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Original Article

Hydrogen sulfide prevents postoperative adhesion in a rat uterine horn model

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ABSTRACT

Objective: Abdominal adhesions are primarily severe postoperative complications that can cause gynecological problems such as infertility and chronic pelvic pain. Inflammatory mediators are significantly related to adhesion formation, and hydrogen sulfide plays a significant anti-inflammatory role in multiple physiological processes. Therefore, the effect of NaHS, a hydrogen sulfide donor, on postoperative adhesion formation was examined in a rat uterine horn model.

Materials and methods: A rat uterine horn model was created to evaluate whether NaHS, a hydrogen sulfide donor, could decrease postoperative adhesion formation. Rats were randomly grouped and administrated with different doses of NaHS, where DL-propargylglycine and low-molecular-weight heparin acted as negative and positive controls, respectively. The extent and severity of adhesions were assessed on the 14th postoperative day. Serum of rats was sampled for the determination of 27 cytokines using a chip.

Results: The severity and total scores of adhesion in rats given 112μM/kg and 56μM/kg NaHS were significantly less compared with those of the control group ($p < 0.01$). Scores for the extent of adhesion re-formation in the DL-propargylglycine and control groups did not differ ($p > 0.05$). At least six cytokines were involved in the procedures for the prevention of adhesion formation, as they varied significantly among different groups.

Conclusion: Administration of NaHS could apparently reduce postoperative adhesion in the rat uterine horn model. This preventive effect may be associated with the variation of cytokine that is related to inflammatory.

Introduction

Abdominal adhesions are primarily severe postoperative complications, developed by internal scars that misconnect tissues when organs are handled and shifted temporarily from their normal positions after laparotomy. Adhesion-related twisting and pulling of internal organs can result in quite a lot of common complications such as abdominal pain or intestinal obstruction, as well as gynecological problems such as infertility and chronic pelvic pain [1,2].

An unfortunate fact is that adhesions are difficult to prevent and unavoidable in surgery, and currently the main treatment for adhesions is mostly through surgery. Inflammatory reactions are generally assumed to play an important part in abdominal adhesions, despite the fact that their pathogenesis has not yet been revealed. Many important substances are involved in adhesion formation. For instance, certain mediators, such as interleukins and transforming growth factors, increase the formation of adhesions through decreasing the fibrinolytic capacity of the peritoneum [3,4]. To the best of our knowledge, some of the drugs were reported to effectively suppress inflammatory reactions through inactivation of the nuclear transcription factor (NF)-κB signaling pathway and subsequently prevent abdominal adhesions [4,5].

Hydrogen sulfide (H_2S), originally considered to be a malodorous and toxic gas, is produced endogenously from cysteine

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by cystathionine-β-synthase, cystathionine-γ-lyase, and 3-mercaptopyruvate sulfurtransferase. A rapidly expanding body of studies has reported the critical role of H_2S in a multitude of pathophysiological processes, including maintenance of cardiovascular homoeostasis [6–8], oxidative stress, mitochondrial function, inflammation, apoptosis [9,10], vasodilatation, angiogenesis, ion channel signaling, and interaction with NO. Although H_2S has a significant anti-inflammatory effect in a multiple physiological processes, and inflammatory mediators are remarkably related to adhesion formation, a potential effect of H_2S on abdominal adhesions has not been investigated. Thus, a rat uterine horn adhesion model was prepared to explore the effect of H_2S on adhesion prevention. The antiadhesion effect of NaHS on the rat uterine horn adhesion model was investigated, with the low-molecular-weight heparin (LMWH) as a positive control and DL-propargylglycine (PAG) as the inhibitor. The standard score system was used to evaluate the adhesion on the rat uterine horn adhesion model, and the effect of H_2S on the animal model was further examined by scores of adhesion. Moreover, to test the connection between the inflammatory process and the effect of H_2S in the model, the serum of rats was sampled for cytokine detection by a chip. As a result, we first found that H_2S reduced postoperative adhesion in the rat uterine horn model through its interaction with specific inflammatory mediators.

Materials and methods

Animals

All procedures during the feeding and experiment complied with the Regulations of Experimental Animal Management and Care of Fudan University, and approval was obtained from the Institutional Review Board before conducting the study.

For this study, 64 nonpregnant 8-week-old female Wistar-Albino rats of special pathogen free grade (SPF grade), weighing 180–210 g, were provided by the Animal Center of the Pharmacy School, Fudan University. They were used as model animals for the experimental induction of postoperative intra-abdominal and uterine horn adhesions. Prior to and after the surgery, the animals were housed in cages (4 per cage) with free access to water and food in an SPF environment. They were kept under controlled conditions of temperature (21–24°C), humidity (40–60%), and light (12-hour light/12-hour dark regime).

Prior to the induction of the animal model, the rats were randomly assigned to eight groups, with each group containing eight rats. All the groups were given 2 days of continuous medication in advance before the day of model induction. The normal group, the model group, and the sham-operation group received physiological saline before the surgery. Three groups for model induction were given different concentrations (112μM/kg, 56μM/kg, and 28μM/kg) of NaHS solution by hypodermic injection (i.h.). One of the groups for model induction received LMWH of 1.5 mg/kg (i.h.). The last group was applied with PAG of 10 mg/kg (i.h.).

Establishment of animal models

After 2 days of different medications in these groups, a modified rat uterine horn adhesion model was used to induce intra-abdominal adhesion formation [11,12]. All the operations were performed by the same person who was blind to animal allocation. All the rats were fasted for 12 hours before the operation. Each rat was anesthetized with chloral hydrate (7%, 0.4 mL/100 g, intraperitoneal injection) and fixed in the supine position for surgery. The operation was limited to 10 minutes for each rat to control the effect of room-air tissue drying, and handling of other tissues was minimized and care taken to avoid gross bleeding from injured sites. The middle lower abdomen of each rat was shaved and sterilized with iodine solution. A lower midline vertical incision, approximately 3 cm in length, was made. The peritoneal cavity was kept moist with copious amounts of saline solution throughout the surgery. Both peritoneal sidewalls were scraped using blades 10 times, taking care not to harm the retroperitoneal structures except mild bleeding. Unipolar electrocautery at a temperature of 380°C was used to traumatize the antimesenteric surface of the bilateral uterine horns at nine to 10 spots. The scraped peritoneal sidewalls and the cauterized uterine horn were then sutured approximately, from the top and bottom ends, at intervals of about 1 cm. The midline incision was closed with two layers of 4/0 Prolene sutures.

The sham-operated group, which contained eight rats, was prepared using the same procedures as those conducted for the model groups, except for the nondamage to the tissues by unipolar electric cautery and blades.

Scores standards

To ascertain the objectivity and impartiality, the scoring criteria of Linsky et al [13] and Knightly et al [14] were modified and applied for the evaluation of adhesion formation. The severity of adhesions to the uterine horn was measured as follows: 0, no adhesion; 1, tiny filmy adhesions, easy to separate without tension or injury of the involved tissues; 2, mild adhesion, moderate force for separation; 3, dense adhesion, which leads to serosal injury during lysis or needs to be divided with scissors; and 4, severe adhesion, cohesive attachment of the uterine horn to the ipsilateral abdominal sidewall. Scores for the extent of adhesions was evaluated (characterized, accessed) as follows: 0, no uterine adhesion; 1, 1–25% of traumatized area; 2, 26–50% of traumatized area; 3, 51–75% of traumatized area; and 4, 76–100% total involvement. The sum of both parameters was used as the overall score for each uterine horn.

Score and cytokine determination

After model induction, all the rats of the different groups continued to be administered the daily doses in the same way as that in the initial period, until the second laparotomy. No antibiotic prophylaxis was applied during or after the surgery.

Two weeks postoperation and after the administration of different experimental agents, the rats were anesthetized with chloral hydrate (7%, 0.4 mL/100 g, intraperitoneal injection) and fixed in the supine position for second laparotomy. Then, the abdominal wall scar was examined. Adhesions of the peritoneal sidewall with the uterine horn were scored according to the scoring system, which was performed by a person who was unaware of the allocation and medication of the rats.

The blood was collected through the aorta abdominalis after scoring the adhesions during the second laparotomy. Different batches of serum were prepared through centrifugation of these clotted blood, and stored in a refrigerator at −20°C. Measurement of cytokines inside the serum was performed using the determination kit of Raybiotech (Norcross, GA, USA) according to its standard procedure.

Statistical analysis

Statistical evaluation was performed using SPSS software (version 13.0; SPSS Inc., Chicago, IL, USA). Data are expressed as median (min.–max.). Statistical analysis was performed by the Kruskal–Wallis test for multiple independent groups except the normal and sham groups, and by Wilcoxon rank sum test with

Bonferroni correction for the comparison of control group with any of the other treated groups. A two-tailed p value < 0.05 was considered to be statistically significant for the Kruskal–Wallis test. After Bonferroni correction, a p value < 0.01 was considered to be statistically significant for Wilcoxon rank sum test.

Results

Establishment of animal models

Except for the different adhesion forms between groups, no difference was found in the experimental results between any two groups of animals, in terms of weight increase, coat color, activity, and food or water intake. In our experiments, more than 90% of the rats in the model group formed adhesions of varying severity and extent between the uterine horns and the peritoneum during the second laparotomy. It was confirmed both in the preliminary and in the formal experiment. The scores of adhesions were approximately 2–3 in severity, 2–3 in extent, and 5–6 in sum, which were coherent between different batches for model induction. The results were consistent with those of the previously reported literatures [15], indicating perfect induction of the rat uterine horn adhesion model.

Scores after model induction

To ascertain the objectivity and impartiality in the evaluation of adhesion, scores were assigned by the staff not involved in the group and medication processes. As shown in Figure 1, the status and scores of the induced lesion model were listed.

Scores for different groups

Table 1 shows the scores of adhesions of the groups under the administration of different experimental agents, examined during the second laparotomy. The Kruskal–Wallis test and Wilcoxon rank sum test with Bonferroni correction were performed and used for difference test through their final p value. Only the p value of <0.01 was considered to be significantly different between groups. No adhesion was formed in the normal control and sham operation groups, so their scores are all zero. The model control group given the physiological saline showed the highest scores in the adhesion evaluation. Although LMWH acted as a positive drug in our experiment, the groups receiving LMWH showed no difference from the model control group in terms of severity, extent, and total scores. The same results have been observed when comparing the group receiving NaHS 28μM/kg with the model control group. Compared with the model control group, the groups receiving NaHS of 56μM/kg and 112μM/kg showed significantly lower severity and total scores, but no statistical difference in terms of the extent of adhesions. The group receiving PAG of 10 mg/kg (i.h.) showed no statistical difference when compared with the model control group. NaHS of three different concentrations was applied to different groups of animals to find out the concentration of relatively more obvious effect. The group with a medium dose showed better effect than the low-dose group, while the effect did not increase in the high-dose group, which indicates that the effective dose gradient could range between the low and medium doses.

Cytokine determination

As the group receiving 56μM/kg NaHS showed the most effect, among the groups receiving three different concentrations of NaHS, blood sample of this group was sent for cytokine determination, as well as that of other three groups (the normal, sham, and model groups). In this study, 27 cytokines inside the serum were determined using a specified chip. They are B7-2, beta-nerve growth factor, cytokine-induced neutrophil chemoattractant (CINC)-1, CINC-2, CINC-3, ciliary neurotrophic factor, fractalkine, granulocyte-macrophage colony-stimulating factor, intercellular adhesion molecule-1, interferon gamma, interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, LIX, L-selectin, monocyte chemoattractant protein-1, platelet-derived growth factor-AA, prolactin, receptor for advanced glycation end products, cytokine-activated T, tissue inhibitors of metalloproteinases-1, tumor necrosis factor-α, and vascular endothelial growth factor. Among them, 26 cytokines except tissue inhibitors of metalloproteinases-1 exhibited a low expression in the normal group, while in the sham and model groups, they were of high expression (2–10 times higher than that of the former). This indicates that, no matter whether the uterine horn model was inducted or not, the operation of the rat abdomen has caused substantial inflammation and led to a high expression of cytokines in the rat blood. However, as shown in Figure 2, six of the 26 cytokines in the model group appeared to have 1.2 times higher expression than those in the sham group, which should be considered to be highly related to adhesion (for the sham group, as the abdomens of the rats were only opened up and their uterine horn fixed to their abdominal wall without any damages, no adhesions were formed). It should be noted that the expression of the six cytokines in the NaHS groups was significantly lower than that in the model group, which was close to or lower than that of the sham group.

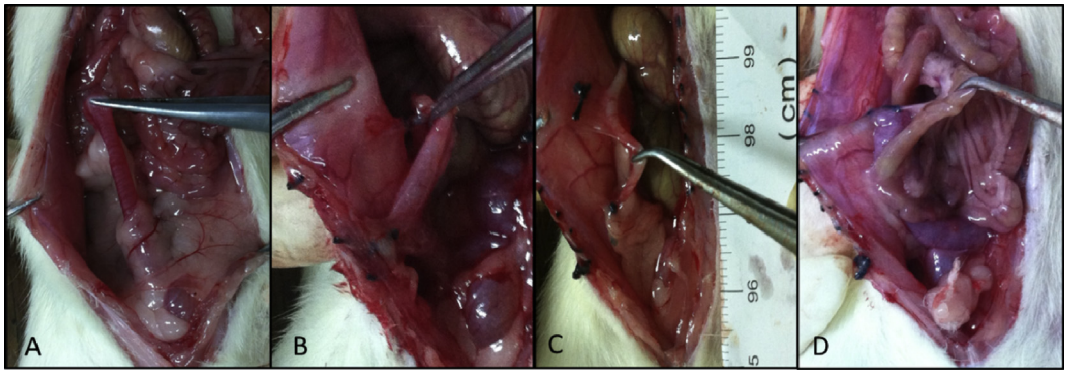

Figure 1. Formation of adhesion with different scores after operation: (A) normal rat uterus and peritoneum, where uterine horn is free from the peritoneum; (B) no adhesion, scores 0 in severity and 0 in extent according to the standard of score; (C) tiny membrane adhesion, easy to tear with slight pull, adhesion formed in 26–50% of the lesion area, scores 1 in severity and 2 in extent; and (D) adhesion blended with the intestine tissue and formed in more than 75% of lesion area, scores 4 in severity and 4 in extent according to the standard of score.

Table 1
Adhesion scores among different rat uterine model groups.

	Uterine horn (n)	Adhesion scores		
		Extent (median; min.–max.), mean ± SE	Severity (median; min.–max.), mean ± SE	Total score (median; min.–max.), mean ± SE
Normal group	16	(0; 0–0), 0 ± 0	(0; 0–0), 0 ± 0	(0; 0–0), 0 ± 0
Sham group	16	(0; 0–0), 0 ± 0	(0; 0–0), 0 ± 0	(0; 0–0), 0 ± 0
Control group	16	(4; 0–4), 3.19 ± 0.33	(3; 0–4), 2.50 ± 0.29	(7; 0–8), 5.69 ± 0.58
LMWH 1.5 mg/kg	16	(3; 0–4), 2.63 ± 0.38	(2; 0–3), 1.63 ± 0.20*	(5; 0–7), 4.25 ± 0.56*
NaHS 112μM/kg	16	(2; 0–4), 2.14 ± 0.41*	(1; 0–3), 1.29 ± 0.24**	(4; 0–6), 3.43 ± 0.49**
NaHS 56μM/kg	16	(2.5; 0–4), 2.19 ± 0.43*	(1; 0–2), 1.06 ± 0.19**	(4; 0–6), 3.25 ± 0.60**
NaHS 28μM/kg	16	(3; 1–4), 2.94 ± 0.30	(1; 1–4), 1.63 ± 0.30*	(5; 2–8), 4.56 ± 0.38
PAG 10 mg/kg	16	(4; 1–4), 2.88 ± 0.34	(2; 1–4), 2.19 ± 0.31	(5; 2–8), 5.06 ± 0.47

* $p < 0.05$ when compared with control.
** $p < 0.01$ when compared with control.
LMWH = low-molecular-weight heparin; max. = maximum; min. = minimum; PAG = DL-propargylglycine; SE = standard error.

Discussion

There are many methods for lesion induction of rat uterine horn adhesions [16,17]. After checking the literature and conducting the preliminary experiment, we induced lesions in the rats by traumatization and sutures on bilateral horns and the peritoneum [18]. During the preliminary and formal experiments for model establishment, scores of the model control group of different batches varied between 5 and 6, which indicated a controllable lesion induction during the experiments. Therefore, the method for lesion induction was a mature method, and the objectivity in our study was warranted.

From the results of scoring of these surgery groups, we can see clearly the effect of administration of different agents on rat uterine horn adhesions. It is apparent that NaHS reduced the extent and severity of rat uterine horn adhesions. LMWH, as a positive drug in the therapy of abdominal adhesions, which was extensively demonstrated, was applied to one of the lesion-induced groups [19,20]. PAG, as an inhibitor of cystathionine-γ-lyase, showed no statistical difference from the model control group in any score. This may be attributed to the existence of other synthases in female reproductive systems, and they were not inhibited. As a result, the generation of endogenous H_2S was not blocked completely [21,22].

Adhesions are proposed as scars formed during normal physiological protective reaction for restraining activated stimuli and healing tissue injury. During the recovery and fibrosis of pathological wounds where inflammation occurs on the surface of abdominal organs or the peritoneal lining, adhesions reach a peak. Meanwhile, it is widely accepted that the unbalanced state between procoagulant reactions and fibrinolytic activity is a decisive factor for the consequence of the wound recovery process. During the critical period of inflammation and wound recovery, hormones and growth factors mediate tissue oxygenation, and further determine the result of adhesion formation. When the procoagulant reactions prevail in the tissue reaction, fibrin is produced, which connects the two adjacent structures around the damaged tissues. Fibrin, like glue, seals injury sites and builds initial adhesions. The evolution of inflammatory mediators could play an important role in the adhesion process, as excessive inflammation may strengthen the adhesion formation. Currently, a large number of pharmaceutical studies are dedicated to the trial of drugs with anti-inflammatory effects on various animal models and their use in clinical settings.

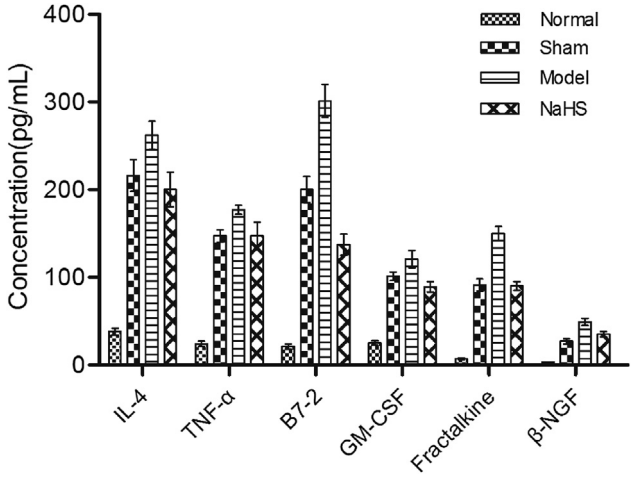

Figure 2. Expression of cytokines of obvious difference between groups. Data are presented as mean ± standard deviation. β-NGF = beta-nerve growth factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; IL = interleukin; TNF-α = tumor necrosis factor-α.

Since H_2S plays an important role in anti-inflammatory of different pathological processes and inflammatory is the dominant step in the formation of abdominal adhesions, we try to find out the association between H_2S and peritoneum adhesions. In the investigation, expressions of cytokines inside the serum of different groups after the administration of different experimental agents were determined, which helps evaluate the inflammatory and finally the anti-inflammatory effects of H_2S and the prevention of adhesion formation. As a result, we found at least six cytokines (B7-2, beta-nerve growth factor, fractalkine, granulocyte-macrophage colony-stimulating factor, IL-4, and tumor necrosis factor-α) involved in the procedures for the prevention of adhesion formation, as they varied significantly among different groups.

The fact that the inflammation-related cytokines were reduced in the model group receiving NaHS suggested an obvious anti-inflammatory effect of NaHS on the prevention of rat uterine horn adhesions. There are several ways of anti-inflammatory effect of H_2S in different physiological processes reported previously, which may be involved in the prevention of rat uterine horn adhesions. Hydrogen or its donors were reported to prevent leukocyte transmigration and adherence, downregulate inflammatory mediators, and enhance some anti-inflammatory actions of macrophages [23]. They can also modulate the expression of genes for many proinflammatory cytokines, chemokines, and enzymes that have largely been linked to effects on NF-κB activity [24,25]. Our group also found [26,27] that S-propargyl-cysteine (a slow H_2S-releasing donor) produced an anti-inflammatory effect through the cystathionine-γ-lyase/H_2S pathway by impairing IκBα/NF-κB signaling and by activating the PI3K/Akt signaling pathway. In the current study, we believe that NaHS has prevented the formation of rat uterine horn adhesions partly through anti-inflammatory mechanisms, since the involved cytokines were found to vary in different experiment groups. Whether one of the abovementioned

mechanisms or another new pathway played an important role will be investigated in our future research.

Although H_2S has been implicated to play a proinflammatory role in systemic inflammation [28–32], a majority of elegant studies strongly suggest that H_2S is a potent anti-inflammatory molecule in various models [33–36]. The observed pro- and anti-inflammatory effects of H_2S may be influenced by the exact nature of the model of inflammation (e.g., acute vs. chronic inflammation), and the timing of the administration of a H_2S donor or an inhibiting compound. Generally speaking, H_2S has been reported to play a proinflammatory role in acute inflammation. Examples of acute inflammation are septic shock, multiple organ failure, and pancreatitis.

In summary, we have examined the potential effect of H_2S on the prevention of adhesion. Results showed that H_2S could apparently reduce postoperative adhesion in the uterine horn model. The prevention effect is strongly associated with the variation of cytokines that are related to inflammation. It is certain that H_2S inhibits adhesions via interaction with the inflammatory pathway. The novel result may help explore new therapeutic ways for abdominal adhesions after laparotomy. For a deeper understanding of the role of H_2S in abdominal adhesion, a further study on the detailed signal pathway related to the prevention effect is underway.

Conflicts of interest

The authors have no conflicts of interest relevant to this article.

Acknowledgments

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