

Role of Hydrogen Sulfide, Biologically-Active Compound, During Cell De-Differentiation and Differentiation Processes



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Abstract

H₂S has been shown to act as a biologically active compound in mammalian cells; H₂S may also involve cardio protective or cardiovascular therapeutic effects. The concentrations of NaHS, used instead of H₂S at μM - mM , in most of previous studies are higher than the IC₅₀ or Ki of cytochrome c oxidase (COX) activity by H₂S. However we found COX is inhibited by only 500 nM H₂S, reactive oxygen species causing DNA double-strand breaks are produced, and the mitochondrial membrane is depolarized. Following the above redox reactions, the p53 pathway is activated. Consequently, apoptosis is initiated. If the lowest concentration of H₂S (1 nM) is applied for hepatic or pancreatic differentiation from human-tooth pulp, the differentiation or proliferation is heavily promoted through WNT signaling and PI3K-AKT signaling pathways. A possibility of regenerative medicine or reversal ageing using H₂S at nM level is also suggested. On the other hand previous studies clearly indicated that the accuracy of dose-response studies using NaHS or Na₂S at μM - mM are questionable, we cannot produce constant concentration of H₂S using NaHS. NaHS is easily vaporized, and the dissociation constant of H₂S is not equal to that of NaHS. This presents a huge discrepancy affecting investigations of redox biology. The concentration of H₂S used for *in vitro* or *in vivo* experiments is strongly recommended to be determined by a precise and suitable measure. The review focuses on effects of H₂S on apoptosis, typical de-differentiation process, differentiation of the stem cells, and regenerative medicine.

Keywords: Hydrogen sulfide; Biologically-active compound; Reactive oxygen species; Apoptosis; Stem cell; Tooth

Abbreviations: H₂S: Hydrogen Sulfide; COX: Cytochrome C-Oxidase; MDA: Malon-di-Aldehyde; ROS: Reactive Oxygen Species; CBS: Cystathionine β -Synthase ; CSE: Cystathionine γ -Lyase; DCFH-DA: Di-Chloro-Fluorescein Diacetate; HGF: Human Gingival Fibroblasts; Caspase: Cysteiny-Aspartic-Acid-Proteases; NO: Nitric Oxide; CO: Carbon Monoxide; MAPK: Mitogen-Activated Protein Kinase; ERK: Extracellular Signal-Regulated Kinase ; iPS: Induced Pluripotent Stem; ES: Embryonic Stem

Introduction and Background

Hydrogen sulfide (H₂S) is a poison gas like cyanide; both are strong inhibitors of cytochrome c oxidase (COX; EC 1.9.3.1), which is a crucial enzyme for the respiratory chain in mitochondria [1-3]. Thus, H₂S readily causes cell suffocation leading to death; people attempt suicide using home-produced H₂S [4]. H₂S causes human death rapidly but the IC₅₀; half maximal (50%) inhibitory concentration of COX activity caused by H₂S in animals is 0.13 μM - 0.30 mM [5-7]. H₂S has been also shown to be a gasotransmitter, meaning that H₂S is one of the biologically-active compounds required for initiating or supporting biochemical processes in mammalian cells [8]. Gasotransmitters are able to enter into the cell. Cell membranes present no barrier to them [9]. This means that H₂S is unlike many of the regular transmitters, which need receptors on the cell membrane to control the signal transduction

systems in the cells [8]. The barrier for H₂S permeation on the Gibbs energy profile is negligible, and the hydrophobicity of H₂S allows it to get into the paraffinic interior of the membrane freely [10]. H₂S might control or regulate the process more easily than the regular transmitters do, since H₂S permeates cell membranes so easily [10]. Thus, H₂S at low concentrations may be involved in many biological activities in mammalian cells.

There is some doubt about the H₂S concentrations utilized in studies determining the biological activity of H₂S, if H₂S is provided by exogenous NaHS or Na₂S. Most of the studies employed 50-1000 μM of NaHS in place of H₂S for their *in vitro* studies [11-13]. Some have even employed Na₂S instead of H₂S. The concentrations utilized in these studies are much higher than the IC₅₀ of COX activity by H₂S. Even animal studies employed higher concentration of NaHS than

the IC_{50} determined by Nicholson et al. [5]. Therefore, cell toxicity of H_2S is expected, but the studies have been reported only the positive activities of H_2S rather than its cytotoxicity. In measuring malondialdehyde (MDA) content Geng et al. [14] found that NaHS at 100 μM , which is higher than the IC_{50} determined by Nicholson et al. [5], almost completely scavenged 0.3% H_2O_2 oxidative stress. Other studies employing NaHS as exogenous H_2S have demonstrated that a high concentration of H_2S of over 50 μM , also higher than the IC_{50} , reduces oxidative stress in mammalian cell culture in which reactive oxygen species (ROS) were increased by chemical or other pathological interventions [13,15-19].

On the other hand, no changes were found in cells having a healthy level of ROS (without any intervention) after exposure to high concentrations of NaHS [16,19]. Hence, anti-oxidative activity of 50 μM H_2S is uncertain, the H_2S concentration in these reports may not be same as active free H_2S concentration. However, antioxidant activity by H_2S has been strongly suggested in mammalian tissues. H_2S endogenously produced by cysteine reduces ROS, but its concentration is not clear [20]. The usefulness of NaHS in treating cardiac disorders, especially myocardial injury, has been reported [8, 13-17, 19, 21-24], many studies utilized much higher concentration of H_2S than the IC_{50} . It has been clearly indicated the potential ability of H_2S to control or prevent these conditions, although convinced concentrations of free active H_2S has not yet determined. Very high concentration of NaHS at the mM level causes COX inhibition followed by depolarization of mitochondrial membrane [12], leading to the production of large amounts of ROS. Such a concentration of H_2S produces oxidative stress through ROS production [11].

This speculation seems to be correct, since a H_2S concentration higher than that causing anti-oxidative activities increases oxidative stress as reported by others [8,11,13-15]. These results were supported by Sun et al. [25]. It has been claimed that most tissues contain H_2S at the concentration range of approximately 30- 300 μM [15]. However, the cells will not be able to survive in the mM level of H_2S . There is a further contradiction here: such a high concentration of H_2S at 30-300 μM is higher than the IC_{50} of COX activity by NaHS [5]. Moreover, the K_i , the inhibitor constant, of H_2S to COX is only 0.2 μM [26]. Furne et al. [26] reported that the real concentration of H_2S is only around 15 nM in many tissues, less than one thousandth of the concentration previously claimed, although they spent much time for sampling: this means the production of extra H_2S may happen. But they used a precise gas chromatograph with flame photometric or chemiluminescence detector to determine H_2S concentration, moreover they produced gas samples for H_2S determination rather than tissue-fluid samples containing much non-free H_2S . So Nicholson et al. [5] and Furne et al. [27] detected only the free form of H_2S .

Furthermore amount of H_2S produced by both NaHS and Na_2S is not constant. H_2S production rate depends on pH [28]. Hence, as Nicholson et al. [5] or Furne et al. did, a gas chromatograph measurement is needed to find the exact concentration of H_2S . Further discussion regarding the inconsistency of H_2S concentration in the tissues may be required. Besides NaHS vaporizes quickly.

Although a reliable sensor with perfect specificity to H_2S has not yet developed [29,30], DeLeon et al. [30] found much faster loss of H_2S with using a semiconductor sensor, and reported their inability to maintain H_2S concentration; also half time, the time required to lose half of the H_2S , is only 5 min or less in several experimental conditions: they concluded that the accuracy of dose-response studies are questionable. Part of the basis for the above comments is that a stock solution of NaHS utilized for the H_2S studies is never recommended for H_2S studies [14]. On the other hand Ariyaratnam et al. [31] have reported that NaHS causes dilatation of human pulmonary arteries and attenuates cardiac dysfunction.

This fact is undisputed; however, the concentrations of H_2S actually utilized in those experiments might be different from those reported, because of the inability to determine the true concentration of NaHS present [32]. The method of Gilboa-Garber [33] is frequently employed to determine H_2S concentration [8,19,34,35]. However the method of Gilboa-Garber [33] quantifies the concentration of inorganic sulfide including H_2S , the method is not specific to detect H_2S . A problem lies in that they used NaHS, but they were unable to determine how much of the free form of H_2S was present before Nicholson et al. [5], Furne et al. [26] or Yaegaki et al. [29] described how to measure concentrations of H_2S in the free form with using a gas chromatography with a flame photometric detector. Yaegaki et al. [29], Calenic et al. [36] and Li et al. [37] determined the certain concentration of H_2S effecting on several tissues with using a H_2S gas generator producing a constant concentration instead of NaHS (Figure 1), and the effects of H_2S at a nM level on cell metabolism, the respiratory chain and on apoptosis have been determined.

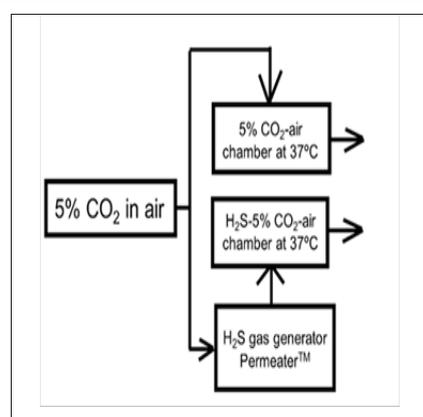


Figure 1: H_2S gas-producing system for keeping a constant concentration. NaHS instead of H_2S gas is utilized for most other studies, but these do not determine the concentration of the free form of H_2S in the medium. As NaHS is easily gasfied, there must be a large loss of SH. Using both our system and a gas chromatograph we can produce less than 100 nM H_2S constantly. Instead of the Permeator™ (GASTEC, Kanagawa, Japan), the Dynacalibrator (VICI Metronic, Houston, USA) can also be uti.

The role of H_2S during de-differentiation is relatively well investigated as mentioned above. On the other hand, information on its role during differentiation is very limited. H_2S exhibits an important role in the cell viability through controlling redox

homeostasis by cysteine/glutathione metabolism including cystathionine β -synthase (CBS) and cystathionine γ -lyase (CSE) [13,38,39], and by mediation through ion channels [40]. As well, activation of the PI3K-Akt pathway inhibits apoptosis. However, neither the relationship between H_2S and stem-cell differentiation nor the role of H_2S during differentiation has been clearly determined. The efficacy of the gasotransmitter H_2S in regenerative medicine using human-tooth-pulp stem cells has been determined, and a clear positive effect on cell differentiation in regenerated tissues was found [37, 41-43]. The role of H_2S during differentiation is also discussed in this review.

ROS Production, SOD Activity and H_2S

It has been previously reported that high concentrations, around 100 μM of NaHS, increased ROS scavenging activities in mammalian cells [8,13-15,20]. The following hypothesis is still under discussion: H_2S evokes a strong antioxidant activity against ROS in mammalian cells, consequently reducing oxidative stress [8,13-15,20]. It has also been suggested that d-cysteine may protect tissues, especially heart, from ROS and even from ischemia, due to H_2S produced from d-cysteine by CBS, CSE or 3-metacaptopyruvate sulfurtransferase [8,13-20]. Hence Yang et al. [13] have been implied that H_2S decreases the amount of pathologically-elevated ROS by inhibiting the ROS-activated NF- κB pathway or by increasing the activity of superoxide dismutase (SOD) [15]. Hence, pathologically elevated ROS might be decreased by administrating NaHS as an exogenous form of H_2S [15,19], although the real concentration of active H_2S is not clear. On the other hand, NaHS does not increase ROS in mammalian cells that are in a normal or healthy condition [19]. Based on those studies there is another hypothesis: it is claimed that H_2S administration might be a novel cardiovascular therapeutic procedure [8,16].

The assertion that such a high concentration (hundred μM level) NaHS can protect the cells from ROS rather than causing damage to the cells may need to be taken under careful reconsideration and further research for the following reasons: Ago et al. [44] reported that a 100 μM sulfur introduced by H_2S inhalation causes human death. Cysteine, around 100 μM , is known to cause cell death, and cysteine significantly elevates in patients who suffer early stroke deterioration, cysteine converts to H_2S , indicating that H_2S may be acting as a mediator of ischemic brain damage [45]. Inhibition of H_2S formation was also suggested as a novel approach in acute stroke therapy [45]. Mann et al. [46] implied a cause of sudden infant death syndrome (SIDS): the H_2S detoxifying system at the colon surface may not have matured by the age of 3 months, so H_2S could possibly be absorbed, resulting in SIDS. It is clear that H_2S inhibits COX, leading to cellular asphyxia [1,47,48]. In the mitochondrial respiratory chain COX is the terminal enzyme (complex IV), SIDS might increase ROS production dramatically in a burst [48,59,50].

There is another outstanding question concerning the protective or curative effect of NaHS in situations like heart or myocardial conditions, i.e. both pathways of AMPK and AKT are activated by H_2S for cardio protection, but AKT negatively controls

AMPK [19,49,52]. These contradictions might happen because they did not make accurate and exact measurements of active H_2S concentration [32]. To confirm the hypothesis that H_2S has protective/treatment effects on heart conditions, further studies are required to determine the safe and effective concentration of H_2S that will prevent or treat these conditions. On the other hand, application of 500 nM H_2S , which is less than that found in human gingival crevicular fluid in periodontal conditions producing much amount of volatile sulfur compounds as the colon does [53], and which is less than one hundredth of the exogenous NaSH utilized in previous researches, increased the amount of ROS, including all mitochondrial ROS present, and inhibited SOD activity [54], which results are totally different from those found by other researchers [11,13-15,55]. However, all of them used μM level or more of NaHS. Sun et al. [15] and Eghbal et al. [11] and Yang et al. [13] utilized 2',7'-dichlorofluorescein diacetate (DCFH-DA) to determine ROS value.

DCFH-DA detects mainly hydrogen peroxide or hydroxyl and peroxy radical in cytosol even though the manufacturer's instructions claim that the product is for detecting ROS [56]. Geng et al. [14] detected malondialdehyde which is byproduct from ROS, they did not directly measure ROS. Shibuya et al. [55] did not determine amount of O_2^- . But Yaegaki et al. [54] detected O_2^- in mitochondria. Furthermore H_2S at 500 nM does not increase any activity of ROS scavengers [36,54,57-61]. On the other hand 500 nM H_2S inhibited SOD strongly and increased ROS greatly [54]. If such a low concentration (500 nM) of H_2S inhibits SOD and produces ROS, much higher H_2S concentrations (μM levels) may not be able to scavenge ROS. The effects of exogenous NaHS on ROS production and the process are not clear. CuZn-SOD (E.C.1.15.1.1; Sigma-Aldrich, Oakville, ON, Canada), Mn-SOD (E.C.1.15.1.1; Wako Pure Chemical, Tokyo, Japan) and also SOD in human gingival fibroblasts (HGF) homogenate were exposed to 500 nM H_2S [54]. The percentage inhibition of CuZn-SOD and Mn-SOD was found to be around 70-80 % for 1h incubation and increased in proportion to incubation time.

The inhibition of HGF-SOD was 80% [54]. They found that such a low concentration of H_2S inhibits SOD activity rather than protecting the cells from ROS. All cells in their studies are human cells. It is very unlikely that a concentration as high as 50-100 μM of NaHS would reduce oxidative stress. There is a possible ironic explanation of why 50-100 μM of NaHS reduces ROS: since a high concentration of NaHS reduces cell vitality, biological products including ROS might also decrease. Part of the toxicity of H_2S to mammalian cells is ROS production, which may cause DNA damage [11,12,62]. Further studies would be required to confirm if H_2S increase or decreases oxidative stress.

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reported the anti-apoptotic effect of 100 μM H_2S in cardiomyocytes by antioxidative activity of H_2S through the SIRT1 pathway. Zhen et al. [17] found that NaHS at 500 μM induces cell proliferative or anti-apoptotic effects by activating the NF- κB pathway. But 100-500 μM of NaHS was utilized as the exogenous H_2S source in these studies is much higher than IC_{50} or Ki. On the other hand Yang et al. [68] investigated whether H_2S caused apoptosis in human aorta smooth-muscle cells. Exogenous H_2S originated from NaHS did not induce significant cell necrosis and LDH release, but very high concentration NaSH (500 μM) increased the number of condensed apoptotic nuclei, accompanied by the typical morphological changes of apoptosis.

Furthermore, NaHS also increased the number of TUNEL-positive cells, and DNA fragmentation was confirmed by DNA ladder agarose gel electrophoresis. NaHS induced apoptosis in human aorta smooth-muscle cells. They found around 10% incidence of apoptosis after incubating the cells with 200 μM NaHS for 12 h. Also similar apoptosis happened in neurons [69]. Takeuchi et al. [70] carried out cell-cycle analysis after exposure to H_2S expected to be lower than 1 μM , using the same H_2S -exposure system as Yaegaki et al. [54] used (Figure 1). The proportion of cells in G (1) phase was significantly increased and the cells in S phase significantly decreased. Moreover Rb phosphorylation was reduced and p21 (Cip1) expression was increased after exposure to H_2S . They concluded that H_2S inhibits cell proliferation and induces cell-cycle arrest through p21 (Cip1) in the HeLa cell line. It was also suggested that H_2S might cause apoptosis in human-oral keratinocytes.

Yaegaki et al. [54], Calenic et al. [36,57,58,61] found that a similar apoptotic process determined in the HeLa cells also happened in human-skin keratinocyte stem cells and human primary oral keratinocyte stem cells. Human oral-keratinocyte stem cells were separated from human biopsies using a protocol established by Calenic et al. [58]. p53 expression was induced by 500 nM H_2S in the cells [57]. As previously demonstrated, the number of apoptotic cells among these cells was significantly increased after exposure to 500 nM H_2S [57,58,59]. In the apoptotic process DNA fragmentation indicating apoptosis was increased, ROS production was also increased, mitochondrial membrane potential was collapsed, and there was a significant amount of cytochrome c released into the cytosol. Caspase-9 and -3 activities were also significantly increased by H_2S exposure, but caspase-8 activity was not increased except osteoblasts MC3T3-E1 from mouse [67].

Calenic et al. [57,58] measured p53 and the related gene BAX to determine if the p53 pathway is stimulated by DNA damage caused by 500 nM H_2S . A member of the Bcl-2 protein family, Bax is the primary response gene to p53, and starts after p53-mediated apoptosis [71]. Total p53, phosphorylated p53 Serine 46 and Bax were significantly increased after H_2S incubation. In particular, both total and p53 phosphorylated p53 at serine 46 were dramatically increased after exposure to H_2S . These data demonstrated that the p53 pathway plays the most important role in apoptosis caused by H_2S . A simple apoptotic pathway caused by 500 nM H_2S is summarized in Fig. 2. The expression of all genes appearing in

the p53 pathway activated by 500 nM H_2S was determined; more details of the p53-pathway-related cell cycle and DNA repair are found (Figure 3) [61]. From these reports it was suggested that the gasotransmitter H_2S , of which the concentration might be much less than previously expected, may cause apoptosis.

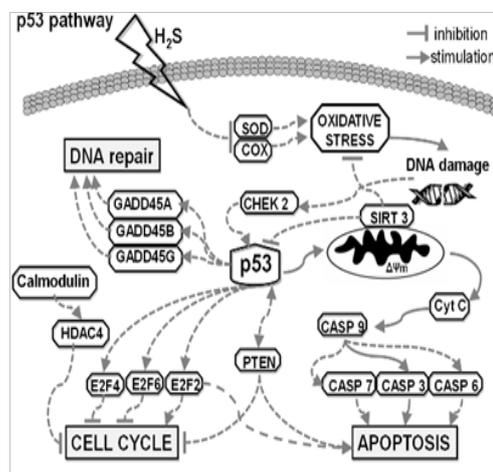


Figure 3: Cell cycle, DNA repair and apoptotic pathways initiated by 500 nM H_2S [61].

H_2S Differentiates Osteoclasts Rather Than Demonstrating its Toxicity

Zhou et al. [49] found that 100 μM NaSH inhibited mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase p38 pathways, and then activated PI3K/Akt signaling in cardiomyocytes, when H_2S reduced high-glucose-induced apoptosis both in vitro and in vivo. On the other hand Imai et al. [72] found that 500 nM H_2S inhibits the proliferation of osteoblasts through the MAPK pathway as shown in Fig. 4. This contradiction might depend on the differences in the concentrations or the tissues. It was also found that 500 nM H_2S causes apoptosis in osteoblasts, as described above [67]. Li et al. [37] determined the effects of H_2S on osteoclast differentiation. When the murine macrophage cell line RAW264 was exposed to only 1 nM H_2S instead of 500 nM, osteoclasts were differentiated from the cell without nuclear factor-kappa B ligand (RANKL) [37].

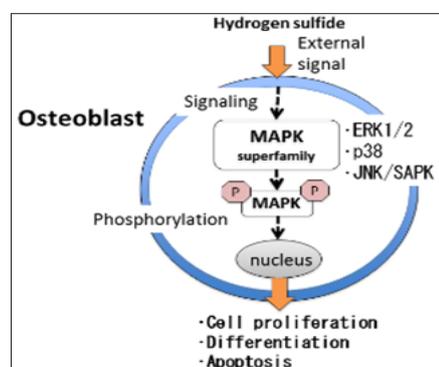


Figure 4: H_2S controls the proliferation of human osteoblasts through MAPK pathway [72].

A recent review has demonstrated that the strength of osteoclast

differentiation by a receptor-activator of RANKL is dependent on the redox state of the precursor cells, macrophages, that is controlled by H₂S [73]. Li et al. [37] determined the effects of MAPK inhibitors on RAW264 cell differentiation by H₂S, extracellular signal-regulated kinase (ERK) inhibitor and p38 inhibitor suppressed osteoclast differentiation by H₂S. They concluded that H₂S at 1 nM in the medium differentiated osteoclasts from RAW264 through MAPK [37]. Lee et al. [74] determined the effects of NaHS on nicotine- and LPS-induced osteoblastic and osteoclastic differentiation, and found that the differentiations were inhibited by MKP-1 enzyme inhibitor (vanadate) and expression inhibitor (triptolide). These findings strongly suggest that H₂S has effects on the differentiation of stem cells, especially adult stem cells.

H₂S and Ion Channel

H₂S influences a number of biological compounds, one of them being T-type Ca²⁺ channels. The T-type Ca²⁺ channel is the low-voltage activated channel activated by depolarization of the plasma membrane. The T-type Ca²⁺ channel involves three isoforms: C_v3.1, Ca_v3.2 and Ca_v3.3. H₂S appears to affect Ca_v3.2 channel activity especially using human embryonic kidney cells [75]. Ca_v3.2 channel is blocked by zinc, since it binds to a histidine residue on the channel protein [76]. H₂S activates Ca_v3.2 channel by interacting with zinc binding to the histidine residue at the channel [77]. H₂S also activates the KATP channel through cysteine sulfhydration, resulting in relaxation of vascular muscle [40]. Neurite outgrowth and altered electrophysiological changes, i.e. an increase of voltage-gated Na⁺ and/or Ca²⁺ channels, in neuronal progenitor or stem cells indicate neuronal differentiation of the progenitor or stem cells [78]. Very high concentrations of H₂S (mM level of NaHS), which would not exist in mammalian cells, induced neuronal differentiation from the progenitor or stem cells, since neurite outgrowth and increase of the high voltage-activated current in the cells through the channels were observed [79]. Consequently, activating Ca_v3.2 T-type Ca²⁺ channels with H₂S induces neuronal differentiation of the progenitor cells.

H₂S and Neuronal Differentiation

The relationship between H₂S and neuronal differentiation was also brought into focus [40] but this has not yet been done for other tissues. It has been suggested that endogenous H₂S protects neurons from oxidative stress [38]. Oxidative stress is a plausible reason for neuronal damage or conditions in the brain, including stroke, epilepsy, and Alzheimer's disease [80,81]. Oxytosis, oxidative stress caused by glutamate, is a typical condition for neural tissues. H₂S protects primary neuron cultures of rat cortex from cell death caused by oxytosis [38]. Glutathione is produced by enhancing the activity of γ -glutamylcysteine synthetase and up-regulating cysteine transport. Cysteine is the substrate of glutathione synthesis. Glutathione normally decreases oxidative stress caused by H₂O₂. Glutamate inhibits transport of cysteine into the cell, thus oxytosis is caused by glutamate. They found that NaSH at 100 μ M increases the glutathione concentration [38]. In this way H₂S protects neurons from oxytosis. Moreover, Jiang et al. [82] reported that H₂S protected neuronal cells from formaldehyde-

induced oxidative stress through the BDNF-TrkB pathway, reducing oxidative stress and controlling apoptosis by increasing Bax expression and decreasing Bcl-2 expression.

Because of the anti-oxidative activities of H₂S, a positive effect on differentiation from neural stem cells can be expected. Liu et al. [81] examined the effects of H₂S on mouse neural stem cells, and the underlying molecular mechanisms. NaHS at 0.5 - 5 μ M promoted proliferation and neuronal differentiation of neural stem cells, NaHS-induced proliferation was caused by activating ERK. Neuronal proliferation was also activated through expression of the basic helix-loop-helix transcription Factors: neurogenin 1, NeuroD2 and mammalian achaete-scute homologue 1 [81, 83]. Zhao et al. [84] reported that NaHS increased the proliferation of human iPS-cell-derived mesenchymal stromal cells via the PI3K-Akt pathway. Wang et al. [21] also determined the effects and mechanism of endogenous H₂S on the in vitro proliferation and differentiation of neural stem cells. They found that L-cysteine stimulated proliferation and increased the differentiation potential of neural stem cells through cystathionine β synthase/ H₂S and ERK pathways. In an in vivo study, NaHS administration increased the number of proliferating cells in the hippocampus of mouse after hypoxia. Moreover the administration of NaHS allowed recovery from cognitive impairment in mice subjected to hypoxia. Hence, a H₂S donor could be used for neuronal treatment, and to improve endogenous neurogenesis [81].

Effects of H₂S on Regenerative Medicine: Hepatic and Pancreatic Differentiation from Human-Tooth Pulp

It has been shown that transplantation of hepatocyte-like cells differentiated from human tooth pulp perfectly treated liver cirrhosis produced in rats [85]. It was never happened before, although adult stem cells are being used to treat diseases. On the other hand, embryonic stem (ES) cells and induced pluripotent stem (iPS) cells demonstrate the largest capacity for multilineage differentiation. However, the former involves an ineluctable ethical problem and the great possibility of producing a malignant tumor. The latter may have the same problems, and genetic modification might cause further trouble in the future: its possibility of producing malignancy may be higher than in adult stem cells [86]. In fact, Institute of Physical and Chemical Research (Japan) send-off their second retina transplantation using human iPS cells because of DNA sequence modification [87]. Regenerative medicine using stromal mesenchymal stem cells is much safer than using ES or iPS cells. Human-tooth pulp cells showed expression of CD117, nanog, nestin, CD44, alkaline phosphatase, Oct3/4, CK18, CK19, osteonectin and P63. Nanog and Oct3/4 are markers of ES or iPS cells [88]. Approximately 70% of the primary culture showed stem-cell markers, and the CD117⁺ fraction, which forms 50% of the primary culture, is now utilized for hepatic or pancreatic differentiation.

The protocol for hepatic differentiation directly from deciduous- and wisdom-tooth pulp was established [88], and then a new protocol using non-serum medium for hepatic differentiation

was developed [89]. It was also suggested that H₂S may directly affect hepatic and pancreatic differentiation [37,41-43]. On the other hand the relationship between H₂S and human-tooth-pulp stem cells has also been well reported. The CD117⁺ fraction of deciduous-tooth-pulp stem cells were incubated with 1 nM H₂S produced using a Permeator™ (GASTEC, Kanagawa, Japan), as shown in Figure 1. All the cells differentiated into hepatic cells [41]. Glycogen storage happens in hepatocyte-like-cells; it is the

distinguishing function of hepatocytes. Increased accumulations of glycogen were shown in the cells exposed to 1 nM H₂S after several days (Figure 5). Urea concentrations in the medium of the cells increased after H₂S exposure [41]. It was concluded that H₂S exposure produces more matured hepatocyte-like cells which have much less possibly of producing malignancy compared with initialized stem cells or direct transplantation of stromal stem cells without in vitro differentiation.

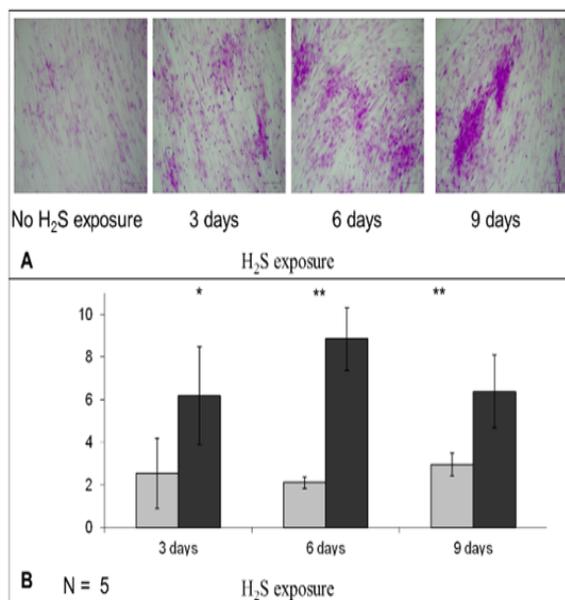


Figure 5: Glycogen storage in hepatocyte-like cells. (A) Cytoplasmic glycogen storage after the differentiation period (PAS staining, ×200). (B). The area was evaluated with Image J, and the proportion of positive cells in samples exposed to H₂S was compared with that in non-exposed samples [41]. *p<0.05, **p<0.001.

They have also compared the effects of H₂S exposure on human deciduous-tooth-pulp stem cells with those on human-bone marrow stem cells [42,43]. They confirmed that the expression level of stem-cell-related transcription factors (112 factors) was almost the same between CD117⁺ tooth-pulp stem cells and bone-marrow stem cells in six groups: ectodermal-lineage markers, mesodermal-lineage markers, endodermal-lineage markers, stem-cell/embryonic-development markers related to late embryonic

development, axis/symmetry/segmentation-markers associated with early embryonic development, and other pluripotency markers supporting pluripotency and regenerative abilities. DLX2 and MSX2 showed significant increases in tooth-pulp stem cells compared with bone-marrow stem cells; significant decreases were found in EGR3 and NANOG. Expression of OLIG2, GATA6, EGR3 and ESR1 was found only in bone-marrow stem cells [43].

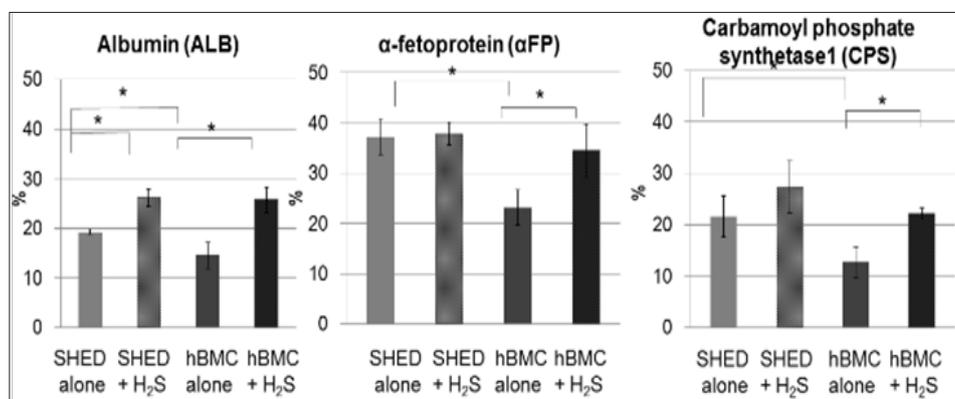


Figure 6: Flow cytometric analysis. Almost 100% of cells were positive to ALB, α- FP and CPS by microscopic examination; however, flow cytometric data showed smaller numbers, possibly because of a higher detection threshold. It was expected that more matured cells would be detected by a flow cytometer. Significant differences amongst CD117⁺ tooth-pulp stem cells, bone-marrow stem cells and H₂S treatment were found [42]. *p < 0.01.

Almost 100% of hepatic differentiated cells were positive for albumin, fetoprotein and Carbamoyl phosphate synthetase1 by microscopic examination, hence quantitative flow-cytometric data was obtained and showed smaller numbers than those obtained by microscopic examination, perhaps because of the higher detection threshold (Figure 6). It had been anticipated that more matured cells would be detected by a flow cytometer. Human-tooth showed more positive cells for albumin, fetoprotein and carbamoyl phosphate synthetase1 than human-bone-marrow stem cells. H₂S increased the number of positive cells in both types (Figure 6) [42].

It was concluded that H₂S promotes hepatic differentiation from human-tooth pulp stem cells, and thus more matured hepatocyte-like cells are obtained. Hepatocyte-like cells differentiated under exposure to H₂S might result in great clinical success when these cells are utilized to regenerate the liver in future. As described above, even implantation of non-H₂S-treated cells was able to treat liver cirrhosis produced in rats (Figures 7&8) [85], but no preclinical study using hepatocyte-like cells has succeeded before. H₂S-treated cells might be able to transform the regenerated liver into mature. Ishkitiev et al. [90] differentiated pancreas-like cells from the CD117⁺ fraction of human-tooth-pulp, describing

Concentration of urea in the culture medium was determined after hepatic differentiation. Urea concentration for H₂S-treated cells significantly increased in the media of both cell types, compared with non-H₂S-treated control cells. H₂S increased urea concentrations in a time-dependent manner. After 9 days of H₂S exposure, the urea concentration had increased almost one-and-a-half fold compared to non-H₂S-treated cells. Urea production in non-H₂S-treated tooth pulp was also found to be significantly higher than non-H₂S-treated bone-marrow cells.

the pathways of pancreatic transcription factors and hormones in differentiating the cells into pancreatic cells (Fig. 9); they also determined the effects of H₂S on pancreatic differentiation from human-tooth, and how H₂S affects it through WNT and PI3K-AKT signaling pathways [43]. Flow-cytometric analysis for cells expressing insulin and glucagon was carried out. H₂S increased the number of cells expressing insulin compared to non-H₂S-treated cells, but decreased the number of glucagon-positive cells. Thus the pancreatic cells produced by H₂S are appropriate to treat diabetes mellitus.

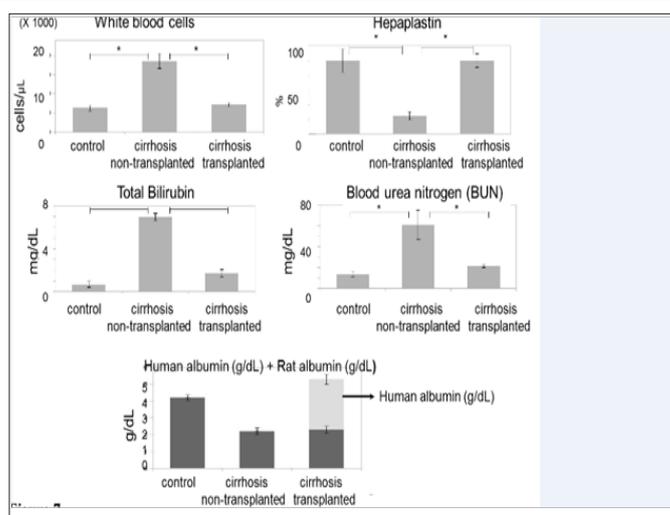


Figure 7: Treatment of liver cirrhosis in rats using regenerated hepatic cells originating from human tooth pulp. Cirrhosis non-transplanted demonstrated the typical results of blood examinations from cirrhotic rats, whereas cirrhosis transplanted, which had received transplantation of hepatocyte-like cells originating from human tooth cells, showed similar results to the control. More than half of the albumin was human albumin [85].

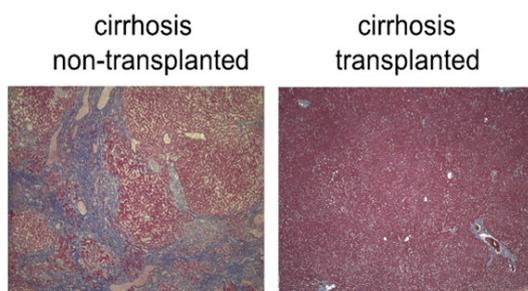


Figure 8: Treatment of liver cirrhosis in rats using regenerated hepatic cells originating from human tooth pulp. Cirrhosis non-transplanted demonstrated typical cirrhosis pathology, but fibrosis had disappeared in cirrhosis transplanted, which had received transplantation of hepatocyte-like cells originating from human tooth cells [85]. Magnification 200x.

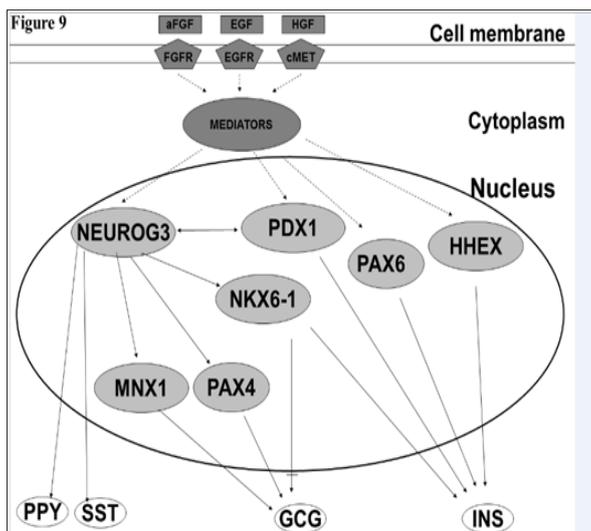


Figure 9: Pathways of pancreatic transcription factors and hormones. Analysis was performed by Ingenuity Pathway Analysis. FGF1, EGF and HGF utilized for the pancreatic differentiation protocol of human dental pulp activate the pancreatic transcription factors PDX1, HHEX, NEUROG3, PAX6, NKX6-1, Pax-4 and MNX1, and result in production of pancreatic hormones. Solid lines represent direct interactions. Dashed lines represent transduction pathways involving different and diverse cytoplasmic mediators activating the transcription factors in the nucleus. PPY: pancreatic polypeptide, SST: somatostatin, GCG: glucagon and INS: insulin [90].

To understand the relationship between H₂S and insulin or pancreas, the excellent review by Wang [91] is useful. He cited Patacchini et al. [92], Yang et al. [93] and Patel and Shah [94], and concluded that H₂S regulates insulin secretion from pancreatic β cells by enhancing the KATP channel and suppressing L-type Ca²⁺ channel activities. KATP channels are activated by H₂S at concentrations, which is more than the IC₅₀ of COX [9,92-94]. Szabo et al. [95] wrote a review of the regulation of mitochondrial bioenergetic function by H₂S. Interestingly, Okamoto et al. [96]

described protective activities of H₂S on pancreatic β cells. Insulin concentration in pancreatic cells or medium was determined when applying glucose stimulation (Figure 10) [43]. Exposure of the cells to H₂S increased intracellular or extracellular insulin concentration under no glucose stimulation. Glucose at 3.3mM increased insulin concentration in both the medium and the cells, and also in both the H₂S-treated and non-treated groups. However, stimulation with 16.7mM glucose decreased insulin production compared with 3.3 mM glucose, except in the medium without H₂S exposure.

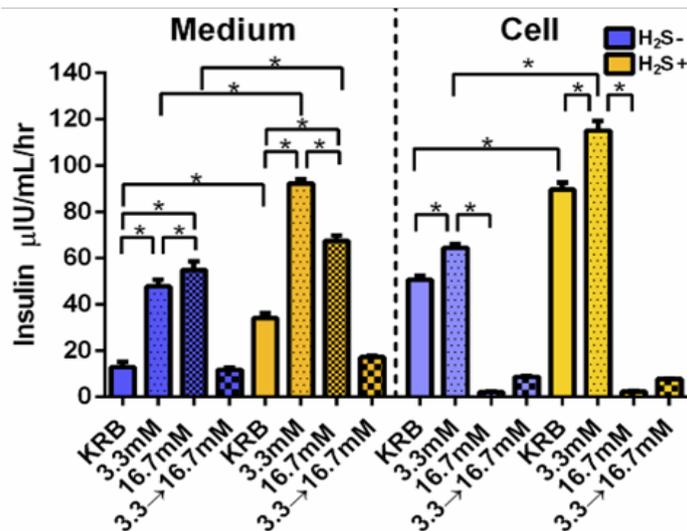


Figure 10: Glucose stimulation of pancreatic cells originating from human tooth pulp. Without glucose stimulation H₂S increased insulin concentration. Glucose at 3.3 mM increased insulin concentration in both the medium and the cells, and also in both the H₂S+ and H₂S- groups. However, 16.7mM glucose stimulation decreased insulin concentration compared with 3.3 mM glucose.

Moreover, 16.7mM glucose stimulation following 3.3mM glucose decreased insulin concentration greatly (Figure 10). However, the

amount of C-peptide inside the cells showed the highest value in those treated with 16.7mM glucose stimulation following 3.3mM

glucose under H₂S exposure. This means that when the first stimulation with 3.3 mM was carried out, the cell was already exhausted [43]. On the other hand it was found their pancreas-like β cell has same function for insulin secretion as shown in the cells derived from human embryonic stem cells [97]. WNT signaling is mainly composed of the canonical pathway, the planar-cell polarity pathway, and a calcium-ion-dependent pathway. All these pathways were activated by H₂S, but expression of WNT-signaling negative-regulation genes was very weak [43]. H₂S increased the level of expression in PI3K-AKT-related genes [43]. This pathway involves the following: AKT and PI3K family members and their regulators, the IGF-1 signaling pathway, the BAD phosphorylation and anti-apoptotic pathways, the inactivation of Gsk3 and accumulation of β-Catenin, and PTEN-dependent cell-cycle arrest and apoptosis. Genes involved in all functions of the PI3K-AKT signaling pathway were significantly expressed after H₂S exposure compared with the non-treated group [43]. It was elucidated that H₂S promotes the differentiation from human-tooth pulp through WNT signaling and PI3K-AKT pathways. The data using both 1 nM of H₂S and adult stem cells showed that lower concentration of H₂S strongly promotes differentiation of adult stem cells, a possibility of reversal ageing using H₂S, i.g. H₂S releasing NSAIDs, is suggested.

Conclusion

H₂S promotes ROS production, DNA damage and p53-mediated apoptosis at much lower concentrations than previously reported. The lowest concentration of H₂S promotes both hepatic and pancreatic differentiation of human-tooth pulp stem cells. The real concentration of H₂S is only around nM level in human tissues, less than one thousandth of the concentration previously claimed. Moreover less than one- thousandth or hundredth of the concentration previously reported showed the toxicities causing apoptosis in many tissues. Low concentration such as 1 nM promotes hepatic and pancreatic differentiation from human tooth through WNT signaling and PI3K-AKT pathways.

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