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Role of H_2S Supplementation on Burn Wound Healing and Molecular Chaperones

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ABSTRACT

Treatment of non-healing burn injuries is a major challenge for the current scientific research. Hydrogen sulfide (H_2S) is an endogenous gasotransmitter, which regulates redox homeostasis and cytoprotection during pathophysiological conditions. Similarly, heat shock proteins (HSPs) are molecular chaperones, which also confer cytoprotection during the wound repair process. Notably, the role of H_2S as a regulator of HSPs during burn wound healing is still elusive. The present study investigated the effects of H_2S supplementation on molecular chaperones during full-thickness, third-degree burn wound healing in the experimental rats. The animals were treated with sodium hydrosulphide (NaHS) as H_2S donor (5 mg/kg body weight, intraperitoneal) daily for 10 days prior to burn-induction and continued till the fifth-day post-wounding. Histopathological analysis (Masson's trichrome) revealed enhanced wound healing evident by increased collagen fiber deposition, cellular proliferation and re-epithelialisation in NaHS administered group as compared to the burn control. Furthermore, immunoblot analyses demonstrated significantly increased protein expression of molecular chaperons viz. HSP90, HSP70, HSP27, and GRP78 in H_2S treated group as compared to control. Therefore, the present study signifies that H_2S supplementation upregulates the protein expression levels of molecular chaperones, which could facilitate the cytoprotection during the tissue repair process and accelerates the burn wound healing.

Keywords: Burn wound healing; Collagen; Cytoprotection; Heat shock proteins; Hydrogen Sulfide; Molecular chaperones

1. INTRODUCTION

Burn injuries are prevalent and debilitating pathophysiological conditions, which confer unbearable pain to the patients. The normal wound repair, a well-orchestrated process encompasses four overlapping phases such as hemostasis, inflammation, proliferation and remodeling. The deviation from the sequential phases of the normal healing process further impedes the progression of burn wound repair. The burn wound healing is delayed owing to many critical factors viz. dysregulated inflammation, neuropathy, vascular system impairment, excessive proteolysis, oxidative stress and septicemia[1-2]. Therefore, the innumerable complexities concomitant with the treatment of intractable burn wounds impose a profound burden on the medical healthcare system. Hence, the prevailing situations have compelled the scientific community to develop effective new therapeutic interventions and treatment modalities to overcome the impediments related to the healing of burn injuries.

Hydrogen sulfide (H_2S) is one of the major gasotransmitters produced in mammalian cells through complex enzyme system[3]. Recent in-vivo and human studies have demonstrated that H_2S plays an important function in various diseases associated with neurovascular, cardiovascular, and gastrointestinal systems[4,5]. Furthermore, H_2S exerts its biological effects via activation and inactivation of molecular targets involved in multiple cellular processes such as redox homeostasis[6], neovascularisation[7], cell death and cytoprotection[8]. These cellular processes are also involved in the regulation of the healing of chronic wounds like burns. Increased levels of endogenous H_2S scavenge ROS and alleviate oxidative stress by activating multiple antioxidants such as reduced glutathione, superoxide dismutase and catalase[9]. A study demonstrated that H_2S treatment prevents neurodegeneration by activating anti-inflammatory and antioxidant pathways in Parkinson's disease[10]. Recently our group also reported that H_2S imparted cytoprotection to cardiomyocytes during β-adrenergic stress response via activation of glucose-6-phosphate dehydrogenase (G6PD) pathway[11]. Contrary, other studies have also shown that H_2S upregulated the inflammatory response in various diseases[12]. Although, H_2S is known to regulate multifarious cellular processes in various pathological conditions, however, the role of H_2S in burn wound healing is still not properly understood.

Heat shock proteins (HSPs) are molecular chaperones involved in the regulation of multiple cell survival and cell protection pathways under stress conditions. Excessive cellular stress denatures proteins and actuates cell signaling pathways associated with the regulation of cell death/survival. Under stress conditions, HSPs bind to the target proteins and protect their structural and functional integrity. Thus, HSPs play a very important role in maintaining metabolic and regulatory functions by keeping a check on cellular protein

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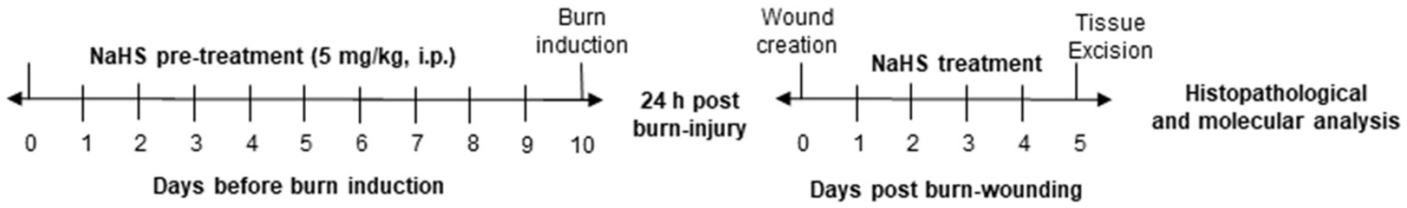

Figure 1. Schematic experimental design for supplementation of sodium hydrosulfide (H_2S donor) (NaHS, 5 mg/kg body weight; i.p) administered in experimental rats for 10 days before burn induction and till fifth-day post-wounding. Histological and molecular parameters were analyzed in the excised wound tissue at the fifth-day post-wounding.

turnover. Similarly, HSPs also play a critical role during the process of wound healing by regulating various stages of redox homeostasis, inflammation, apoptosis, and proliferation[13]. A study reported that inhibition of the K_{ATP}/p38/hsp27 signaling axis decreased the H_2S-mediated proliferation of endothelial cells (angiogenesis) and also decreased wound healing in mice[7]. Another study also showed the association between H_2S-mediated cytoprotective effects and hsp70/HO-1/hsp27 signal transduction pathways during hypoxic stress (ischemia) in rat model[14]. As cellular proliferation and cytoprotection are critical factors of the wound repair process, hence, it prompted us to explore the role of HSPs in burn wound healing after H_2S supplementation. Therefore, the present study investigated the effects of H_2S supplementation on molecular chaperones during full-thickness, third-degree burn wound healing in the experimental rats.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Adult male Sprague-Dawley rats (180 ± 20 g) were maintained at 25 ± 1°C in the Institute's animal house facility with a relative humidity of 55 ± 5 per cent and light: dark cycle of 12 h: 12 h with *ad libitum* access to food and water. Animal experimental procedures conform to the guidelines of the Institutional Animal Ethical Committee (IAEC/DIPAS/2017-14) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

2.2 Burn Wound Creation

A cocktail of ketamine-xylazine (90 mg/kg and 10 mg/kg, body weight, respectively; i.p.) was used to anaesthetise the animals. Hairs from the dorsal skin surface were removed using an electric clipper. A full-thickness, third-degree burn wound was created by using the metal rod of 1.5 cm diameter, heated to 85 °C for 20 s as described previously[15]. Animals were allowed to recover and after 24 h, the dead skin tissue was excised using sterile surgical. The animals were euthanised by administrating an overdose of thiopental (90 mg/kg, i.p.) at the fifth-day post-wounding. Wound tissues were excised to evaluate molecular markers and histopathological analyses.

2.3 Experimental Study and Drug Administration

Thirty animals were selected and randomised into five experimental groups viz. uninjured skin (US), burn control (BC), NaHS administered group (H_2S), povidone-iodine ointment treated (PI, reference care), and combination (H_2S+PI) group. Sodium hydrosulfide (NaHS; Cayman Chemical, USA) was freshly prepared (2 mg/ml) in PBS (0.15 M, pH 7.4) and used as an H_2S-donor and administered intraperitoneal at the dose of 5 mg/kg body weight, in respective groups for 10 days, before the burn induction followed till 5 days post-wounding (Fig. 1). The PI ointment was used as reference care (5 % w/v, Cipladine, Cipla, India).

2.4 Masson's Trichrome Staining

The wound tissue was collected on the fifth-day post wounding to perform Masson's trichrome staining. The excised tissue was fixed in the buffered formalin (10 %) for 24 hrs. The fixed tissue was dehydrated by treatment through alcohol gradation series (10 %, 30 %, 50 %, 70 %, 90 %, and 100 %). The dehydrated tissue was cleared in xylene and embedded in paraffin wax at 56 °C. Sections of 5-µm thickness were obtained from paraffin-embedded tissue through microtomy and kept on gelatin-coated glass slides. Slide sections were deparaffinised in xylene and rehydrated by passing through in alcohol gradation series (100 %, 90 %, 70 %, 50 %, 30 %, and 10 %) and deionised water. Each immersion step was performed for 5 min. The sections were left overnight in Bouin's fixative at room temperature (RT). After incubation sections were washed under running tap water to remove the yellow color of picric acid. Tissue sections were immersed in Weigert's hematoxylin solution for 10 mins to stain the nuclei (black) followed by rinsing with deionised water for 5 min. The sections were dried and stained with Biebrich Scarlet-acid Fuschin reagent for 5 min. to stain muscle and cytoplasm (red) followed by rinsing in water. The sections were eventually immersed in Phosphotungstic/phosphomolybdic acid for 10 min. and subsequently stained with aniline-blue dye (5 min.) to stain the collagen (blue) followed by rinsing in distilled water. The slides were placed in 1 per cent acetic acid (1 min.), air-dried, and cleared in xylene before mounting using DPX. The MT stained slides were examined under bright-field microscopy (Nikon Eclipse Ti2, USA) followed by photomicrography.

2.5 Preparation of Tissue Homogenate

The wound tissue was collected and homogenised using a Polytron homogeniser (PT 3100, Switzerland) in an ice-cold radioimmunoprecipitation (RIPA) buffer. Protease inhibitors cocktail (Sigma-Aldrich, USA) was also added prior to homogenisation. The tissue homogenates were centrifuged at 4000×g for 30 min at 4 °C. The supernatant was taken and immediately stored at -80 °C. The protein concentration was measured according to the method described by Lowry *et al.*[16]

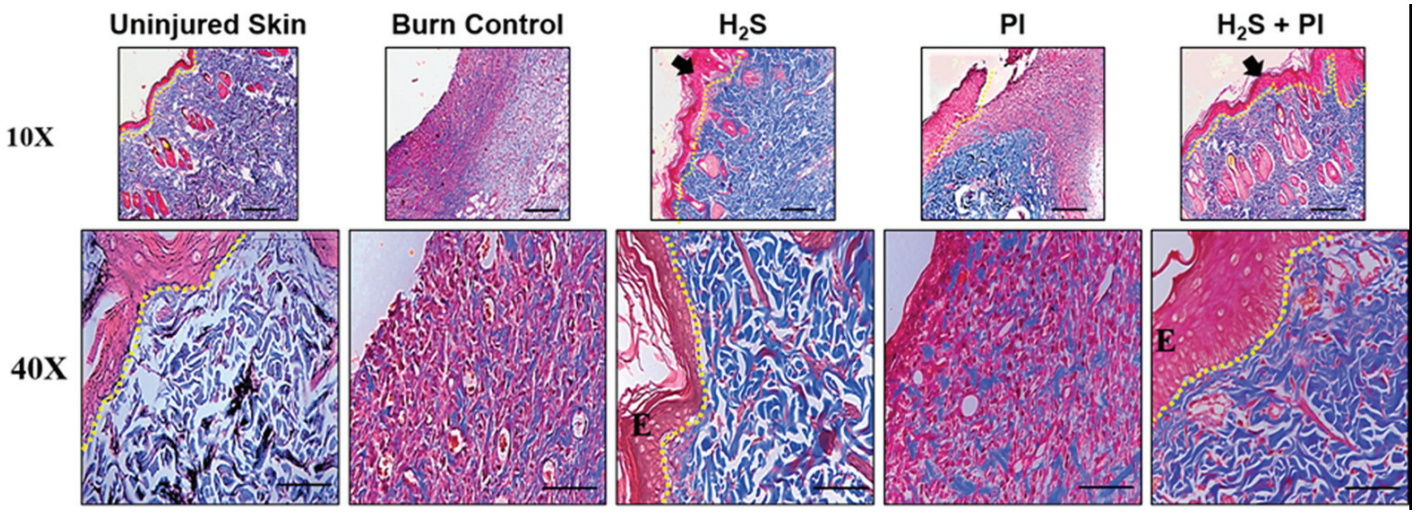

Figure 2. Masson's trichrome (MT) staining of the burn wound skin on the fifth-day post-wounding at 10X and 40X magnification in un-injured skin, burn control, sodium hydrosulfide treated group (H_2S group) (NaHS, 5 mg/kg body weight; i.p), Povidone-iodine (PI) ointment (reference care) and combination group demonstrated the extent of collagen deposition and re-epithelialization (illustrated by a yellow dotted line). H_2S treated groups showed fibrosis and increased re-epithelialization as compared to burn control. However, rats in the burn control and PI treated group demonstrated reduced collagen formation. Epithelial migration is indicated by a black arrow; 'E' denotes the regenerated skin epithelium. Scale bar, 10 µm.

2.6 Immunoblotting

The total proteins were separated on 10 per cent SDS-PAGE and transferred onto the nitrocellulose membrane (Whatman, Germany)[2]. The membrane was further blocked with 5 per cent BSA (overnight at 4 °C) in TBST buffer (0.15 M NaCl; 0.01M Tris-HCl, pH 7.4 and 0.05 per cent Tween-20). The following day, the membranes were incubated with primary antibody solution for 2.5 h (1:1000) at RT followed by washing in TBST buffer (3 times for 5 min). The primary antibodies used were HSP70, HSP90, HSP27, and GRP78 (Abcam, USA). Then, the membranes were probed with HRP-conjugated respective secondary antibodies (1:10000) for 2 h at RT. After washing (3 times for 5 min), the enhanced chemiluminescent substrate (Sigma-Aldrich, USA) was used to develop immunoreactive bands. β-actin (loading control) was used for normalisation. The densitometric analysis was performed by Fiji (ImageJ) software and results were expressed as mean intensity (per cent control).

2.7 Statistical Analysis

Data were expressed as mean ± SE. One-way analysis of variance (ANOVA) with Dunnett's *posthoc* test was performed to determine statistical significance between the experimental groups and burn control. GraphPad Prism 6.0 (La Jolla, CA, USA) software was employed for statistical analyses. A $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1 Histological Analysis (MT staining)

The extent of wound healing between the experimental groups was examined through histopathological analysis. MT staining illustrated increased collagen deposition, enhanced fibrogenesis and prominent re-epithelialisation in NaHS treated groups compared to burn control on fifth-day post-wounding (Fig. 2). However, histological observations of the PI treated group and burn control wounds exhibited less collagen accumulation and reduced epidermal progression. Thus, the histological observations substantiated the potential efficacy of H_2S supplementation in burn wound healing.

3.2 NaHS Administration Activates HSPs Machinery During Burn Wound Repair

Molecular chaperones promoted and maintain the structural integrity of newly formed proteins such as collagen, during the regenerative and proliferative phases of the healing process. HSPs are also involved in activating cytoprotective pathways. The abrogation of activation of chaperone machinery may impede the healing process. We observed a significant increase in expression levels of HSP90, HSP70, HSP27, and GRP78 in the NaHS treated group compared to the burn control (Fig. 3A-E). Our results corroborate that H_2S treatment has modulated the expression levels of HSPs on fifth-day post wounding and confer cytoprotection to accelerate the process of burn wound repair.

4. DISCUSSION

For the past few decades, H_2S is known as a major endogenous gaseous signaling molecule along with other gaseous molecules like nitric oxide (NO)[17]. Many studies have attempted to understand the mechanistic insight of H_2S during the inflammatory process and redox homeostasis in various pathophysiological conditions. However, the role of H_2S in the pathophysiology of burn wound healing is still not defined. Nevertheless, the potential role of H_2S in regulating the process of angiogenesis, oxidative stress, and inflammation has provided the cues for the possible involvement of H_2S mediated signaling pathways during burn wound repair. Similarly, HSPs also play a crucial role during cellular regeneration by

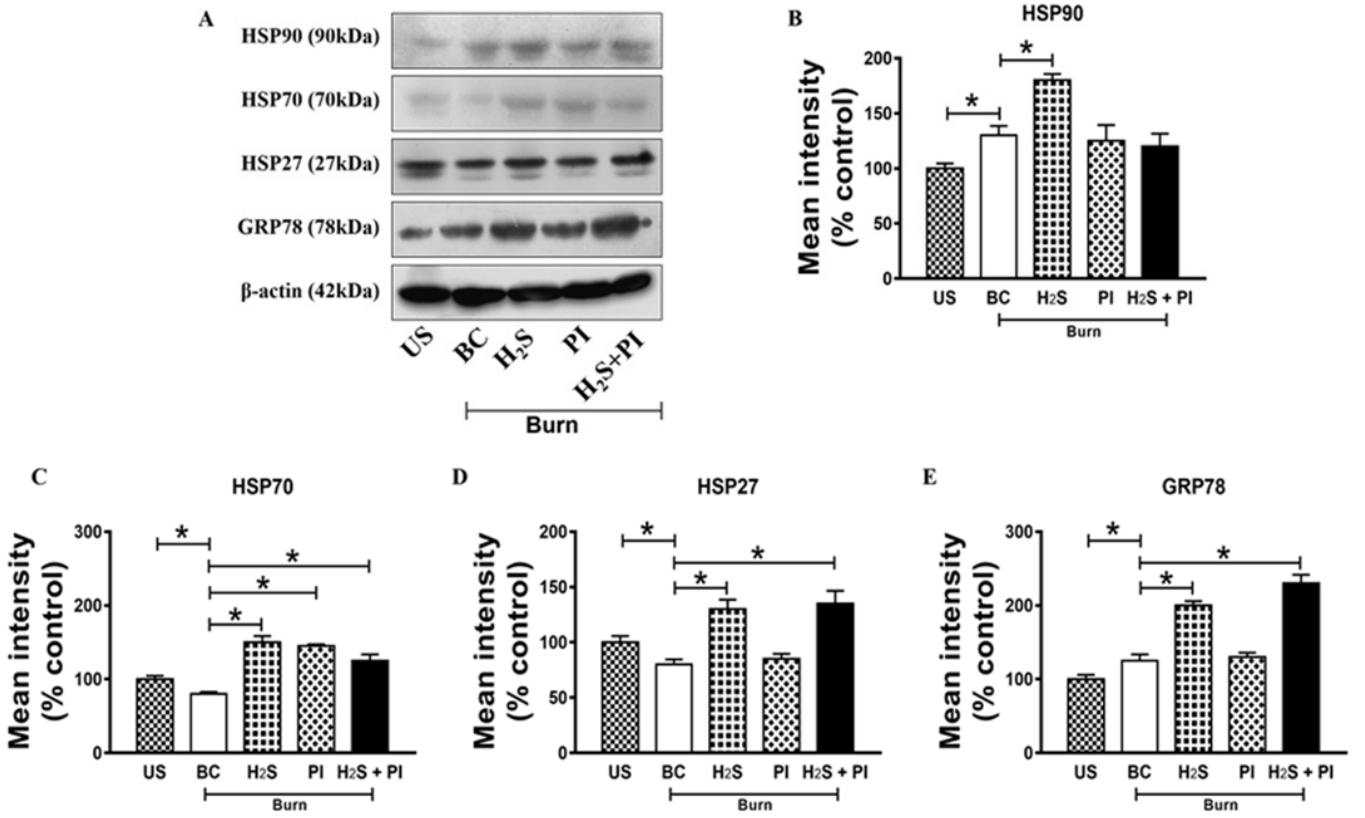

Figure 3. Supplementation of sodium hydrosulfide (H_2S group) (NaHS, 5 mg/kg body weight; i.p) treatment increased the protein expression levels of molecular chaperones in the H_2S group as compared to the burn control. (A-E) Immunoblot protein expression profile of HSP90, HSP70, HSP27, GRP78 after NaHS intervention on the fifth-day post-wounding. Data are mean ± SE (n=6), *$p < 0.05$ as compared to the burn control.

conferring cytoprotection to the newly synthesised proteins. HSPs also support cellular proliferation via mitigating apoptosis during the process of wound healing[13]. In the current study, we explored the role of H_2S supplementation on burn wound healing and molecular chaperones in the experimental rats.

Systemic inflammatory response syndrome (SIRS) and sepsis are the characteristics of third-degree burns, which constitute the total body surface area (TBSA) of more than 25 per cent[18]. A study showed that burn wound induction in mice led to up-regulation of H_2S levels in the plasma, which further promoted the systemic inflammatory responses[19]. In contrast, another study had shown that exogenous supplementation of H_2S accelerated the cutaneous wound healing in rats via inhibiting oxidative stress and upregulating angiogenesis[20]. Therefore, the molecular mechanism of H_2S for the regulation of burn wound healing is still elusive. Taking cognizance of the current situation, we have examined the effect of H_2S intervention on burn wound healing and the expression of HSPs, which are regulatory factors of the wound repair process.

In the current study, histopathological observations through MT staining and subsequent analysis substantiated that NaHS supplementation promoted cellular proliferation and enhanced collagen accumulation, evident by the well-aligned, densely packed, thick and the parallel arrangement of collagen fibers in the H_2S group as compared to burn control. Furthermore, the histological analysis also evinced prominent epidermal migration and reduced congestion in NaHS treated group as compared to burn control. Whereas, the burn control group of rats exhibited loosely packed and a lesser amount of collagen accumulation in wound tissue on fifth-day post-wounding. Notably, these findings are in concurrence with the previous study reported that administration of sodium bisulfite (Na_2S, 2 % w/v) accelerates diabetic wound healing concomitant with increased levels of granulated tissue, angiogenesis, reduced inflammation, and decreased oxidative stress[21].

HSPs constitute the family of proteins, which play a vital role during cellular stress conditions. HSP ubiquitously functions as a catalytic chaperone, binds to the denatured proteins and refold them to maintain their structural and functional integrity during stress. The excessive accumulation of denatured and misfolded proteins in burn wounds can induce the stern endoplasmic reticulum (ER) stress response. The elevated ER stress further activates the inflammatory response and cell-death pathways, which impedes the progression of the normal wound healing process. Therefore, the up-regulation of HSPs promotes the healing process by mitigating inflammation, oxidative stress, and apoptosis while promoting cell proliferation and cytoprotection[22]. A recent study had demonstrated the protective effect of H_2S via inducing crosstalk between HSP70, heme oxygenase-1 (HO-1), and HSP27, which alleviated oxidative stress, inflammation, and apoptosis that rescued the system from pathological consequences of ischemia-reperfusion injury (IRI) in rat model[14]. In our study, we have also demonstrated the significantly up-regulated

expression levels of HSP70, HSP90, HSP27, and GRP78 in NaHS treated groups as compared to burn control at the end of the fifth-day post-wounding. Therefore, it could be hypothesised that activated HSPs would have ensured the proper folding of the newly synthesised protein and activated the cell survival pathways, during the proliferative phases to accelerate the process of burn wound healing.

Based on our current observations, it can be summarised that the supplementation of NaHS before burn wound plausibly had primed the adaptive stress responses in the animal's physiological system. Furthermore, the treatment of NaHS for 5 days post-wounding led to the activation of HSPs machinery. The activated HSPs, possibly, would have suppressed the oxidative stress, reduced inflammation, and inhibited cytotoxic processes, which eventually led to accelerated healing of burn wounds as compared to the burn control. Therefore, our study could aid to envisage the therapeutic significance of H_2S by underscoring the molecular responses mediated by the activation HSPs during burn wound healing. However, further molecular studies are required to assess the cellular processes like redox hemostasis and inflammation to corroborate the therapeutic efficacy of H_2S in burn wound healing. Moreover, HSP regulatory downstream targets and signaling events could be taken further to study their association with H_2S mediated wound healing.

5. CONCLUSION

Altogether, the present study demonstrated that supplementation of H_2S enhanced collagen accumulation, cellular proliferation and re-epithelialisation during burn wound healing in rats. Moreover, the supplementation of H_2S significantly upregulated protein expression levels of molecular chaperones such as HSP90, HSP70, HSP27, and GRP78. This study substantiated that H_2S could play an important role in cytoprotection via regulating HSPs during burn wound healing.

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