Research Paper

Hydrogen Sulfide Promotes Proliferation of HT-29 Colon Cancer Cells in a Mitochondria-independent Pathway

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Several studies reported the carcinogenic and anticarcinogenic effects of hydrogen sulphide. The present study evaluated the role of mitochondria in mediating the anti/pro-carcinogenic effect of hydrogen sulphide on colon cancer cells as mitochondrial K_{ATP} channel and mitochondrial electron transport chain are one of the promising targets for cancer treatment. The colon adenoma cell line and normal small intestinal epithelial cell lines were used to study the antiproliferative effect of hydrogen sulphide in the presence of enzyme inhibitors, mitochondrial K_{ATP} channel modulators and in presence of inhibitors of endogenous hydrogen sulphide metabolizing enzymes namely cystathionine-β-synthase and cystathionine-γ-lyase. Antiproliferative effect of hydrogen sulphide was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, crystal violet, sulforhodamine B and lactate dehydrogenase assays with its donor sodium hydrogen sulphide in both HT-29 and IEC-6 cells, where only IEC-6 cells showed significant cytotoxic effect at a concentration of 49.88 µg (IC_{50}) but HT-29 failed to exhibit cytotoxicity with the same hydrogen sulphide concentration. In order to identify the mitochondrial role, several electron transporting chain inhibitors and K_{ATP} channel modulators were used, but still hydrogen sulphide could able to enhance the colon adenoma cell line growth indicating mitochondrial in-dependency in the pro-carcinogenic effect. However, anticarcinogenic effect of hydrogen sulphide was observed only when the cells were incubated in the presence of cystathionine-β-synthase and cystathionine-γ-lyase inhibitor, indicating their influential role in determining the exogenous hydrogen sulphide toxicity in colon adenoma cell line cells.

Key words: Hydrogen sulphide, mitochondria, cystathionine-β-synthase, HT-29, IEC-6

Colorectal cancer is a heterogeneous group of disease, and is one of the leading causes of cancer related deaths in Western countries[1]. A series of genetic changes that controls specific signalling pathway involving the inactivation of tumor suppressor gene and DNA repair gene along with the activation of oncogene is responsible for the pathophysiology[2]. Survival rate statistics in developing countries showed that early detection stage of the cancer can reduce mortality rate, where the disease is highly curable, with five-year survival rates of about 90 %[3]. However, many people delay seeking medical care in the early stage due to their embarrassment of symptoms related to their bowels. Moreover, the risk increases significantly after age 50 and continues to increase with age.

Although targeted therapies have slowly taken over the conventional chemotherapeutic agents, the latter still has its significance in the therapeutic regimen of colon cancer[4]. Targeted therapy with the use of inhibitors of survival pathways, along with immune cells, differentiation agents, and cytotoxic drugs to block the growth of cancer cell by interacting with specific molecules like Wnt regulators, potassium channels are promising, but limitations like resistance to the therapy due to mutations[5] can deteriorate its efficiency. Hence, recently, therapeutic approach that utilizes targeted therapies in combination with conventional chemotherapeutic agents is found to be promising.

Advances in technology to curb cancer identified mitochondrial K_{ATP} channel and electron transport chain (ETC) as one of the promising targets[6]. In that aspect, hydrogen sulphide (H_2S) is used because it can modulate

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ETC and mitochondrial K_{ATP} channel effectively at lower concentrations[7]. Also, biological interest in understanding the effect of H_2S in the management of cancer pathology is gaining interest primarily due to the increased expression of H_2S synthesizing enzyme like cystathionine-β-synthase (CBS) in colon and ovarian tumor mass[8]. Few studies have shown that H_2S can promote proliferation and migration of human cancer cells (SW 480) by up regulating SIRT 1[9], and by inhibiting nuclear factor kappa-B signalling[10]. But many other studies have demonstrated that H_2S can induce colorectal cancers[11], bring on hyper proliferation in human colon mucosa[12], modulate cell cycle and cause cancer in rat intestinal epithelial cells. But the continuous exposure of H_2S to cancer cell decreased its cell survival, induce genomic DNA damage[13] as compared to the non-cancer cells. Some of the H_2S releasing vegetables like garlic[14], onion[15], were also reported to have anticancer property. Thus, all the information from the above studies highlights the discrepancies in the mode of action of H_2S on colon cancer cells, which needs to be addressed.

Cancer cell metabolism primarily relies on mitochondria for its function and its dysfunction[16] is considered to be one of the hallmarks of the cancer. In the present study, the therapeutic efficacy of H_2S in colon cancer cells, HT-29 was evaluated and compared with normal small intestinal epithelial cells, IEC-6 with a notion that H_2S can act as either carcinogen or anticancer agent. Further, the role of mitochondria in the ambiguous nature of H_2S on cancer cell was investigated by studying the cytotoxic effect in presence of ETC inhibitor, endogenous H_2S metabolic enzyme inhibitor and mitochondrial potassium channel inhibitor and opener.

MATERIALS AND METHODS

The colorectal cancer cell line, HT-29 and small intestinal epithelial cells, IEC-6, obtained from National Center for Cell Science, Pune, India, were cultured in Dulbecco's modified Eagle medium supplemented with 10 % fetal calf serum, 1 % antibiotics/antimycotic (complete medium) and incubated in a 5 % CO_2 incubator at 37°. Sodium hydrosulfide (NaHS), sulforhodamine B (SRB), rotenone, diazoxide, propargyl glycine (PAG) and amino oxy acetic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal calf sera, and antibiotics/antimycotics were obtained from Invitrogen; Thermo Fisher Scientific, Inc. All other reagents were purchased from HiMedia.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction assay:

The MTT reduction assay protocol of Kupcsik *et al*. with a slight modification was used to measure cell viability by determining the level of metabolic activity, where blue formazan was formed from MTT by the action of mitochondrial dehydrogenase[17]. Briefly, the HT-29 cells were grown on a 96 well plate with a seeding density of 8000 cells/well and pretreated with various concentrations of NaHS, a donor of H_2S for 24 h after allowing the cells to get attached to plate and having a confluence of 75 % cells. At the end of incubation, MTT (5 mg/ml) was added to each well and the formazan product was dissolved in 100 µl of dimethyl sulfoxide after fixing the cell with paraformaldehyde. The absorbance of each well was measured at 570 nm using multimode reader (Biotek, synergy H1, Mumbai).

Crystal violet (CV) assay:

HT-29 cells were grown in a 96 well plate and pretreated with various concentrations of NaHS at their 75 % confluence. The cells were then washed with phosphate-buffered saline (PBS) and fixed with 4 % paraformaldehyde. The cells were then incubated with 0.5 % CV (Sigma-Aldrich Corp., St. Louis, MO, USA) in 30 % methanol for 10 min at room temperature. Excess dye was removed by washing with tap water. The cells were lysed in a 1 % sodium dodecyl sulphate solution. The absorbance of the solution was measured using Biotek multimode reader at a wavelength of 570 nm[18].

SRB assay:

SRB assay protocol as described by Vichai *et al*.[19] was followed. Briefly, HT-29 cells were seeded in 96 well plate with 7000 cells/well and once it attains 75 % confluency, the cells were washed with PBS and treated with NaHS of different concentrations from 5 µM to 50 mM for 24 h. At the end of incubation, the medium was removed and washed with PBS. The cells were fixed with 10 % trichloroacetic acid, stained with SRB (1 %), solubilized in 10 mM Tris base and measured for the survival of cells using Biotek multimode reader for fluorescence at excitation of 488 nm and emission of 585 nm.

Lactate dehydrogenase (LDH) assay:

The release of LDH from HT-29/IEC-6 cells was measured using a procedure as described previously[20].

After treating the cells with various concentrations of NaHS in a 96 well plate, the supernatant was used to measure the leaked LDH activity. The reaction was initiated by mixing the supernatant with NADH and sodium pyruvate in phosphate buffer. The change in conversion of NADH to NAD was measured at 340 nm.

Clonogenic assay:

HT-29 cells were plated in 24 well plate at a seeding density of 40 000 cells and once the cells are fixed, they were incubated with various concentrations of NaHS for 24 h. After trypsinizing the cells, they were replated in 6 well plate at a seeding density of 20 000 cells and maintained under normal growth condition for 7 to 8 d at 37° in CO_2 incubator to form colonies[21]. The colonies were measured via CV assay as described above.

Caspase 3 activity:

Caspase 3 activity was measured using fluorometric assay protocol as described previously[22]. After incubating the cells with NaHS, the cell lysate was prepared and incubated with 10 µl of fluorogenic peptide substrate Ac-DEVD-AMC 37° in the dark for 2 h. The activation of caspase was measured using Biotek Synergy H1 multimode reader at excitation of 390 nm and emission of 500 nm.

Influence of ETC, K_{ATP} channel and endogenous H_2S on the growth of cells in the presence of H_2S:

To study the dependability of H_2S over the mitochondrial cellular bio-energetics, rotenone and azide were used to inhibit ETC complexes I and IV. Also, the change in mitochondria membrane permeability pore transition (MPTP), being the key regulator of apoptosis, the effects of H_2S were analysed on mitochondrial potassium ATP channel, that regulate the MPTP, by pre-treating it with a channel opener (diazoxide) and a blocker (glibenclamide). In order to distinguish the effect of exogenous and endogenous H_2S, DL-PAG was used as an irreversible inhibitor of cystathionine-γ-lyase (CSE) and aminooxyacetic acid (AOA), a CBS inhibitor prior to the H_2S treatment.

Statistical analysis:

All data were presented as the mean±SEM for the three independent experiments. A one-way analysis of variance was performed using the prism statistical package (GraphPad Software, USA). $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

H_2S is reported to have both procarcinogenic and anticarcinogenic effect[10]. The procarcinogenic effect was explained through the endogenous H_2S action on angiogenesis, cell cycle progression and blockade of apoptosis mechanism[23]. The anticancer effect of H_2S was explained as, at high concentration H_2S induces cell cycle arrest[24]. Advancements in cancer research identified mitochondrial K_{ATP} channel and ETC as one of the promising targets in the cancer treatment. Many studies have shown that cellular physiological H_2S can modulate ETC[25] and mitochondrial K_{ATP} channel effectively[26]. In the present study, the critical role played by mitochondria in distinguishing the ambiguous impact of H_2S on HT-29 cells was addressed. Major findings of the study were, i) unlike in normal colon cells, H_2S promoted colon cancer cell proliferation and its action was independent of mitochondria and ii) exogenous H_2S-mediated cancer cell growth depended on the level of endogenous H_2S.

The toxic property of H_2S was identified around 300 y ago and recent studies have provided enough evidences for its genotoxicity, mutagenicity and carcinogenicity[13,27]. The effects of different concentration of H_2S were examined on the cell viability of HT-29 cells for 24 h by MTT assay, CV assay, SRB assay and LDH activity (fig. 1). H_2S did not show any inhibition in their growth with different concentrations (5 µM to 50 mM), except 50 mM. All cytotoxicity assays showed similar results suggesting nontoxic nature of H_2S in HT-29 colon cancer cells oncogenesis. Furthermore, the clonogenic assay (fig. 1) was carried out to assess the cell proliferative potential of H_2S and the results suggest no inhibitory action of H_2S on HT-29 cell growth. In addition, caspase 3 (an indicator of apoptosis) activity was measured at the end of the experiment and the results suggest relatively no significant increase across the different concentration, supporting the proliferative capacity of H_2S (fig. 1). Evidence from the literature supported the cell proliferative capacity of H_2S in oral cancer cells[28], C6 glioma cells[29] and human colon cancer cell[11]. Results reported by Cai *et al.* on HCT 116 demonstrated proliferative effects of NaSH up to 1 mM without mentioning its impact on increasing concentrations further[11], which was addressed in the present study.

Another cell line, IEC 6 was used to assess the impact of NaSH on the proliferative capacity. Accordingly it was found that H_2S (up to 10 µM) did not show any

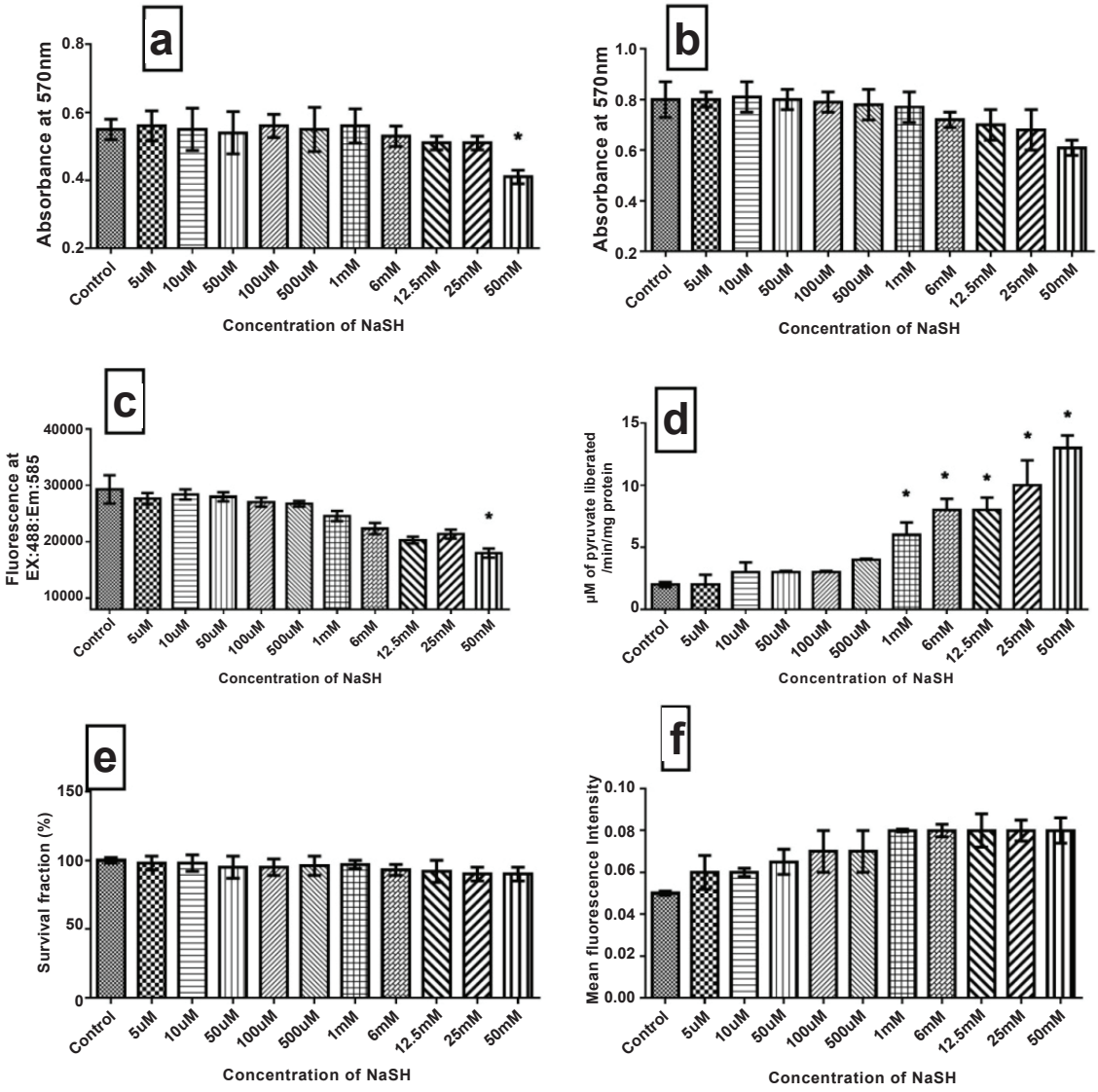

Fig. 1: Role of H_2S on the cell proliferation and apoptosis in HT-29 cells

Cytotoxic effect of H_2S on HT-29 cells evaluated by a) MTT assay; b) CV assay; c) SRB assay and d) LDH release by the cells. Cell proliferation in presence of H_2S is presented as e) clonogenic activity of H_2S in HT-29 cells. Level of apoptosis is represented in f) caspase 3 activity. Data expressed as mean±SD of the experiments done in triplicates. *p<0.05 vs. control group

significant inhibitory effect after 24 h exposure (fig. 2). But, above those concentrations, H_2S inhibited the IEC 6 cell viability in a dose-dependent manner measured via MTT and CV assays. SRB and LDH assay results complemented the above result. Clonogenic assay was used to reconfirm the result and it was found that clonogenic capacity was affected only after 100 µM concentration (fig. 2).

The toxic effect of H_2S is well-established and different cell types experienced different toxic level depends on the expression level of its endogenous enzyme like CBS and CSE. Accordingly, it had been generalized that an intracellular concentration ranging between 0.01 to 1 µM is nontoxic; instead it might trigger ATP production via electron supply to complex V. Concentrations 3 to 30 fold higher were considered to be toxic[30]. Hence, in the present investigation whether H_2S-mediated cytoprotection of HT-29 cells was dependent on mitochondrial cellular bio-energetics (H_2S is reported to modulate mitochondria and control cellular energetics) was studied. It is well-established that the cancer cells have altered respiratory chain complex activities when compared to the normal cell, where the latter is considered to be one of the major sources of reactive oxygen species under pathological conditions. Accordingly, rotenone and azide were used to inhibit ETC complexes I and IV, respectively. The results demonstrated that rotenone and azide had a significant inhibitory effect on cell growth by 40 and 20 %, respectively. According to the early scientific

reports, mitochondrial complex I and III are the major source of ROS. Hence by inhibiting complex I and III via rotenone and antimycin, respectively might generate elevated ROS production, as reported by many[31], can kill or reduce the growth of HT-29. However, no similar effects were observed when the cells were incubated with H_2S prior to ETC inhibition. The results showed that there was no influence of these ETC enzymes over the cytoprotective effect of H_2S on HT-29 cell, measured via MTT and CV assays (fig. 3). Based on the above results, it is confirmed that cytoprotective effect of H_2S on HT-29 cancer cell was independent of ETC.

Recent strategy to combat cancer growth included those agents that can specifically compromise the structural and functional integrity of mitochondria, by modulating the respiratory chain, DNA biogenesis, potassium channels, the Bcl-2 protein and the permeability transition pores, provided that these molecules can be selectively delivered to tumor sites[32]. H_2S, being metabolized inside mitochondria can influence the MPTP transition that regulate apoptosis via mitochondrial K_{ATP} channel. Evading apoptosis is one of the hall marks of cancer cell[33]. The change in mitochondria MPTP is the key regulator of apoptosis and the effects of H_2S on mitochondrial potassium ATP channel was analysed, that regulate the MPTP using a channel opener, diazoxide and a channel blocker, glibenclamide. Pre-treatment of cells with diazoxide had an inhibitory effect on cell growth by 40 % whereas glibenclamide had no effect on cell viability as given in fig. 4. However, cytotoxicity evaluated in HT-29 in presence of both H_2S and mitochondrial K_{ATP} channel modulators showed similar insignificant change when compared with the control, indicating that absence of mitochondrial dependency of H_2S in promoting the proliferation in HT-29 cells.

Evidence reported in the literature suggested that H_2S can promote cell proliferation via activating COX2/AKT/ERK1/2 axis[28]. In another study with neuroblast cells (SH-SY5Y), it was reported that H_2S could inhibit rotenone-induced cell apoptosis via regulation of mitochondrial K_{ATP} channel/p38- and JNK-MAPK pathway[34]. More experimental evidence on the biological effect of H_2S suggested that in normal cells, two forms of sulphur store can release H_2S that included bound sulfane sulphur (under reducing condition) and acid labile sulphur. Exogenous administration of H_2S normally get absorbed into the cell as bound sulfane sulphur and this form can act as the source for sulphur in iron sulphur centre in ETC redox centre[35]. Szabo and associate have shown that CBS derived H_2S can stimulate cell proliferation, maintain bioenergetics and promote angiogenesis[12]. The endogenous gasotransmitter H_2S is generated from L cysteine via the action of CBS and CSE[36]. In order to distinguish the effect of exogenous and endogenous H2S, PAG was used as an irreversible inhibitor of CSE and AOA, a CBS inhibitor. Administration of the inhibitors induced 60 % reduction in cell viability as shown in fig. 5,

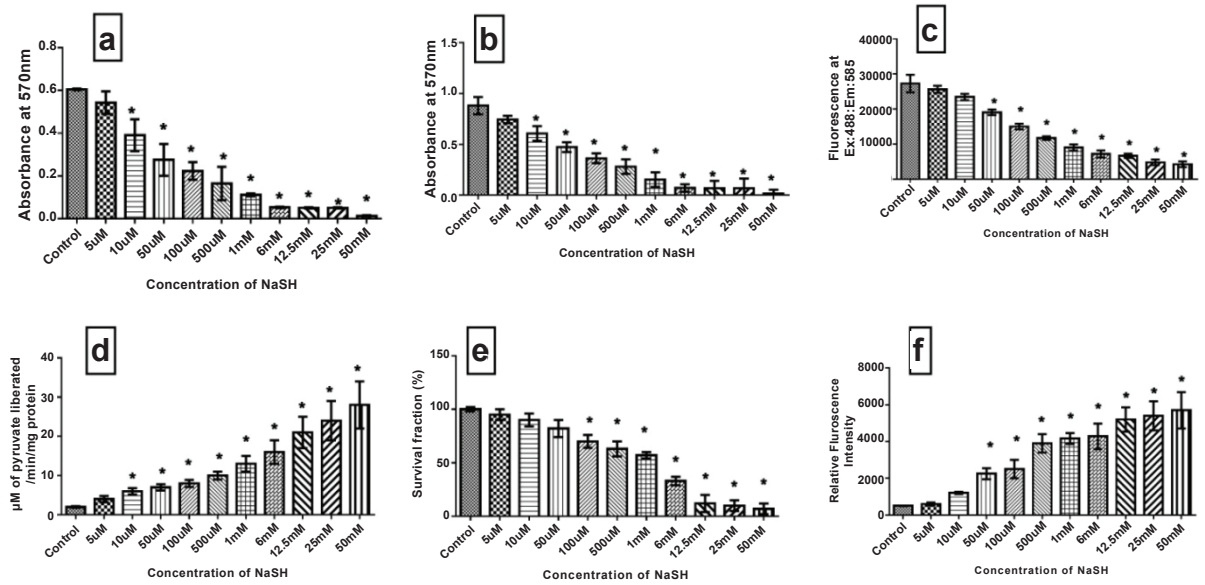

Fig. 2: Effect of H_2S on intestinal cell line, IEC-6 cells

Cell viability of IEC-6 cells when treated with H_2S is presented in a) MTT assay; b) CV assay; c) SRB assay; d) LDH release by the cells; e) cell proliferative potential of IEC-6 cells in the presence of H_2S by clonogenic assay and f) apoptosis level by caspase activity. Data expressed as mean±SD of the experiments done in triplicates. *p<0.05 vs. control group

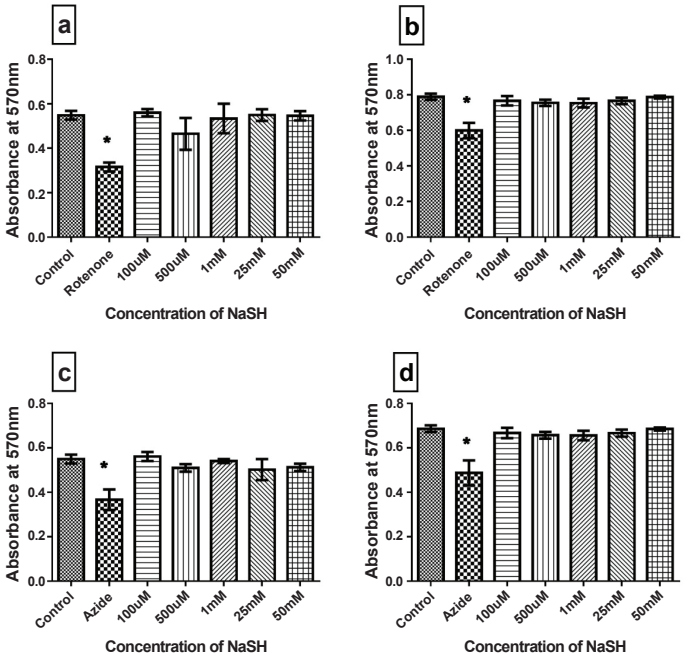

Fig. 3: Influence of ETC inhibitors on the growth of HT-29 cells in the presence of H_2S

Cell viability of HT-29 cells in the presence of H_2S when treated with electron transport chain (ETC) inhibitors I and IV are presented. a) MTT assay with pretreatment of rotenone (complex I inhibitor) and H_2S; b) CV assay with pretreatment of rotenone (complex I inhibitor) and H_2S; c) MTT assay with pretreatment of azide (complex IV inhibitor) and H_2S; d) CV assay with pretreatment of azide (complex IV inhibitor) and H_2S. Data expressed as mean±SD of the experiments done in triplicates. *p<0.05 vs. control group

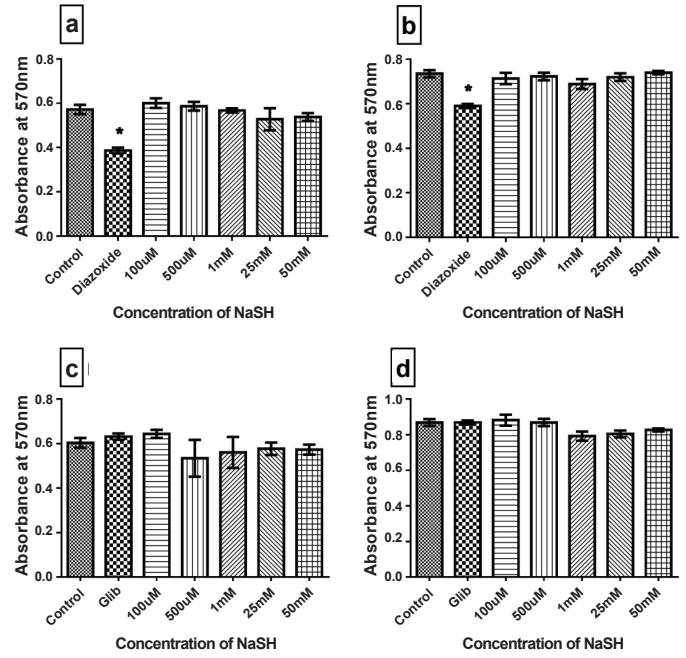

Fig. 4: Role of mitochondrial K_{ATP} channel in the growth of H_2S-treated HT-29 cells

Cell viability of HT-29 cells in the presence of H2S when treated with a K_{ATP} channel opener (diazoxide) or a K_{ATP} channel closer (glibenclamide) in a) MTT assay with pretreatment of diazoxide and H_2S; b) CV assay with pretreatment of diazoxide and H_2S; c) MTT assay with pretreatment of glibenclamide and H_2S; d) CV assay with pretreatment of glibenclamide and H_2S. Data expressed as mean±SD of the experiments done in triplicates. *p<0.05 Vs control group

indicating the impaired utilization of H_2S as fuel and the importance of endogenous H_2S in the cell survival. However, when the cells were pretreated with higher concentration of exogenous H_2S (above 100 μM) for 24 h along with the inhibitors, the cell viability was significantly improved by 45 %, when compared with PAG/AOA control group. In fact, the low dose of H_2S administration along with PAG/AOA did not improve

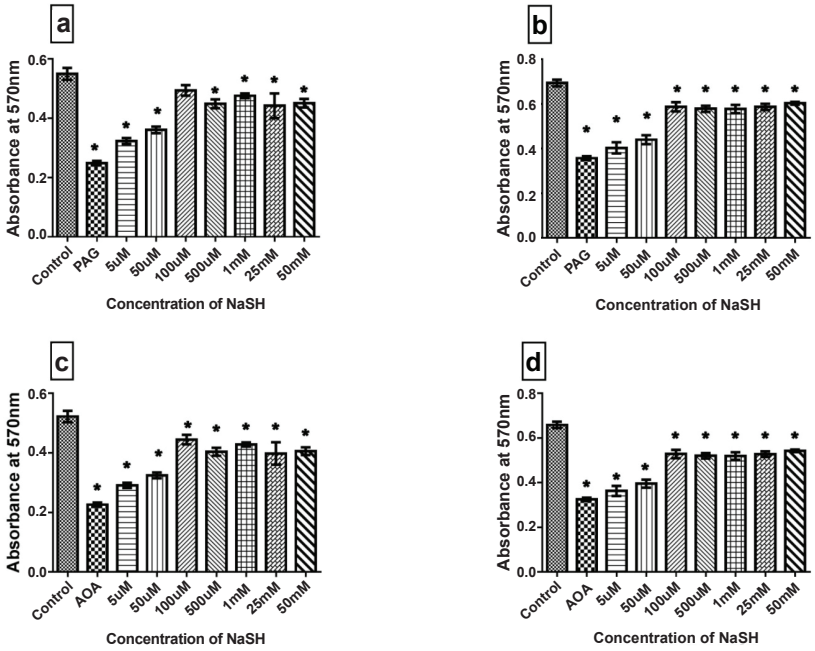

Fig. 5: Influence of endogenous H_2S on exogenous H_2S-mediated cell growth in HT-29 cells
The role of endogenous H_2S on exogenous H_2S-mediated cell growth of HT-29 cells was studied using a) MTT assay with pretreatment of PAG (H_2S inhibitor) and H_2S; b) CV assay with pretreatment of PAG and H_2S; c) MTT assay with pretreatment of AOA and H_2S; d) CV assay with pretreatment of AOA and H_2S. Data expressed as mean±SD of the experiments done in triplicates. *p<0.05 Vs control group

the cell viability. This emphasizes the prominent role played by endogenous H_2S in colon cancer cell growth or the endogenous H_2S enzyme regulation by exogenous H_2S administration. In this line of thought, several researchers suggest that PAG/AOA treatment to cancer cell can reduce basal cellular respiration thereby reduce ATP synthesis and attenuated the spare respiratory capacity. It is worth mentioning that H_2S mediated two opposing effects on mitochondria, i) H_2S suppressed mitochondrial function in mammalian tissue by inhibiting cytochrome c oxidase and ii) H_2S acted as an inorganic substrate and electron donor for electron transport system that generate ATP. Thus the variations in H_2S function depended on the metabolic status of the cell. Accordingly, high cell death with H_2S in presence of PAG/AOA is most likely related to the impaired utilization of H_2S as fuel or impaired transporter (malate aspartate shuttle)/transaminase reaction via pyridoxal dependent enzyme by AOA.

Based on the above observations it could be concluded that H_2S is toxic to normal colon epithelial cells above 10 µM but could be well utilized by colon cancer cell even with 50 mM. The ability of HT-29 to utilize H_2S depends on the level of endogenous H_2S and independent to electron flux in ETC and change in mitochondrial K_{ATP} channel.

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Conflict of interest:

The authors declare no conflict of interest.

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