Hydrogen Sulfide Suppresses H2O2-induced Proliferation and Migration of HepG2 Cells Through Wnt/β-catenin Signaling Pathway

Hongzhi Zhao (✉ zhaohongzhi@purotong.com.cn)  
Chongqing Emergency Medical Center  
https://orcid.org/0009-0009-2824-4211

Liang Zhao  
Chongqing Emergency Medical Center

Lin Wu  
Chongqing Emergency Medical Center

Sheng Hu  
Chongqing Emergency Medical Center

Yangmei Huang  
Chongqing Emergency Medical Center

Wei Zhao  
Chongqing Emergency Medical Center

Research Article

Keywords: Hepatocellular carcinoma, hydrogen sulfide, H2O2, Wnt3a/β-catenin signaling system

Posted Date: May 25th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2920792/v1

License: ☕️ ⌚️ This work is licensed under a Creative Commons Attribution 4.0 International License. Read Full License

Version of Record: A version of this preprint was published at Medical Oncology on June 28th, 2023. See the published version at https://doi.org/10.1007/s12032-023-02091-w.
Abstract

Both H$_2$S and H$_2$O$_2$ affect many cellular events, such as cell differentiation, cell proliferation and cell death. However, there is some controversy about the roles of H$_2$S and H$_2$O$_2$, since the detailed mechanisms they are involved remain unclear. In this study, low concentration of H$_2$O$_2$ (40 µM) increased the viability of hepatocellular carcinoma cells HepG2, while both H$_2$S and high concentration of H$_2$O$_2$ decreased the cell viability in a dose-dependent manner. Wound healing assay indicated that 40 µM H$_2$O$_2$ promoted migration of HepG2 cells, which was suppressed by exogenous H$_2$S. Further analysis revealed that administration of exogenous H$_2$S and H$_2$O$_2$ changed the redox status of Wnt3a in HepG2 cells. Altered expression of proteins including Cyclin D1, TCF-4, and MMP7, which are downstream of the Wnt3a/β-catenin signaling pathway, were found after treatment with exogenous H$_2$S and H$_2$O$_2$. Compared with H$_2$S, low concentration of H$_2$O$_2$ showed opposite effects on these protein expression levels in HepG2 cells. These results suggest that H$_2$S suppressed H$_2$O$_2$-induced proliferation and migration of HepG2 through regulating Wnt3a/β-catenin signaling pathway.

1 Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors, of which the global average annual incidence is on the rise [1, 2]. Although therapeutic strategies, such as liver transplantation, surgical resection, radiofrequency ablation (RFA) and transcatheter arterial chemoembolization (TACE), have greatly improved the prognosis of HCC, the 5-year survival rate remains below 20% [3]. Therefore, identification of etiological mechanisms and novel strategies are urgently needed for the prevention and treatment of this prevalent malignancy.

The occurrence of HCC is the result of unlimited proliferation and migration of liver cells, among which many signal transduction pathways play important roles [4]. The majority of HCC therapeutic strategies are designed to abrogate these pathways. The main signaling pathways involved in HCC are as follows: Wnt/β-catenin pathway [5, 6], MARK pathway[7, 8], PI3K/Akt/mTOR and PKB/Akt pathway [9–11], HGF/C-MCT pathway [11, 12], JAK pathway [13–15], and VEGF pathway [16].

H$_2$S is proved to be an important endogenous modulator, which has been qualified as the third gasotransmitter after NO and CO [17, 18]. H$_2$S exerts multiple physiological and pathological effects on cell growth, differentiation and proliferation as an anti-peroxidative agent in oxidative stress and angiogenesis [19–21]. A number of studies have investigated the role of H$_2$S and evidence has been presented that this gas molecule can affect cancer cell survival using sulfide salts as donor agents [22, 23].

However, the effects of H$_2$S on cancer cells are controversial. Several in vivo and in vitro studies showed that H$_2$S is beneficial for cancer cell growth, proliferation, migration, and invasion [17, 24, 25]. These effects may rely on the NF-kB, PIK3K/AKT, and STAT3 pathways [17]. Meanwhile, other studies found that
H$_2$S exerted its potential anticancer effects on several human cancer cell lines, such as Hela, HCT-116, MCF-7 [17]. A H$_2$S donor, GYY4137, inhibits proliferation of HCC cells via inactivation of the STAT3 pathway [23]. Another finding implies that H$_2$S may act as a double-edged sword in the proliferation of HCC through the EGFR/ERK/MMP-2 and PTEN/AKT signaling pathways [25]. Thus, the effects of H$_2$S on cancer cells are complicated and the mechanisms involved remain unclear.

Reactive Oxygen Species (ROS) are recognized as cytotoxic agents, and they can serve as second messengers to control many cellular events of gene expression, differentiation, cell proliferation and cell death [26, 27]. Normally, cells keep ROS level in balance between their generation and elimination by a redox reaction system. However, disorder of ROS gives rise to a lot of human diseases, including cancers.

H$_2$O$_2$, as an individual ROS, is proved to be an extracellular and intracellular signaling molecule in regulation of cancer cell proliferation and migration [28, 29]. Compared with normal cells, cancer cells show increased generation rate of H$_2$O$_2$, resulting in a higher level of H$_2$O$_2$ in the tumor tissues [30]. The role of H$_2$O$_2$ in regulation of cancer cell proliferation and migration has been reported, but its mechanism has not been well investigated. Breast cancer cells is inhibited by H$_2$O$_2$ with cell cycle arrest and apoptosis which were correlated with altered expression of cyclin D1, cyclin E, and BAX [31, 32]. Increasing evidences show that H$_2$O$_2$ has dual effects on cancer cell proliferation depending on its concentration [31–34]. Since the accumulation of H$_2$O$_2$ can reflect the development of many cancers, novel strategies for the treatment of cancer basing on the use of H$_2$O$_2$ may be helpful in reducing the mortality [35].

Despite the importance of H$_2$S and H$_2$O$_2$ in cancer cell proliferation and migration, the direct effects of exogenous H$_2$S and H$_2$O$_2$ on human cancer cells have not been well elucidated. In order to investigate how H$_2$S and H$_2$O$_2$ affect HCC and their therapeutic potential for HCC, we examined the effects of varying doses of NaHS and H$_2$O$_2$ on the growth of human HCC cells in vitro and clarified the associated molecular mechanisms. We found that exogenous NaHS could suppress low concentration H$_2$O$_2$-induced cell proliferation and migration, and protect HepG2 cells from H$_2$O$_2$-induced oxidative stress by reducing the damage of Wnt3a and inhibiting the production of downstream proteins of the Wnt3a/β-catenin signaling pathway.

2 Materials and method

2.1 Cell culture

Human HCC cell line HepG2 was obtained from the Cell Bank of Chinese Academy of Sciences. All cells were cultured in high-glucose Dulbecco's modified Eagle's medium containing 10% high-quality fetal bovine serum, and 100 units/mL penicillin at 37°C in a humidified atmosphere with 5% CO$_2$.

2.2 Cell viability assay
HepG2 cells were seeded into 96-well plates at a density of $5 \times 10^3$ cells/well, and incubated at 37°C. After overnight incubation, cells were respectively treated with various treatments for 24h, including 0-5000 µM NaHS (Macklin Inc., Shanghai, China) and 0-500 µM H$_2$O$_2$ (Beyotime Biotechnology, Shanghai, China). Then, cell viability was evaluated by the Cell Counting Kit (CCK-8) (Beyotime Biotechnology, Shanghai, China). 10 µL CCK-8 was added to each well and incubated for 4 h at 37°C before measurement. Six parallel wells were used for each treatment. The absorbance was detected at a wavelength of 450 nm on a microplate reader (Thermo Fisher, Waltham, USA). Cell viability and inhibition were expressed as a percentage of the untreated control.

2.3 Wound healing assay

The cells were cultured for 24 h after seeding $2 \times 10^5$ cells per cell in 6-well plates. Then, the cellular layer was scratched straightly with a sterile micropipette tip. The migration distance was measured using Image J software (National Institute for Health, Bethesda, USA) after incubation separately with 0 µM NaHS for 24 h, 2000 µM NaHS for 24 h, 40 µM H$_2$O$_2$ for 24 h, 2000 µM NaHS for 12 h followed by 40 µM H$_2$O$_2$ for 12 h, and 40 µM H$_2$O$_2$ for 12 h followed by 2000 µM NaHS for 12 h. The migration rate (MR) was calculated as MR (%) = $[(A - B)/A] \times 100\%$, where A is the width at 0 h, and B is the width at 24 h or 48 h. The experiments were conducted in triplicates independently.

2.4 Cell apoptosis assay

Propidium iodide (PI)/ Annexin V assay kit (Beyotime Biotechnology, Shanghai, China) was used to detect the cell apoptosis of HCC cells. After treatment with various concentrations, the HCC cells were collected and washed twice with PBS. Then, the HCC cells were resuspended with Annexin V-FITC binding buffer at a concentration of $1 \times 10^5$ cells/ml. 100 µl cell suspension was transferred to a 1.5 ml tube and stained with 5 µl Annexin V-FITC for 10 min. Then, 5 µl PI was added and incubated for 10 min in dark. Finally, flow cytometry was performed with the CytoFLEX system (Beckman, USA) according to the manufacturer's instruction.

2.5 Western blotting

Western blotting was performed to detect the expressions of Cleaved Caspase-3, Wnt3a, β-catenin, Cyclin D1, TCF-4, MMP7, β-actin, and GAPDH in HepG2 cells. Total proteins were extracted from HepG2 cells after various treatments for appropriate times. Then, these lysates were diluted in PBS buffer separately. 30 µg of each total protein was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE). Proteins from SDS-PAGE were transferred to nitrocellulose membranes. Membranes were blocked by 5% non-fat milk (w/v) in PBS buffer for 1 hour before incubation with antibodies at room temperature for 2 h. Membranes were incubated with secondary antibodies after rinsing with PBS for three times. Then, the membranes were detected by the ECL detection.

The primary antibodies, including rabbit anti-human total protein, Cleaved Caspase-3, Wnt3a, β-catenin, Cyclin D1, TCF-4, MMP7, β-actin, and GAPDH were purchased from abcam Biotechnology Inc (abcam Biotechnology Inc., Shanghai, China). The secondary antibody, horseradish peroxidase-conjugated sheep
anti-rabbit IgG was purchased from Beyotime Biotechnology (Beyotime Biotechnology, Shanghai, China). The bands were semi-quantified with Image J software. The experiment was carried out three times independently.

### 2.6 Measurement of sulfhydryl modified Wnt3a

Sulfhydryl modified Wnt3a were determined by biotin conversion assay using an *EZ-Link™ HPDP* kit (Thermo Fisher Biotech Inc., San Diego, USA). Total proteins were extracted from HepG2 cells after incubation separately with 0 µM NaHS for 24 h, 2000 µM NaHS for 24 h, 40 µM H₂O₂ for 24 h, 2000 µM NaHS for 12 h followed by 40 µM H₂O₂ for 12 h, and 40 µM H₂O₂ for 12 h followed by 2000 µM NaHS for 12 h. Then, sulfhydryl modified proteins from HepG2 cell were purified by biotin conversion assay according to the standard protocol of *EZ-Link™ HPDP* kit. The sulfhydryl modified Wnt3a was detected by western blot using anti-human Wnt3a protein.

### 2.7 Measurement of oxidized Wnt3a

Oxidized Wnt3a were determined by biotinylated iodoacetamide assay using an EZ-Link Iodoacetyl-PEG2-Biotin kit (Thermo Fisher Biotech Inc., San Diego, USA). Total proteins were extracted from HepG2 cells after incubation separately with 0 µM NaHS for 24 h, 2000 µM NaHS for 24 h, 40 µM H₂O₂ for 24 h, 2000 µM NaHS for 12 h followed by 40 µM H₂O₂ for 12 h, and 40 µM H₂O₂ for 12 h followed by 2000 µM NaHS for 12 h. Then, Wnt3a protein from those total proteins were precipitated separately by anti-Wnt3a protein A-agarose beads (Thermo Fisher Biotech Inc., San Diego, USA). At last, oxidized Wnt3a protein was isolated by EZ-Link Iodoacetyl-PEG2-Biotin kit. The oxidized Wnt3a protein was detected by western blot further.

### 2.8 Statistical analysis

Data are presented as means ± standard error of the mean (SEM). The differences between multiple groups were analyzed by Student’s t-test. A *p*-value of less than 0.05 was considered to be statistically significant. Three or more separated experiments were performed.

### 3 Results

#### 3.1 The viability of HepG2 cells were affected by NaHS and H₂O₂

In order to test the effect of NaHS on human liver cancer cells, HepG2 cells were treated with 200–5000 µM NaHS for 24 h. Then, the cell viability was tested by using the cell counter kit (CCK-8) to reflect the viable cells. The cell viabilities are shown in Fig. 1A, and the corresponding inhibition rates are shown in Fig. 1B. The proliferation of HepG2 cells was obviously inhibited by applications of exogenous NaHS in the concentration range of 200–5000 µM, and the inhibitions were enhanced with the increase of concentration.
The effect of $\text{H}_2\text{O}_2$ on human liver cancer cell viability was also tested with varying doses (10, 20, 30, 40, 50, 100, 200, 500, and 800 µM). As shown in Fig. 1C and 1D, the doses of $\text{H}_2\text{O}_2$ below 40 µM increased HCC cell viability slightly. However, high concentrations of 50–500 µM $\text{H}_2\text{O}_2$ decreased HCC cell viability markedly. According to these experimental results which were consistent with previous reports, $\text{H}_2\text{O}_2$ has dual effects on the cell viability of HepG2 cells depending on its concentration.

### 3.2 The apoptosis of HepG2 cells induced by NaHS was rescued by $\text{H}_2\text{O}_2$

Interestingly, increasing evidences have shown that $\text{H}_2\text{O}_2$ has dual effects on cancer cell proliferation depending on its concentration. Thus, we wondered whether $\text{H}_2\text{S}$ could affect $\text{H}_2\text{O}_2$ signaling in HepG2 cells. Therefore, we used 2000 µM NaHS and 40 µM $\text{H}_2\text{O}_2$ for the next experiments. To evaluate the effects of NaHS and $\text{H}_2\text{O}_2$ on the cell apoptosis separately, HepG2 cells were subjected to different treatments and cell apoptosis analysis was performed by flow cytometry.

As shown in Fig. 2A and 2B, 2000 µM NaHS promoted the cell apoptosis while 40 µM $\text{H}_2\text{O}_2$ decreased cell apoptosis slightly. Notably, the cell apoptosis induced by NaHS was partially rescued by adding $\text{H}_2\text{O}_2$. Besides, the cell apoptosis of cells treated with 40 µM $\text{H}_2\text{O}_2$ was not obviously affected by the addition of NaHS. Then, a cell apoptotic marker, activated caspase 3, was analyzed by western blot. As shown in Fig. 2C, the activated caspase 3 level was significantly elevated in HepG2 cells treated with 2000 µM NaHS, and was decreased under 40 µM $\text{H}_2\text{O}_2$. Importantly, the elevated activated caspase 3 level induced by NaHS is suppressed by $\text{H}_2\text{O}_2$ treatment. These results showed that the apoptosis of HepG2 cells induced by NaHS could be partially rescued by $\text{H}_2\text{O}_2$.

### 3.3 The migration of HepG2 cells was affected by $\text{H}_2\text{O}_2$ and $\text{H}_2\text{S}$ oppositely.

The migration distance of HCC cell was reported to be affected significantly after treatment with $\text{H}_2\text{S}$. To further test the effects of $\text{H}_2\text{S}$ and $\text{H}_2\text{O}_2$ on cell migration, wound healing assay was used. The human HCC HepG2 cell lines were seeded into 6-well plates at $2\times10^5$ cells/well. After incubation separately with 0 µM NaHS for 24 h, 2000 µM NaHS for 24 h, 40 µM $\text{H}_2\text{O}_2$ for 24 h, 2000 µM NaHS for 12 h followed by 40 µM $\text{H}_2\text{O}_2$ for 12 h, and 40 µM $\text{H}_2\text{O}_2$ for 12 h followed by 2000 µM NaHS for 12 h.

As shown in Fig. 3, the migration in HepG2 was promoted after treatment with 40 µM $\text{H}_2\text{O}_2$ for 24 h and 48 h. The migration of HepG2 cells was slightly inhibited by treatment with 2000 µM NaHS for 24 h and 48 h. Therefore, we then investigated whether NaHS could protect HepG2 cells from $\text{H}_2\text{O}_2$-induced cell migration.
This enhancement could be partially inhibited by addition of NaHS followed by incubation for another 36 h. Besides, the addition of H₂O₂ could not enhance the migration of HepG2 cells after the treatment with NaHS. These results indicated that the migration of HepG2 cells could be enhanced by H₂O₂, which might be suppressed by NaHS.

3.4 Expressions of Wnt3a and β-catenin induced by H₂O₂ could be reversed by NaHS

The Wnt/β-catenin signaling pathway is one of the most conserved pathways in organism evolution and play key roles in liver cancer. Wnt3a was proved to be involved in HCC development. So, we investigate whether the Wnt/β-catenin signaling pathway was involved in the effects of NaHS and H₂O₂ on the viability and migration of HepG2 cells. Expression levels of Wnt3a and β-catenin under different treatments were detected and western blot results were shown in Fig. 4. Data shows that 2000 µM NaHS had little effect on the expression of Wnt3a and β-catenin. However, the addition of 40 µM H₂O₂ could induce the expressions of Wnt3a and β-catenin in HepG2 cells. Notably, the expressions of Wnt3a and β-catenin induced by 40 µM H₂O₂ could be observably reversed after adding 2000 µM NaHS.

3.5 NaHS and H₂O₂ affect the redox status of Wnt3a in HCC

Wnt3a encodes a secretory glycoprotein with a length of 350–400 amino acids, characterized by 22–24 conserved cysteine residues. Data shows that mutation of any individual cysteine of Wnt3a results in covalent Wnt oligomers which diminishes or abolishes Wnt signaling [36, 37]. So, we wanted to know if the redox status changes caused antagonistic effects of NaHS and H₂O₂ on HepG2 cells. We detected the redox status of Wnt3a under different treatments with NaHS and H₂O₂. Sulphydryl and oxidized Wnt3a in each experimental group were examined as shown in Fig. 5. It was found that sulphydryl Wnt3a was accumulated in HepG2 cells treated with exogenous NaHS (Fig. 5A and 5B), and this effect could be reversed by adding H₂O₂. Similarly, the oxidized Wnt3a was accumulated in HepG2 cells treated with exogenous H₂O₂ (Fig. 5C and 5D), which could also be reversed by adding NaHS. These results suggested that NaHS and H₂O₂ play opposite roles in the redox status regulation of Wnt3a.

3.6 NaHS inhibited expressions of Cyclin D1, TCF-4 and MMP7 induced by H₂O₂

Cyclin D1, TCF-4 and MMP7 were the downstream proteins of Wnt3a/β-catenin signaling pathway, which supports cell growth and facilitates cell motility. Expression of Cyclin D1, TCF-4 and MMP7 in HCC cells were further examined. As shown in Fig. 6, after treatment with 2000 µM NaHS in HCC cells, expressions of Cyclin D1, TCF-4 and MMP7 were inhibited. While exogenous H₂O₂ promoted the protein expressions of Cyclin D1, TCF-4 and MMP7. The effect of H₂O₂ on Cyclin D1, TCF-4 and MMP7 expression levels could be reversed by pretreatment with 2000 µM NaHS. These evidences show that NaHS and low concentration of H₂O₂ regulate the proliferation and migration of HepG2 cells through Wnt3a/β-catenin signaling pathway, and have antagonistic effects on each other.
4 Discussion

H₂S, along with NO and CO, forms part of a group of biologically active gases [38, 39]. And, their roles have attracted attention in several signaling processes. Previous studies have shown that H₂S endogenously promotes tumor cell growth and proliferation [17, 24]. However, the roles of H₂S have also caused great controversy. In this study, CCK-8 and cell migration assay were used to detect the viability and migration ability of HepG2 cells. And, data showed that 100–5000 µM NaHS inhibited the proliferation in a dose-dependent manner (Fig. 1A and B). Meanwhile, migration of HepG2 cells was inhibited by treatment with 2000 µM NaHS (Fig. 3). The inhibition of proliferation and migration of HepG2 cells may be related to the increased cell apoptosis induced by NaHS (Fig. 2). Those results were consistent with previous researches [23, 25]. GYY4137, a slow-releasing H₂S donor, exhibits anti-cancer activity and was proved to block IL-6-induced STAT3 cascade leading to the suppression of cell growth [23]. Another study found that the growth and migration of HCC cells were enhanced by 10–100 µM NaHS and dose-dependently inhibited by 600–1000 µM NaHS [25]. H₂S perhaps acts as a double-edged sword in HCC cells.

The precise molecular mechanism of H₂S function in HCC is still complicated and ambiguous. Previous studies showed that over-activation of endogenous H₂S promotes the proliferation of liver cancer cells, and this effect was related to inhibition of p53, p51, JNK, caspase-3, PARP, Bax/Bcl-2, EGFR, and ERK1/2 [17]. Treatment of HCC cell with high concentration of NaHS prevents the migration and proliferation of HCC cell via PI3K/Akt/mTOR signaling pathway and STAT3 pathway [17, 23]. Here, our studies found that after treatment with NaHS, proteins downstream of Wnt3a/β-catenin signaling pathway, including Cyclin D1, TCF-4, and MMP7, were affected (Fig. 5). So, H₂S could also regulate HCC cell growth and migration through Wnt3a/β-catenin signaling pathway.

H₂O₂ is one kind of ROS and has been frequently applied to induce oxidative stress [40]. Several studies show that H₂O₂ also has dual effects on the growth and migration of HCC cells [31–34]. For instance, low concentrations (1–10 µM) of H₂O₂ were proved to promote the proliferation and migration of hepatoma 7721 cells and colon cancer cells HT-29, while high concentration of H₂O₂ inhibits the proliferation of human breast cancer cells MCF-7 and colon cancer HT-29 [41]. Here, our data shows that 40 µM H₂O₂ promoted the proliferation and migration of HepG2 cells(Figs. 1 and 3). Meanwhile, H₂O₂ affected the cell apoptosis induced by NaHS (Fig. 2).

Previous studies have prompted us to investigate the potential role of H₂S as a cardio-protective reagent. Cancer cells show increased generation rate of H₂O₂, resulting in a higher level of H₂O₂ in the tumor than normal tissues. So, we consider whether endogenous H₂O₂ could affect the signaling pathway of H₂S. Interestingly, the cell apoptosis induced by NaHS was inhibited after pretreatment with H₂O₂ and migration ability of HepG2 cells in the NaHS + H₂O₂ group was decreased comparing with H₂O₂ treatment group, which also indicated that the promotion effect of H₂O₂ could be inhibited by adding NaHS (Figs. 2
and 3). So, effect of H$_2$O$_2$ on HepG2 cells could be suppressed by applying high concentration of exogenous H$_2$S. Thus, this antagonistic effect might be useful for clinical treatment of liver cancer.

The mechanism of H$_2$O$_2$ in regulation of cell proliferation and migration has not been well investigated. Several studies show that H$_2$O$_2$ regulated proliferation and migration of cancer cells via DLC1/RhoA signaling pathway [32]. Here, we found that the addition of exogenous H$_2$O$_2$ could induce the expressions of Wnt3a and β-catenin in HepG-2 cells (Fig. 4). Also, H$_2$O$_2$ could promote the protein expressions of Cyclin D1, TCF-4 and MMP7, which are downstream of Wnt3a/β-catenin pathway (Fig. 6). Interestingly, the increased expression levels of Wnt3a, β-catenin, Cyclin D1, TCF-4 and MMP7 could be reversed by exogenous NaHS (Figs. 4 and 6). So, exogenous NaHS and H$_2$O$_2$ regulate the proliferation and migration of HepG2 cells through Wnt3a/β-catenin signaling pathway, and have antagonistic effects on each other.

Wnt proteins have conserved cysteine-rich domain and have been demonstrated to be crucial for regulation of Wnt/β-catenin downstream target genes [37]. The Wnt/β-catenin pathway begins with the binding of a Wnt ligand which can promote β-catenin nuclear translocation and enable β-catenin to interact with TCF/LEF to regulate gene expression, such as c-Myc, MMP-7 and cyclin D1 [42]. Wnt disulfide bonds and subdomains were proved to be critical and play distinct roles in secretion and activity [43].

Wnt3a is a ligand of the Wnt signaling pathway and was proved to show higher expression in tumor tissues than normal liver tissues in HCC patients [44]. Down-regulation of Wnt3a expression inhibited cell viability via decreased expression of cyclin D1 and c-Myc [42]. Wnt3a recruited TCF4 to bind to the Wnt response region of LEF1 promoter [42]. Wnt3a protein has 24 conserved cysteine residues spread across the entire length of the molecule [43]. Loss of any conserved cysteine in Wnt3a results in high molecular weight oxidized Wnt oligomers, which are formed through inter-Wnt disulfide bonding. Mutation of any individual cysteine of Wnt3a results in covalent Wnt oligomers through ectopic intermolecular disulfide bond formation and diminishes Wnt signaling. Cys-77 of Wnt3a has been studied extensively, and its mutant is secreted normally but displays diminished activity [43].

Here, we investigated the redox status of Wnt3a after treatment with NaHS and H$_2$O$_2$ in HepG2 cells. It was found that the sulphydryl Wnt3a was accumulated in HepG2 cells after treatment with NaHS (Fig. 5). However, oxidized Wnt3a accumulated in HepG2 cells after treatment with H$_2$O$_2$. Therefore, oxidation and reduction reactions of Wnt3a have different functions in HepG-2 cells, which can activate/inhibit the activity of Wnt3a and regulate the proliferation and migration of HepG2 cells through Wnt3a/β-catenin signaling pathway. H$_2$S upregulates antioxidant signaling pathway and mediates the “S-sulfhydration modification” of cysteine. H$_2$S may regulate the thiothiolation of Wnt3a cysteine residues through “S-sulfhydration modification”, making the cysteine residues of Wnt3a free from “oxidative modification”, and results in the inhibition of Wnt3a secretion and activation. Thereby, the proliferation and migration of HCC would be inhibited. When the endogenous H$_2$S production of liver cancer is reduced, and the tumor is in a relative ischemic anoxic state. The cysteine residues of Wnt3a are “oxidized modified”, secrete a
large amount of Wnt3a, and activate Wnt/β-catenin signal pathway to promote the proliferation and migration of liver cancer cells.

The principal findings of this study relate to how NaHS and H$_2$O$_2$ affect Wnt3a/β-catenin signaling pathway during hepatic cell proliferation and migration. Exogenous NaHS and H$_2$O$_2$ could regulate redox status of Wnt3a in HepG2 cells, thereby activating/inhibiting the activity of Wnt3a. Proteins downstream of Wnt3a/β-catenin signaling pathway were affected, including Cyclin D1, TCF-4, and MMP7. In conclusion, H$_2$S-mediated suppression of H$_2$O$_2$ effects on HepG2 cells may be due to the alteration of oxidation/reduction reaction of Wnt3a which affects the Wnt3a/β-catenin signaling pathway.

**Abbreviations**

HCC, Hepatocellular carcinoma; H2S, hydrogen sulfide; RFA, radiofrequency ablation; TACE, transcatheter arterial chemoembolization; JAK, Janus protein tyrosine kinase; CCK-8, Cell Counting Kit; NO, nitric oxide; CO, carbon monoxide

**Declarations**

**Acknowledgments**

This work was supported by The Chongqing Natural Science Foundation Project (No. CSTC2020jcyj-msxmX0912) and the Yuzhong District Basic Research and Frontier Exploration Project of Chongqing (No. 20200136). We also thank Dr. Xunling Han for help with pathological examinations.

**Contributions**

ZH designed the study. ZH performed the experiments and analyzed the data, with assistance from ZL, WL and HS. ZW analyzed the data. ZH wrote the manuscript which was read and revised by all authors.

**Ethics declarations**

**Conflict of interests**

The authors have no relevant financial or non-financial interests to disclose.

**Consent for publication**

The informed consent was obtained from study participants.

**References**


**Figures**

**Figure 1**

**Effects of NaHS and H$_2$O$_2$ on the viabilities of HepG2 cells.**

The cell viability was tested by using the cell counter kit (CCK-8). (A-B) The cell viability and cell viability inhibition rate of HepG2 cells treated with different doses of NaHS (200, 500, 1000, 2000 and 5000 µM) were analyzed. (C-D) The cell viability and cell viability inhibition rate of HepG2 cell treated with different doses of H$_2$O$_2$ (10, 20, 30, 40, 50, 100, 200, 500, and 800 µM) were analyzed. Data are presented as mean...
± SEM of three independent experiments; *: p-value<0.05, **: p-value<0.01. All experimental groups were compared with the control group (0 µM).

**Figure 2**

**Effects of NaHS and H$_2$O$_2$ on the apoptosis of HepG2 cells.**

(A) The cell apoptosis analysis was performed by flow cytometry with Propidium iodide (PI)/ Annexin V assay kit. The human HCC cell line HepG2 was seeded into 6-well plates at 2×10$^5$ cells/well and incubated separately with 0 µM NaHS for 24 h, 2000 µM NaHS for 24 h, 40 µM H$_2$O$_2$ for 24 h, 2000 µM NaHS for 12 h followed by 40 µM H$_2$O$_2$ for 12 h, and 40 µM H$_2$O$_2$ for 12 h followed by 2000 µM NaHS for 12 h. (B) Quantitative analyses of flow cytometry were shown. Data are presented as mean ± SEM of three independent experiments; *: p-value <0.05, **: p-value <0.01, compared with the control group. +++: p-value <0.01, compared with the H$_2$O$_2$ group. (C) Expression levels of activated caspase 3 were analyzed with western blot.
Figure 3

Effects of NaHS and H₂O₂ on the migration of HepG2 cells

(A) Cell migration was measured by wound healing assay. Original magnification 100x. (B-C) The migration rates were calculated as the formula shown in 2.3. Data are presented as mean ± SEM of six independent experiments; *: p-value < 0.05, **: p-value < 0.01, compared with the control group. †: p-value < 0.05, ‡: p-value < 0.01, compared with the H₂O₂ group.
Figure 4

Expression of Wnt3a and β-catenin in HepG2 cells under different treatments of NaHS and H₂O₂

(A) Expression levels of Wnt3a and β-catenin were analyzed with western blot. (B-C) The densitometry analysis of Wnt3a and β-catenin were performed, normalized to the corresponding GADPH level. Values were presented as mean ± SEM of three independent experiments; *: p-value <0.05, **: p-value <0.01, compared with the control group.
**Figure 5**

**Redox status of Wnt3a in HepG2 cells under different concentrations of NaHS and H$_2$O$_2$**

(A) Sulfhydryl Wnt3a levels were analyzed with western blot. (B) The densitometry analysis of sulfhydryl Wnt3a was performed, normalized to the corresponding GADPH level. (C) Oxidized Wnt3a was analyzed with western blot. (D) The densitometry analysis of oxidized Wnt3a was performed, normalized to the corresponding GADPH level. Values were presented as mean ± SEM of three independent experiments; *: $p$-value $<0.05$, **: $p$-value $<0.01$, compared with the control group.

![Image of western blot and densitometry analysis](image)

**Figure 6**

**Expressions of Cyclin D1, TCF-4 and MMP7 in HepG2 cells under different concentrations of NaHS and H$_2$O$_2$**

(A) Expressions of Cyclin D1, TCF-4 and MMP7 were analyzed with western blot. (B-D) The densitometry analysis of Cyclin D1, TCF-4 and MMP7 were performed, normalized to the corresponding GADPH level. Values were presented as mean ± SEM of three independent experiments; *: $p$-value $<0.05$, **: $p$-value $<0.01$, compared with the control group.

![Image of western blot and densitometry analysis](image)