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The anti-inflammatory effect of hydrogen sulphide on acute necrotizing pancreatitis in rats

Kutay Sağlam[1], Etem Alhan[1], Serdar Türkyılmaz[1], Birgül Kural Vanizor[2], Cengiz Erçin[3]

ABSTRACT

Objective: The aim of this study was to investigate the dose-dependent anti-inflammatory effectof the Hydrogen sulfidedonor sodiumhydrosulphide on acute necrotizing pancreatitis in rats.

Material and Methods: A total of 42 male Sprague-Dawley rats were divided into 4 groups: sham+saline (group 1), sham+NaHS (group 2), acute necrotizing pancreatitis+saline (group 3), and acute necrotizing pancreatitis+NaHS (group 4). Acute pancreatitis was induced in rats in groups 3 and 4 with the infusion of glycodeoxycholic acidinto the biliopancreatic canal and infusion of cerulein parenterally. In group 4, 10 mg/kg NaHS was administered intraperitoneally after cerulein infusion.Tests for liver and kidney function, interleukin-6, lactate dehydrogenase in bronchoalveolar lavage, and malonyaldehyde and myeloperoxidase activities in pancreas and lung tissue were performed, and histopathologic examination of pancreas was conducted.

Results: In groups 3, a significant increase in amylase, alanine aminotransferase, urea, interleukine-6, lungmalondialdehydeand myeloperoxidase activities, pancreas myeloperoxidase activity, edema, and necrosis in pancreas tissue and a significant decrease in serum calcium levels were detected ($p<0.05$). In group 4, addition of NaHS resulted in a significant decrease in lactate dehydrogenase level in bronchoalveolar lavage, amount of urea, lung myeloperoxidase activity, and pancreatic edema ($p<0.05$).

Conclusion: Although not in pancreatic necrosis, hydrogen sulphide has an anti-inflammatory effect especially in the inflammatory process in lung and edema in pancreasin acute necrotizing pancreatitis at particular doses. With further studies evaluating the anti-inflammatory effects of hydrogen sulphide, we believe it can be used in the treatment of edematous acute pancreatitis and the related complications in lungs.

Keywords: Hydrogen sulphide, pancreatitis, rats

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[1]Department of General Surgery, Karadeniz Technical University School of Medicine, Trabzon, Turkey
[2]Department of Medical Biochemistry, Karadeniz Technical University School of Medicine, Trabzon, Turkey
[3]Department of Pathology, Kocaeli University School of Medicine, Kocaeli, Turkey

Address for Correspondence
Kutay Sağlam
e-mail: saglamk@gmail.com

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INTRODUCTION

Acute pancreatitis is an inflammatory disorder of the pancreas presenting with abdominal pain and elevated pancreatic enzymes in the blood (1).Gall bladder stones and chronic alcohol usage are the etiologic factors in 80% to 90% of the cases (2). The incidence of pancreatitis ranges from 4.9 to 35 per 100.000. Mortality rates were previously reported 10% to 30%.In necrotizing pancreatitis, mortality rates are reported to increase up to 12% in the case of sterile necrosis, to 30% in infected necrosis, and to 47% in multi-organ failurein previous studies (3, 4).

The pathophysiology of acute pancreatitis is not obvious, although intracellular activation of the digestive enzymes in acinar cells is accepted as the starting point. Free oxygen radicals released from the injured cells and mediators and cytokines from the leukocytes play a major role in the progression of acute pancreatitis and multi-organ failure (5). Autodigestion of pancreas and failure of microcirculation in pancreas are the main mechanisms in the pathophysiology of pancreatitis (6). Acute necrotizing pancreatitis (ANP) is the most severe form of inflammation in pancreas. Coagulation necrosis of the glandular cells and fat tissue are the pathological findings in ANP.

Hydrogen sulphide (H_2S) is a gaseous mediator, which can be endogenously synthesized by cystathionine-δ-lyase (CSE) and cystathionine-β-synthase (CBS) enzymes from L-cysteine aminoacids (7, 8). The synthesis of H_2S is increased in disorders coursing with inflammation like acute pancreatitis, sepsis, and endotoxemia (9). The effects of H_2S on inflammation are conflicting. Inhibition of endogenously synthesized H_2S has previously shown to decrease the inflammatory response (10, 11). With these properties, H_2S was thought to be a pro-inflammatory molecule. The anti-inflammatory property of H_2S was detected in a study conducted with an H_2S-releasing non-steroidal anti-inflammatory drug (NSAID) (diclofenac) whenH_2S provided a more anti-inflammatory response compared with an H_2S non-releasing NSAID (12).

Sidhapuriwala et al. (13) showed the anti-inflammatory effect of H_2S in edematous pancreatitisin their study using H_2S-releasing S-diclofenac. The anti-inflammatory effect of H_2S was also shown

by the inhibition of TNF-α vs. IL-6 in the study of Xu et al. (14) with hemorrhagic shock-induced rats. H_2S inhibits the nuclear factor κβ (NF-κβ), one of the main regulators of inflammation, and decreases proinflammatory cytokines, chemokines, and adhesion molecules (15-17). Further, its antioxidant and anti-apoptotic efficiency have been previously reported (18-20). To the best of our knowledge, no study has evaluated the anti-inflammatory effect of H_2S in necrotizing pancreatitis.

The aim of this study was to investigate the dose-dependent anti-inflammatory effects of H_2S on the histopathology of pancreas and its functions by biochemical parameters in necrotizing pancreatitis in rats.

MATERIAL AND METHODS

This experimental study was conducted with the approval of Ethical Committee of the Surgical Research Laboratory of our hospital.

Forty-two male Sprague-Dawley rats weighing 300-350 g were used in the study. Rats were maintained in routine laboratory conditions, 21°C, 60% to 70% humidity, and 12/12 h light/dark cycle, at our institution'sAnimal Research Laboratory. Rats were divided into four groups. Oral intake was restrictedto water 12 h before the operation. Anesthesia was administered with intraperitoneal 50mg/kg ketamine (Ketalar, Eczacıbaşı) injection. Subsequently, the right internal jugular veins of the rats were catheterized for fluid replacement, and left carotid artery was catheterized for blood sampling.

Group 1 (Sham+saline, n=7): Right jugular vein and carotid artery catheterizationwas performed. Physiologic saline was infused at 8 mL/kg per hour for 24h via the right jugular venous catheter.

Group 2 (Sham+H_2S, n=7): Procedures performed for group 1 were performed and then 10 mg/kg sodium hydrosulphide (NAHS) (Sigma-Aldrich) (Lot No: 06396APV) dissolved in distilled water was administrated intraperitoneally.

Group 3 (ANP+saline, n=15): After jugular vein catheterization, the distal end of thecatheter was placed in the suprascapular region in 15 rats in this group. Subsequently, laparotomy was performed. Bilio-pancreatic duct was catheterized by a transduodenal approach from the antimesenteric sideof the duodenum. Pancreatic fluid was drained with the help of gravity for 5 min. Main hepatic duct was clamped. Then 10-mMol glycodeoxycholic acid (GDOC, Sigma St Louis, 3528) 1.2 mL/kg under 30mmHgpressure was infused via the catheter. This pressure was achieved using a volume-controlled infusion pump (IVAC 7000; United Kingdom Hampshire, Alaris Medical Systems, RG22, 4BS). After infusion, the catheter was removed and the duodenal hole was repaired. Subsequently, cerulein (Sigma & Aldrich Chemie, GmbH, C-9026) was infused for 6 h, 5 µg/kg per hour, with the infusion pump. Following this, serum physiologic was infused at a rate of 8 mL/kg/h for 18 h.

Group 4 (ANPH_2S, n=13): Like group 3, after acute pancreatitis was formed, 8 mL/kg/h serum physiologic and 5µg/kg cerulein was infused; 8 mL/kg Ringer's lactate was infused for the remaining 18 h. After cerulein infusion, 10 mg/kg NaHS was applied to the rats intraperitoneally.

After 24 h, blood samples were collected from the rats for analyzing biochemical parameters and serum IL-6 levels. Blood samples were centrifuged in Eppendorf Centrifuge 5810 machine at 3200 rpm for 10 min for analyzing biochemical parameters. Enzymatic colorimetric analysis of serum samples was conducted for measuring amylase, glucose, urea, creatinine, ALT, and calcium levels using COBAS 6000 machine. Enzyme linked-immunosorbent assay (ELISA) Kit, RayBio®Rat IL-6 (Lot No: 1137545A), was used for measuring IL-6 levels in serum samples. Then thorax was opened by sternotomy. The left lung was clamped from the left main bronchus and a cannula was placed in the trachea. Bronchoalveolar lavage was performed with 2 cc phosphate buffered saline (PBS) solution. Lavage fluid was stored at −20°C in tubes containing EDTA for protein measurement. At the end of the experiment, BAL protein levels were measured by Lowry method (21). COBAS 6000 machine was used for BAL LDH measurement. After this step, pneumonectomy was performed for the left lung, and the extracted tissue freezed in liquid nitrogen for malondialdehyde (MDA) and myeloperoxidase (MPO) measurements.

After all these procedures, laparotomy was performed and the pancreaswas extracted. Previously defined steps performed for the lung were repeated the measurement of MPO and MDA in the pancreas. Part of the pancreas was stored in 10% formaldehyde-containing tubes for histological examination. Tissue analysis in the pancreas and lungwere performed with the method described by Uchiyama and Mihara, by measuring MDA concentration with thyobarbituric acid colorimetric reaction (22). MPO activity was analyzed as described by Bradley et al. (23).

Pathological studies were conducted on slides prepared from pancreas of the rats. Tissue samples were fixed in 10% formaldehyde. The slides were studied under a light microscope to observe necrosis, edema, and granulocyte infiltration. All these pathological changes were histologically evaluated by the same pathologist (Table 1) (24).

Statistical Analyses

For data analyses Statistical Package for the Social Sciences 13.0 (SPSS Inc.; Chicago, IL, USA) was used. Descriptive statistics were summarized with mean and standarderror. Numeric data appropriate for normal distribution were evaluated with Student t test, and those not appropriate for normal distribution were evaluated with Mann-Whitney U test. Appropriateness of normal distribution was evaluated with Kolmogorov-Smirnov test. $P<0.05$ was accepted as statistically significant.

RESULTS

Sham+saline and sham+H_2S groups had no mortality. Four rats in Sham+ANP group and two rats in ANP+H_2S group died (mortality rates 26.6% and 15.2%, respectively). There was a statistically significant difference in 24-h serum glucose, urea, amylase, creatinine, ALT, calcium, and IL-6 levels and hourly urine flow levels between the groups with ANP and those without. H_2S application significantly improved urea and BAL LDH values. Statistical comparison of the groups and significance values are given in Table 2.

Table 1. Histopathological scoring criteria for necrotizing pancreatitis

Score	Description
Edema	
0	Absent
0.5	Focal expansion of interlobar septae
1	Diffuse expansion of interlobar septae
1.5	Same as 1 + focal expansion of interlobal septae
2	Same as 1 + diffuse expansion of interlobar septae
2.5	Same as 2 + focal expansion of interacinar septae
3	Same as 2 + diffuse expansion of interacinar septae
3.5	Same as 3 + focal expansion + intercellular spaces
4	Same as 3 + diffuse expansion + intercellular spaces
Acinar necrosis	
0	Absent
0.5	Focal occurrence of 1Y4 necrotic cells/high power field
1	Diffuse occurrence of 1Y4 necrotic cells/high power field
1.5	Same as 1 + focal occurrence of 5Y10 necrotic cells/high power field
2	Diffuse occurrence of 11Y16 necrotic cells/high power field
2.5	Same as 2 + focal occurrence of 11Y16 necrotic cells/high power- field
3	Diffuse occurrence of 11Y16 necrotic cells/high power field
3.5	Same as 3 + focal occurrence of >16 cells/high power-field
4>	Necrotic cells/high power field (Extensive confluent necrosis)
Inflammation and perivascular infiltrate	
0, 0-1	Intralobular or perivascular leukocytes/high power field
0.5, 2Y5	Intralobular or perivascular leukocytes/high power field
1, 6Y10	Intralobular or perivascular leukocytes/high power field
1.5, 11Y15	Intralobular or perivascular leukocytes/high power field
2, 16Y20	Intralobular or perivascular leukocytes/high power field
2.5, 21Y25	Intralobular or perivascular leukocytes/high power field
3, 26Y30	Intralobular or perivascular leukocytes/high power field
3.5, >30	Leukocytes/high power field or focal microabscesses
4, >35	Leukocytes/high power field or confluent

Malondialdehyde and MPA were measured in pancreas and lung to detect the oxidative injury and to detect the neutrophil infiltration, respectively. MDA and MPO in lung and MPO in pancreas were significantly increased in the groups with pancreatitis, and H_2S application was found to decrease lung MPO. Statistical comparison of the groups and significance values are given in Table 3.

On histological examination, edema, necrosis, and cellular infiltration were significantly increased in the groups with pancreatitis. The effect of H_2S application on the decrease of edema was statistically significant. Statistical comparison of the groups and significance values are given in Table 4.

DISCUSSION

In the present study, NaHS decreases mortality, does not have any effect on pancreatic necrosis, improves the organ functions, and has partial anti-inflammatory effects with regard to pancreatitis in ANP.

Different experimental acute pancreatitis models have been defined previously. In the present study, the method of Schmidt et al. (24) for ANP was used. In this pancreatitis modelconstructed using cerulein and glycodeoxycholic acid,elevated pancreatic enzymes, edema of pancreatic tissue, and acinar cell necrosis were observed. This method is the most widely used, safest, and standardized method. Patients usually present to the outpatient clinic 24 to 36 h after the onset of pancreatitis. Therefore, H_2S was given 6 h after the induction of the experiment.

H_2S is a gaseous mediator, which can be endogenously synthesized. H_2S opens the adenosine triphosphate (ATP)-dependent potassium (K^+) channels and relaxes the blood vessels and smooth muscles in the gastrointestinal system. H_2S has a vasodilatory effect (25, 26).

Inhibitionof endogenous H_2S using CSE inhibitors decreases the inflammatory response, showing the proinflammatory effect of H_2S (18, 27). However, in this study, the anti-inflammatory effect of H_2S was observed by comparing the effects of H_2S-releasing NSAID (diclophenac) and H_2S-non-releasing NSAID (14). H_2S-releasing drugs were shown to have anti-inflammatory effects (28). In the study of Sidhapuriwala et al. (29) investigating the anti-inflammatory effect of H_2S using NaHS, an H_2S donor, at 10 mg/kg, a decrease in the inflammation in pancreas and lungs secondary to edematous pancreatitis was observed. The aim of the present study was to evaluate the efficiency of the same dose in necrotizing pancreatitis in rats.

Pancreatic necrosis is the key point in severe pancreatitis and directly correlates with mortality (30). Histological examination is important in detecting the severity of acutepancreatitis. In the present study, edema, acinar necrosis, hemorrhage, fat necrosis, and inflammation in pancreas was evaluated according to the histopathological scoring scale defined by Lowry et al. (21). We found that edema, perivascular infiltration, and necrosis in the pancreatitis group were significantly higher than those in the non-pancreatitis group ($p<0.05$). In the group with NaHS (group 4), edema significantly decreased ($p<0.05$). H_2S had no effects on pancreatic necrosis. The other factor increasing necrosis is apoptosis (31). In the present study, we did not work on apoptosis. Xu DQ et al. (14) showed the anti-apoptotic property of H_2S in their study.

In the present study, the enzyme activity of the lipid peroxidation product MDA was measured to detect the oxidative stress in pancreas and lung tissue due to pancreatitis and that of MPO was measured to detect neutrophil infiltration. Pancreatitis groups showed MDA and MPO increase in pancreatic tissue. Increase in MPO levels was statistically significant ($p<0.05$). MDA and MPO decreased in the group containing

Table 2. Glucose, amylase, ALT, urea, creatinine, calcium levels in serum and LDH and urine levels in BAL in the 24th hour

	Sham+saline (n=7)	Sham+H_2S (n=7)	ANP+saline (n=11)	ANP+H_2S (n=11)
Amilase (U/L)	2328±49	2051±115	10741±2162*	7849±1334
Glucose (mg %)	239±21	149±10	101±9*	122±10
Urea (mg %)	16±1	13±1.2	39±5*	19±5#
Creatinine (mg %)	0.38±0.1	0.44±0.4	0.34 ±0.5	0.28±0.5
ALT (U/dL)	63±3	66±10	247±60*	205±55
Calcium (mg %)	10±0.3	9.1±0.6	8.2±0.2*	8.5±0.12
BAL LDH (U/dL)	368±58	188±25	590±78	229±34#
IL-6	41.2±1.4	157±56	995±419*	1248±467
Urine (mL/hour)	1.05±0.5	0.7±0.2	0.28±0.5*	0.4±0.3

Data are shown as mean±standard error of mean. *p<0.05: comparision of ANP and non-ANP groups; #p<0.05: comparision of ANP+saline and ANP+H2S; ALT: alanine amino transpherase; BAL: bronchoalveolar lavage; LDH: lactate dehydrogenase; IL-6: interleukine-6; SEM: standart error

Table 3. MPO and MDA measurements in lung and pancreas tissue

	Sham+saline (n=7)	Sham+H_2S (n=7)	ANP+saline (n=11)	ANP+H_2S (n=11)
Lung MPO (U/mg protein)	3.8±0.11	3.7±0.76	5.94±0.61*	4.3±0.27#
Lung MDA (nmoL/mgProtein)	1.00±.004	0.74±0.03	1.47±0.14*	1.1±0.08
Pancreas MPO (U/mg Protein)	1.02±0.14	0.82±0.15	1.89±0.37*	1.53±0.26
Pancreas MDA (nmoL/mg protein)	0.38±0.07	1.6±0.44	0.7±0.14	0.69±0.15

Data are shown as mean±standard error of mean. *p<0.05: comparision of ANP and non-ANP groups; #p<0.05: comparision of ANP+saline and ANP+H_2S; MPO: myeloperoxidase; MDA: malonylaldehyde

Table 4. Evaluation of histological edema, inflammation, and necrosis in pancreas tissue

	Sham+saline (n=7)	Sham+H_2S (n=7)	ANP+saline (n=11)	ANP+H_2S (n=11)
Edema	0.4±0.17	0.42±0.1	1.4±0.17*	0.8±0.15#
Necrosis	0.0±0.0	0.7±0.7	1.5±0.21*	1.7 ±0.45
Inflammation	0.7±0.07	0.7±0.7	1.1±0.1*	1.5±0.26

Data are shown as mean±standard error of mean. *p<0.05: comparision of ANP and non-ANP groups, #p<0.05: comparision of ANP+saline and ANP+H_2S

NaHS, but this was not statistically significant. Sidhapuriwala et al. (29) showed in their study with mice that H_2S decreases pancreatic MPO activity.

Platelet activating factor, TNF-α, IL-1, IL-6, and IL-8 are the major cytokines that have a role in pancreatic injury and are the starters of systemic anti-inflammatory response syndrome. In the present study, we used IL-6 to detect the cytokinerole in inflammatory response. The groups with pancreatitis showed statistically significant increase in IL-6 levels (p<0.05). NaHS addition did not show any decrease in IL-6 levels. However, Xu et al. (14) showed that H_2S inhibits TNF-α and IL-6 in their study.

Serum amylase increases in acute pancreatitis. Serum amylase levels are not relevant for severity of pancreatitis, and it is only used in diagnosis (32). In the present study, serum amylase levels in ANP-induced rats at 24 h were increased significantly (p<0.05). Although not reaching a statistical significance, addition of NaHS decreased the amylase levels. Sidhapuriwala et al. (29) showed that 10 mL/kg dose of NaHS decreased serum amylase levels in their studyon edematous pancreatitis. In the present study, serum glucose levels were significantly increased in the pancreatitis groups (p<0.05), but the effect of NaHS on glucose levels was not significant.

Parameters showing multi-organ injury such as serum urea and ALT levels significantly increased in the pancreatitis groups, whereas serum Ca^{++} and urine flow significantly decreased (p<0.05). These parameters were improved in NaHS-administered group. The improvement in urea was statistically significant (p<0.05).

The most common and severe complications of acute pancreatitis are in the respiratory system. Hypoxemia secondary to ventilation/perfusion failure, atelectasis, pleural effusion, lung edema, and acute respiratory distress syndrome (ARDS) are some of the complications (33, 34). The most dangerous complication is ARDS with morality rates of approximately 50%. Coagulopathies in microcirculation, lipase and phospholipase activities; and arteriovenous shunts opened by the release of kinines are the triggering factors leading to the development of ARDS (33). In the present study, LDH in BAL fluid and MDH

and MPO in lung tissue were analyzed to evaluate lung complications. BAL LDH was used to detect lung and lung endothelial injury (35, 36). BAL LDH levels were elevated in pancreatitis groups. H_2S addition was shown to decrease BAL LDH values significantly ($p<0.05$). In groups with pancreatitis, lung MDA and MPO values were significantly increased in non-pancreatitis groups ($p<0.05$). In the groupswith H_2S, lung MPO values were significantly decreased ($p<0.05$), although changes in MDA levels were not statistically significant. Previous studies showed that H_2S prevents lung injury induced by lipopolysaccharides (14, 37). H_2S was shown to decrease the number of free oxygen radicals in rabbits with lung transplantation (38).

Xu et al. (14) Showed that H_2S prevents lung injury, inhibits apoptosis and decreases inflammatory response in hemorrhagic shock-induced rats. Also, in a study by Liu et al. (39) it was reported that H_2S inhibits Fas pathway and has preventive effectsin acute lung injury-induced rats. In a study by Sidhapuriwala et al. (29) H_2S was shown to reduce lung MPO activity, pulmonary chemokines, and adhesion molecules.

Limitations

The present study had some limitations. Anti-inflammatory effects of H_2S are dose dependent and we only used 10 mg/kg. Studying a wider range of dose levels may providemore information about the effects of H_2S. Also, the effects of H_2S on inflammatory pathways of the other mediators except IL-6 were not evaluated.

CONCLUSION

In conclusion, autolysis of pancreas secondary to intraacinar enzyme activation is the most accepted theory in the pathogenesis of pancreatitis. Activation of leucocytes, released cytokines, and free oxygen radicals, resulting in multi-organ failure, is an important factor in pancreatitis progression. In the present study, we aimed to detect the dose-dependent effects of H_2S on rats with ANP. Although H_2S does have any effects on pancreatic necrosis, it decreases the mortality rates and improves organ functions. These are the dose-dependent partial anti-inflammatory effects of H_2S.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of Karadeniz Technical University (05.05.2011-2011/14).

Informed Consent: Not required in this study.

Peer-review: Externally peer-reviewed.

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