. doi: 10.1001/jama.293.2.217

Sivarajah, A., Collino, M., Yasin, M., Benetti, E., Gallicchio, M., Mazzon, E.,…Thiemermann, C. (2009). Anti-apoptotic and anti-inflammatory effects of hydrogen sulfide in a rat model of regional myocardial I/R. *Shock, 31,* 267-274. doi: 10.1097/SHK.0b013e318180ff89

Sivarajah, A., McDonald, M. C., Thiemermann, C. (2006). The production of hydrogen sulfide limits myocardial ischemia and reperfusion injury and contributes to the cardioprotective effects of preconditioning with endotoxin, but not ischemia in the rat. *Shock, 26,* 154–161. doi: 10.1097/01.shk.0000225722.56681.64

Taskan, M. M., Balci Yuce, H., Karatas, O., Gevrek, F., Isiker Kara, G., Celt, M.,…Taskan, E. S. (2021). Hyaluronic acid with antioxidants improve wound healing in rats. *Biotech Histochem, 96*(7), 536-545. doi: 10.1080/10520295.2020.1832255

Ueck, C., Volksdorf, T., Houdek, P., Vidal, Y. S. S., Sehner, S., Brandner, J. M. (2017). Comparison of In-Vitro and Ex-Vivo Wound Healing Assays for the Investigation of Diabetic Wound Healing and Demonstration of a Beneficial Effect of a Triterpene Extract. *PLoS One, 12*(1), e0169028. doi: 10.1371/journal.pone.0169028

Vacek, T. P., Gillespie, W., Tyagi, N., Vacek, J. C., Tyagi, S. C. (2010). Hydrogen sulfide protects against vascular remodeling from endothelial damage. *Amino Acids, 39,* 1161-1169. doi: 10.1007/s00726-010-0550-2

Wallace, J. L., Dicay, M., McKnight, W., Martin, G. R. (2007). Hydrogen sulfide enhances ulcer healing in rats. *FASEB J, 21,* 4070–4076. doi: 10.1096/fj.07-8669com

Wang, R. (2003). The gasotransmitter role of hydrogen sulfide. *Antioxid Redox Signal, 5,* 493- 501. doi: 10.1089/152308603768295249

Wang, R. (2004). *Signal Transduction and the Gasotransmitters: NO, CO, and H_2S in Biology and Medicine.* Springer Science & Business Media.

Wang, M. J., Cai, W. J., Li, N., Ding, Y. J., Chen, Y., Zhu, Y. C. (2010). The hydrogen sulfide donor NaHS promotes angiogenesis in a rat model of hind limb ischemia. *Antioxid Redox Signal, 12,* 1065–1077. doi: 10.1089/ars.2009.2945

Wang, G., Li, W., Chen, Q., Jiang, Y., Lu, X., Zhao, X. (2015). Hydrogen sulfide accelerates wound healing in diabetic rats. *Int J Clin Exp Pathol, 8*(5), 5097-5104. www.ijcep.com

Wang, J., Wang, W., Li, S., Han, Y., Zhang, P., Meng, G.,…Ji, Y. (2018). Hydrogen Sulfide As a Potential Target in Preventing Spermatogenic Failure and Testicular Dysfunction. *Antioxid. Redox Signal, 28,* 1447. doi: 10.1089/ars.2016.6968

Xie, L., Gu, Y., Wen, M., Zhao, S., Wang, W., Ma, Y.,… Ji, Y. (2016). Hydrogen Sulfide Induces Keap1 S-sulfhydration and Suppresses Diabetes-Accelerated Atherosclerosis via Nrf2 Activation. *Diabetes, 65,* 3171. doi: 10.2337/db16-0020

Yang, C. T., Chen, L., Chen, W. L., Li, N., Chen, M. J., Li, X.,…Liu, J. (2019). Hydrogen sulfide primes diabetic wound to close through inhibition of NETosis. *Mol Cell Endocrinol, 480,* 74-82. doi: 10.1016/j.mce.2018.10.013

Zhang, G. Y., Wu, L. C., Dai, T., Chen, S. Y., Wang, A. Y., Lin, K.,…Li, Z. J. (2014). NADPH oxidase-2 is a key regulator of human dermal fibroblasts: a potential therapeutic strategy for the treatment of skin fibrosis. *Exp Dermatol, 23,* 639–644. doi: 10.1111/exd.12479

Zhao, H., Lu, S., Chai, J., Zhang, Y., Ma, X., Chen, J.,… Liu, Y. (2017). Hydrogen sulfide improves diabetic wound healing in ob/ob mice via attenuating inflammation. *J Diabetes Its Complicat, 31,* 1363–1369. doi: 10.1016/j.jdiacomp.2017.06.011