Nitric Oxide 90 (2019) 37–46

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

The gasotransmitter hydrogen sulfide inhibits transepithelial anion secretion of pregnant mouse endometrial epithelium

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ARTICLE INFO

Keywords:
H_2S
Ion transport
Anion secretion
CFTR
Endometrial epithelium
Embryo implantation

ABSTRACT

Endometrial epithelium exhibits a robust ion transport activity required for dynamical regulation of uterine fluid environment and thus embryo implantation. However, there still lacks a thorough understanding of the ion transport processes and regulatory mechanism in peri-implantation endometrial epithelium. As a gaseous signaling molecule or gasotransmitter, hydrogen sulfide (H_2S) regulates a myriad of cellular and physiological processes in various tissues, including the modulation of ion transport proteins in epithelium. This study aimed to investigate the effects of H_2S on ion transport across mouse endometrial epithelium and its possible role in embryo implantation. The existence of endogenous H_2S in pregnant mouse uterus was tested by the detection of two key H_2S-generating enzymes and measurement of H_2S production rate in tissue homogenates. Transepithelial ion transport processes were electrophysiologically assessed in Ussing chambers on early pregnant mouse endometrial epithelial layers, demonstrating that H_2S suppressed the anion secretion by blocking cystic fibrosis transmembrane conductance regulator (CFTR). H_2S increased intracellular Cl^- concentration ($[Cl^-]_i$) in mouse endometrial epithelial cells, which was abolished by pretreatment with the CFTR selective inhibitor $CFTR_{inh}$-172. The cAMP level in mouse endometrial epithelial cells was not affected by H_2S, indicating that H_2S blocked CFTR in a cAMP-independent way. *In vivo* study showed that interference with H_2S synthesis impaired embryo implantation. In conclusion, our study demonstrated that H_2S inhibits the transepithelial anion secretion of early pregnant mouse endometrial epithelium via blockade of CFTR, contributing to the preparation for embryo implantation.

1. Introduction

Embryo implantation, a crucial process in mammalian reproduction, involves the intimate interaction between an implantation-competent blastocyst and a receptive endometrium in a limited time span known as the “window of implantation” [1,2]. Ion channels, a group of transmembrane proteins allowing ions to flow across plasma membranes or intracellular organelle membranes, emerge as fundamental players in this complex process [3,4]. A variety of ion channels have been discovered in luminal and glandular endometrial epithelium, which actively regulate the uterine fluid environment and endometrial receptivity by mediating ion transport [3,5]. For example, the epithelial sodium channel (ENaC) is apically located in endometrial epithelium and mediates Na^+ influx, providing the driving force for water absorption, while the cystic fibrosis transmembrane conductance regulator (CFTR) mediates Cl^- efflux, driving water movement into the lumen [6]. Since the intraluminal uterine fluid secreted by endometrial epithelium at preimplantation and the reabsorption at implantation are essential for embryo transportation and embryo attachment reaction respectively, ENaC and CFTR together dynamically regulate the uterine luminal fluid volume for embryo implantation [3,7]. ENaC may be up-regulated and CFTR may be down-regulated in the presence of progesterone, but vice versa in the presence of estrogen [8,9], which is in accordance with the uterine fluid volume and steroid hormone changing patterns during peri-implantation [5,10,11]. Compounds that possess estrogen-like effects, such as quercetin, increased the uterine fluid volume and adversely affected embryo implantation by up-regulating CFTR and down-regulating ENaC [12]. Abnormal over-

Abbreviations: $[Cl^-]_i$, intracellular Cl^- concentration; MEECs, mouse endometrial epithelial cells

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https://doi.org/10.1016/j.niox.2019.05.011
Received 21 December 2018; Received in revised form 24 May 2019; Accepted 29 May 2019
Available online 05 June 2019

expression of CFTR and subsequent excessive fluid accumulation in uterus was claimed to contribute to *Chlamydia trachomatis*-induced infertility [13]. In addition, by providing pathways for ion transport, various channels are also implicated in several cellular responses of endometrial epithelium and neighboring cells that affect embryo implantation [3]. For instance, dysfunction of endometrial ENaC in ion transport processes has been demonstrated to severely impair embryo implantation by affecting decidualization in stromal cells [14]; blockade of the large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca} channel) in endometrial epithelium almost abolished the embryo implantation [15]. However, complicated as they are, there still lacks a comprehensive understanding of the ion channels and ion transport in endometrial epithelium during the peri-implantation period.

Recently, many studies have indicated that hydrogen sulfide (H_2S) modulated epithelial ion transport proteins to affect the electrolyte transport in various epithelia [16–18]. Recognized as the third major gaseous signaling molecule or gasotransmitter akin to nitric oxide (NO) and carbon monoxide (CO), H_2S plays important roles in a wide spectrum of physiologic processes in various tissues [19,20]. Besides functioning as a vasorelaxant factor by activating of the ATP-sensitive potassium channels (K_{ATP} channels), H_2S has also been engaged as a modulator of ion channels, including Cl^- channels, BK_{Ca} channels, L-type and T-type Ca^{2+} channels, members of the transient receptor potential family of channels, and Na^+ channels [21,22].

H_2S can be endogenously produced by three enzymes in mammals using cysteine as a substrate, including cystathionine β-synthetase (CBS), cystathionine γ-lyase (CTH or CSE), and 3- mercaptopyruvate sulfurtransferase (3-MST) [20,23]. These enzymes are widely located in a variety of tissues including brain, kidney, liver, aorta, heart, oviduct, and vagina [17,21,24]. As the key H_2S-generating enzymes, CBS and CTH are also identified in human myometrium tissues and rodent uterus [25,26], but previous studies mainly focused on the relaxant effect and anti-inflammatory effect of H_2S on the uterus [27]. Whether H_2S plays a role in the ion transport of endometrial epithelium remains elusive. The present study, therefore, aimed to investigate the effect of H_2S on the ion transport of pregnant mouse endometrial epithelium and its possible role in embryo implantation.

2. Materials and methods

2.1. Animals

Adult Kunming (KM) mice (8–10 weeks) were purchased from the Animal Center of Sun Yat-sen University (Guangzhou, China). According to the guidelines of the Sun Yat-sen University Animal Use Committee, animals were allowed food and water *ad libitum* and housed under specific pathogen free (SPF) conditions with a constant room temperature of 20 °C and a 12 L:12D photoperiod prior to the experiments. All procedures were subject to approval by the Animal Ethical and Welfare Committee of the Institutional Animal Care and Use Committee, Sun Yat-sen University (Guangzhou, China) (Approval No: SYSU-IACUC-2019-000040). Adult virgin females (8–10 weeks) were mated with fertile males (8–10 weeks) to induce pregnancy. The morning (09:00 h) on which a vaginal plug was found was considered as day 1 of pregnancy.

2.2. Real-time quantitative reverse transcriptase polymerase chain reaction (qPCR)

Mice from day 1 to day 4 of pregnancy were euthanized with CO_2 and the uteri were isolated immediately. The total RNA was extracted using RNAzol® RT (Molecular Research Center, Cincinnati, USA). The reverse transcriptase (RT) reaction was performed using the PrimeScript RT reagent Kit with the gDNA Eraser Kit (Takara, Tokyo, Japan). qPCR was performed following the manufacturer's instructions. The PCR reaction was performed using the SYBR Green (TOYOBO, Tokyo, Japan) on a LightCycler 480 instrument (Roche, Basel, Switzerland). The PCR conditions consisted of 40 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s, and polymerization at 72 °C for 30 s. The $2^{-\Delta\Delta Ct}$ method was applied for calibrations and normalization. The relative quantities of mRNAs were normalized using glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as the internal control gene. Specific primers were used as follow: *Cbs* forward, 5′-ATCCAATCACGAGACCAGGC-3'; *Cbs* reverse, 5′-GCAGTGACAACCC CAAACAC-3'; *Cth* forward, 5′- GTCTTGCTGCCACCATTACG-3'; *Cth* reverse, 5′- CTGTTGGTGCCTCCATACACT-3'; *Gapdh* forward, 5′- TGCAC CACCAACTGCTTAGC -3'; *Gapdh* reverse, 5′- GGATGCAGGGATGATGT TCT -3'.

2.3. Western blot analysis

Mice from day 1 to day 4 of pregnancy were euthanized with CO_2 and the uteri were isolated immediately. Total protein extract was obtained using Radioimmunoprecipitation Assay (RIPA) lysis buffer containing phenylmethylsulfonyl fluoride (1 mM). The equal amount of proteins loaded in each lane were separated by 12% SDS-polyacrylamide gel and transferred onto a PVDF membrane. The membranes were blocked by 5% (w/v) nonfat dry milk for 1 h at room temperature, and then incubated with mouse monoclonal antibody against CBS (1:1000; clone 3E1; Abnova, Taipei, Taiwan), CTH (1:1000; clone 4E1-1B7; Abnova, Taipei, Taiwan), or rabbit monoclonal antibody against GAPDH (1:1000; Cell Signaling Technology, Massachusetts, USA), overnight at 4 °C. After extensively washing, the membranes were incubated with relevant horseradish peroxidase (HRP)-conjugated second antibodies (EARTHOX, CA, USA) diluted at 1:20000 for 1 h at room temperature. Respective protein bands were visualized by High-sig ECL Western Blotting Substrate (Tanon, Shanghai, China) and digitized by a chemiluminescent imaging system (Tanon, 5200, Shanghai, China). The target protein band intensity was normalized over the intensity of the housekeeping protein GAPDH.

2.4. Measurement of H_2S production rate

The production rate of H_2S in mouse uterus homogenate was measured by the methylene blue method based on methylene blue synthesis from H_2S and *N*,*N*-dimethyl-*p*-phenylenediamine in the presence of acid and $FeCl_3$ as described previously [17,28], with modifications. Briefly, freshly isolated from the early pregnant mice euthanized with CO_2, the uteri were homogenized with potassium phosphate buffer (50 mM, pH = 8.0) on ice and centrifuged at 4 °C with 4500 × *g* for 20 min to collect the supernatant. The supernatants of uterus homogenates from day 1 to day 4 of pregnancy were incubated with pyridoxal-5′-phosphate (2 mM) and the substrate L-cysteine (L-Cys) (10 mM). To verify the contribution of CBS and CTH in H_2S synthesis, the homogenates from day 3 of pregnancy were equally divided into several groups, preincubated with or without *O*-(carboxymethyl) hydroxylamine hemihydrochloride chloride (AOAA, 1 mM), or/and DL-propargylglycine (PAG, 10 mM) at 37 °C for 10 min and then cooled on ice for another 10 min before the application of pyridoxal-5′-phosphate (2 mM) and L-Cys (10 mM). The outer tube containing the homogenate and the chemicals mentioned above was considered as a reaction tube, inside which was a small inner collecting tube containing a piece of filter paper soaked with zinc acetate (1% w/v; 300 μl). The reaction tube was sealed and the reaction was initiated by transferring the tube from ice to a 37 °C shaking water bath for 90 min. Next, trichloroacetic acid (10% w/v; 500 μl) was added into the reaction tube to stop the reaction. Another 60 min was allowed for the complete reaction between H_2S and zinc acetate. In the end, *N*,*N*-dimethyl-*p*-phenylenediamine sulfate (20 mM stock solution; 50 μl) in HCl (7.2 M) and $FeCl_3$ (30 mM stock solution; 50 μl) in HCl (1.2 M) were added to the collection tube to form the methylene blue. After 20 min, the absorbance of the resulting solution was measured at 670 nm with a microplate reader. The H_2S

concentration of each sample was calculated against a calibration curve obtained with sodium hydrosulfide (NaHS; 10–320 μM; Aladdin, Shanghai, China) that was similarly processed. The protein concentration of the supernatant of tissue homogenates was determined using the BCA Protein Assay Kit (CWBIO, Beijing, China).

2.5. Short-circuit current (I_{SC}) measurement

Measurement of I_{SC} was performed following a modified procedure as previously described [29,30]. Native endometrial epithelial preparations were freshly isolated from mice on day 3 of pregnancy which were euthanized with CO_2. The preparations were then kept in ice-cold normal physiological saline solution (N-PSS) composed of (in mM): 137 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES and 10 glucose (pH 7.3), containing 1 μM indomethacin before seromuscular stripping [31]. After 10 min, the uterus was incised longitudinally with the mucosal side up in a Petri dish containing ice-cold N-PSS. The mucosa was dissected from the serosa and muscular layers by carefully scraping the serosal side with the edge of a rectangular glass slide and a pair of fine forceps. The mucosal layer was then clamped vertically between two halves of the Ussing chambers (EM-CSYS-2 Ussing Chamber Systems, Physiologic Instruments, San Diego, USA) with an inner area of 0.031 cm^2, bathing in both sides with normal Krebs-Henseleit solution (normal K–H solution) composed of (in mM): 117 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 24.8 $NaHCO_3$, 1.2 KH_2PO_4, and 11.1 glucose and gassed with 95% O_2/5% CO_2 at 37 °C to maintain a pH of 7.4. The epithelium exhibited a basal transepithelial potential difference, which was measured by Ag/AgCl electrodes with KCl/agar bridges connected to the voltage-clamp amplifier (VCC MC6, Physiologic Instruments, San Diego, USA). For I_{SC} measurement, the transepithelial potential difference of mouse endometrial epithelial layer was clamped at 0 mV. The change of I_{SC} (ΔI_{SC}), defined as the difference between the value at baseline and that at a peak following compound addition, was synchronously displayed via a signal collection and analysis system (BL-420E + system, Chengdu Technology & Market Co. Ltd, Chengdu, China) and normalized by the unit area of the preparation ($\Delta\mu A/cm^2$). At the beginning and the end of each experiment, 1 mV pulse was applied and the current change in response was used to estimate transepithelial resistance according to Ohm's law.

In Cl^- free K–H solution, NaCl and KCl was replaced with equimolar sodium gluconate and potassium gluconate, respectively. 2.5 mM $CaCl_2$ was replaced by 11 mM calcium gluconate to counteract the Ca^{2+} chelation effect of gluconate [32,33]. HCO_3^- was replaced by N-2-hydroxyethylpiperazine-N-2-ethane sulphonic acid (HEPES) in HCO_3^- free K–H solution. As an analogy, Cl^- and HCO_3^- was replaced by gluconates and HEPES in Cl^- and HCO_3^- free K–H solution. It is worth notice that when HCO_3^- is absent, it should be aerated with 100% O_2.

2.6. Cell isolation and culture

Murine primary uterine endometrial epithelial cells (MEECs) were isolated and cultured as previously described with some modifications [14,34]. Uteri were isolated from adult female mice euthanized with CO_2, sliced longitudinally, exposing the mucosa, and digested in Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution (HBSS) containing 6 mg/ml dispase (Gibco) and 25 mg/ml trypsin (Gibco) at 4 °C for 1 h and at room temperature for another 1 h. Epithelial cells from loose mucosa can be released by gently pipetting the uteri several times. The disaggregated epithelial cells were collected to centrifuge with 300 × *g* for 5 min and resuspended in K-SFM completed with EGF (5 ng/ml), BPE (0.05 mg/ml), 5% fetal bovine serum and 1% antibiotics. The cells were plated on glass coverslips and cultured at 37 °C in humidified atmosphere containing 5% CO_2 for 3 days for immunofluorescence or intracellular Cl^- concentration ($[Cl^-]_i$) measurement.

2.7. Intracellular Cl^- measurement

$[Cl^-]_i$ in MEECs was measured with a modified procedure as previously described [16]. In brief, MEECs cultured on glass coverslips were loaded with 5 mM *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE; Invitrogen, E3101, Carlsbad, CA, USA) in N-PSS for 30 min at 37 °C. Fluorescence was recorded using an imaging system (Olympus, IX83, Tokyo, Japan) (Ex = 350 nm, Em = 460 nm).

2.8. Implantation site examination

Mice at day 3 of pregnancy were randomly assigned to two groups. On day 3 6:00–8:00 p.m., the mice were anesthetized with pentobarbital sodium (2.5 μl 2% (w/v) water solution per gram of the body weight) intraperitoneally. The uteri were exposed with abdominal surgery under general anesthesia. Saline (20 μl) with or without AOAA (1 mM) and PAG (10 mM) was injected into the lumen of each uterine horn from the uterotubal junction. In the view of the distribution of CTH in myometrium, intraperitoneal injections of saline (100 μl) with or without AOAA and PAG were also conducted at the same time. In midmorning on day 7, the mice were euthanized with CO_2 and the uteri were exposed with abdominal surgery. The number of implantation sites in the uteri were counted.

2.9. Statistics

The mathematical function was employed to fit the concentration-response curve with variable hill confidence given by parameter 'H' through GraphPad Prism 7.0 (GraphPad Software Ins., CA, USA). A1 and A2 represent the value of the bottom asymptote and the top asymptote respectively. The data were presented as means ± SEM. Shapiro-Wilk normality test was applied to justify whether the values came from a Gaussian distribution and all data for subsequent statistical analysis in the present study past normality test. For two groups, a paired or unpaired Student's *t*-test was performed to assess the difference according to experimental design (indicated in corresponding figure legends). For multiple comparisons, a one-way analysis of variance (ANOVA), followed by the Dunnett's test (all groups were compared with 1 control group) or the Tukey's multiple comparisons test (all groups were compared with each other) was performed. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. A H_2S generation pathway exists in early pregnant mouse uterus

The mammalian uterus undergoes comprehensive changes in preparation for embryo implantation, which occurs on day 4 of pregnancy in mice [1]. To evaluate the role of H_2S in endometrial epithelium during the peri-implantation period, initial studies were designed to identify the expression and location of two key endogenous H_2S-generating enzymes, CBS and CTH [35], in early pregnant mouse uterus. As illustrated in Fig. 1A and B, the relative mRNA level of *Cbs* was significantly up-regulated on day 4 of pregnancy, and the expression of *Cth* robustly increased on day 3 and day 4. In accordance with the mRNA levels, CBS and CTH protein abundance presented a rising tendency, which was revealed by western blotting analysis (Fig. 1C, D, and E). Immunohistochemical staining showed that CBS protein was exclusively localized in the pregnant mouse uterine glandular epithelium, whilst CTH protein was present in both uterine glandular epithelium and myometrium (Supplementary Fig. 1). Given that embryos gradually enter the uterus from the oviduct on day 3 of pregnancy in mice, the enhanced expression of CBS and CTH may imply the involvement of H_2S in preparation of embryo implantation.

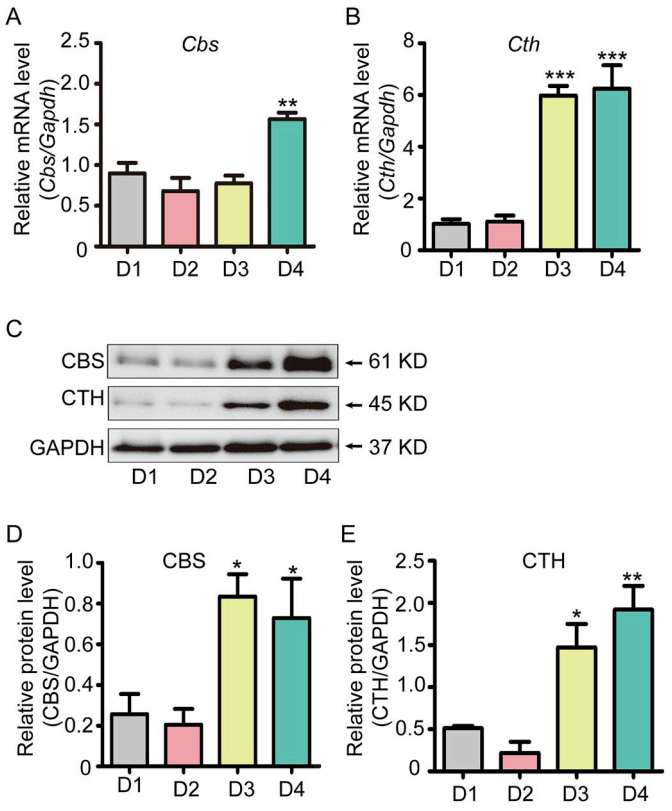

Fig. 1. H_2S generation pathway exists in mouse uterus. (A–B) Statistical analysis showing the relative mRNA levels of *Cbs* and *Cth* in mouse uteri from days 1–4 of pregnancy (n = 4–7). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with uteri on day 1 (ANOVA). (C) Representative Western blot analysis for CBS and CTH protein in mouse uteri from days 1–4 of pregnancy. (D–E) Summary of the relative protein levels of CBS and CTH in mouse uteri from days 1–4 of pregnancy (n = 3–6). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with uteri on day 1 (ANOVA). Symbols and bars indicated the means ± SEM. All data were from at least three independent experiments.

3.2. Homogenates of early pregnant mouse uteri produced H_2S enzymatically

In view of the existence of CBS and CTH in mouse uteri from day 1 to day 4 pregnancy, we then examined the H_2S levels in the early pregnant mouse uterus homogenates in the incubation of L-Cys, the CBS/CTH substrate. As shown in Fig. 2A, H_2S level was heightened on day 3 and day 4 of pregnancy compared to the day 1 group. Pretreatment with AOAA, an inhibitor of CBS, and/or PAG, a selective inhibitor of CTH [36], markedly suppressed the H_2S production of day 3 uterus homogenates incubated with L-Cys (Fig. 2B). Collectively, these results obtained during the peri-implantation period suggested that H_2S might be implicated in embryo implantation.

3.3. H_2S induced I_{SC} response in pregnant mouse endometrial epithelium

To investigate the effect of H_2S on ion transport of mouse endometrial epithelium, mouse uteri from day 3 of pregnancy were stripped off serosa and muscular layers and the I_{SC} was measured with the Ussing chamber technique. The mouse endometrial epithelium exhibited a mean basal I_{SC} of 42.30 ± 2.14 μA/cm^2 (n = 122), a transepithelial resistance of 18.19 ± 1.10 Ω cm^2 (n = 98) and a mean transepithelial potential of 0.55 ± 0.06 mV (n = 122) (apical side negative). Mucosal administration of NaHS, an exogenous donor of H_2S, elicited an abrupt decline in I_{SC} in a concentration-dependent manner, but subsequently increased back to baseline within 25 min (Fig. 3), which was probably due to the progressive dissociation and

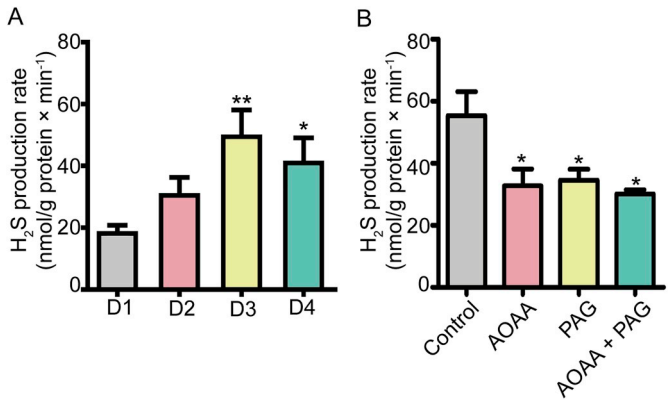

Fig. 2. Pregnant mouse uterus homogenate produced H_2S *in vitro*. (A) Statistical analysis showing the production rate of H_2S by mouse uterus homogenate from days 1–4 of pregnancy when incubated with 10 mM L-Cys (n = 8–11). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with uteri on day 1 (ANOVA). (B) The production rate of H_2S by mouse uterus homogenates from day 3 of pregnancy in the presence or absence of AOAA (1 mM) and/or PAG (10 mM) when incubated with 10 mM L-Cys (n = 3). $^{*}P < 0.05$ compared with the control group (ANOVA). Symbols and bars indicated the means ± SEM. All data were from at least three independent experiments.

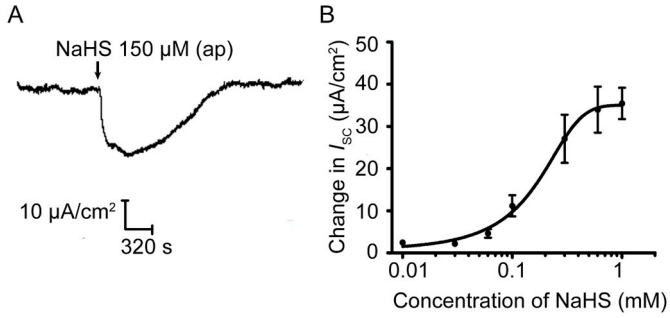

Fig. 3. H_2S dose-dependently decreased short circuit current (I_{sc}) of mouse endometrial epithelium on day 3 of pregnancy. (A) Representative trace of the I_{SC} responses induced by the apical application of H_2S donor NaHS (150 μM). The tissue resistances at the beginning and the end of the experiment were 16.3 and 17.6 Ω cm^2. (B) Concentration-response curve of NaHS-stimulated I_{SC} responses (n = 3–7). Symbols and bars indicated the means ± SEM. All data were from at least three independent experiments.

evaporation of H_2S in the Ussing chamber system [37,38]. The half-maximal effective concentration of NaHS was 133.6 μM. Hence, 150 μM NaHS was applied in the subsequent experiments unless indicated otherwise.

3.4. H_2S-induced I_{SC} response was anion-dependent

The decrease of basal I_{SC} in endometrial epithelium can be induced by inhibiting anion secretion/cation absorption, promoting cation secretion/anion absorption, or the combination of these. As ENaC is demonstrated to play an important role in endometrial epithelial ion transport and embryo implantation [14,39,40], we investigated whether H_2S induced basal I_{SC} decrease by blocking Na^+ absorption. As illustrated in Supplementary Fig. 2, the inhibitor of ENaC, amiloride (100 μM) had no significant effect on NaHS-induced ΔI_{SC}, excluding the participation of Na^+ transport in H_2S-induced I_{SC} decrease. On the other hand, anion substitution was conducted symmetrically in both serosal and mucosal baths to verify the ionic basis of H_2S-induced ΔI_{SC}. Compared to the normal K–H solution group, I_{SC} responses of NaHS were pronouncedly attenuated in Cl^- free K–H solution, HCO_3^- free K–H solution, or both Cl^-/HCO_3^- free K–H solution, respectively (Fig. 4). Notably, these observations illustrated that H_2S-induced ΔI_{SC}

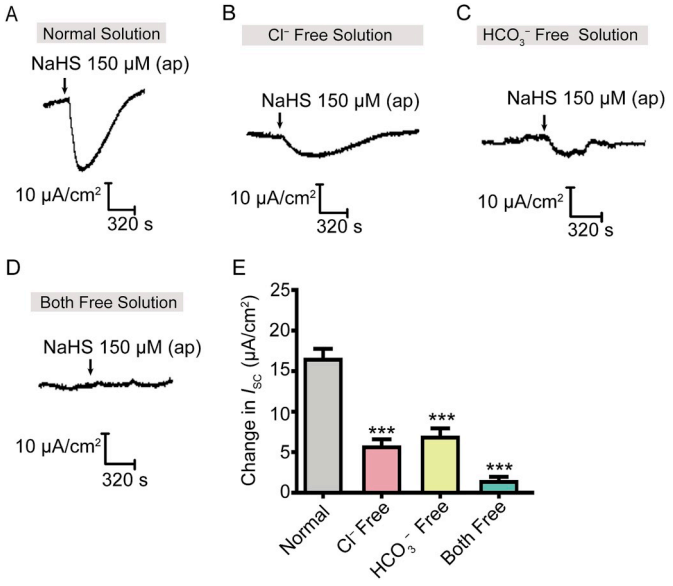

Fig. 4. H_2S-induced I_{sc} response was anion-dependent. (A–D) Representative trace of the I_{SC} responses induced by the apical application of H_2S donor NaHS (150 μM) in normal K–H, Cl^- free K–H, HCO_3^- free K–H, or Cl^-/HCO_3^- both free K–H, respectively. The tissue resistances at the beginning and the end of the experiments were 26.6 and 29.2 Ω cm^2 in (A), 18.7 and 19.9 Ω cm^2 in (B), 9.6 and 5.8 Ω cm^2 in (C), and 18 and 12.6 Ω cm^2 in (D). (E) Comparison of NaHS (150 μM) induced ΔI_{SC} obtained in different component of KH solution (n = 3–4). ***$P < 0.001$ compared with normal K–H group (ANOVA). Symbols and bars indicated the means ± SEM. All data were from at least three independent experiments.

was anion-dependent and probably occurred as a result of suppressing transepithelial anion secretion.

3.5. H_2S-induced I_{sc} response was attenuated by CFTR blocker

A variety of transport proteins located in the apical membrane of endometrial epithelia are responsible for the transepithelial anion transport, including CFTR and calcium-activated Cl^- channels (CaCCs) [41–43]. We sought to identify which anion channel was implicated in H_2S-induced ΔI_{SC}. As shown in Fig. 5A, pretreatment with a non-selective Cl^- channels inhibitor, diphenylamine-2-carboxylic acid (DPC) (1 mM), almost abolished the NaHS-induced I_{SC} response, suggesting the involvement of Cl^- channels. The non-specific blocker of CaCCs, 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) (100 μM), had no significant effect on NaHS-induced ΔI_{SC} (Fig. 5B), while the selective blocker of CFTR, $CFTR_{inh}$-172 (20 μM), remarkably attenuated the ΔI_{SC} induced by NaHS (Fig. 5C). According to these results, we inferred that H_2S might decrease the basal I_{SC} mainly via blockade of CFTR.

3.6. H_2S increased $[Cl^-]_i$ of mouse endometrial epithelial cells

Given that CFTR mediates Cl^- efflux to maintain ionic homeostasis in MEECs, blockade of CFTR by H_2S might lead to an increase in $[Cl^-]_i$. To confirm this hypothesis, $[Cl^-]_i$ was measured using a Cl^- indicator dye MQAE quenched rapidly by Cl^-, the fluorescence reduction of which reflected an increase in $[Cl^-]_i$ [44]. We successfully established a primary culture of MEECs (Supplementary Fig. 3). Notably, NaHS decreased the MQAE fluorescence intensity, that is, increased the $[Cl^-]_i$ of MEECs (Fig. 6A). Administration of 20 μM $CFTR_{inh}$-172 almost abolished this effect, demonstrating that H_2S increased $[Cl^-]_i$ of MEECs by blockade of CFTR (Fig. 6B and C). Collectively, these results indicated that H_2S induced an elevation of $[Cl^-]_i$ mainly by blocking CFTR, resulting in a decrease in I_{SC} response as a consequence of restrained Cl^- secretion.

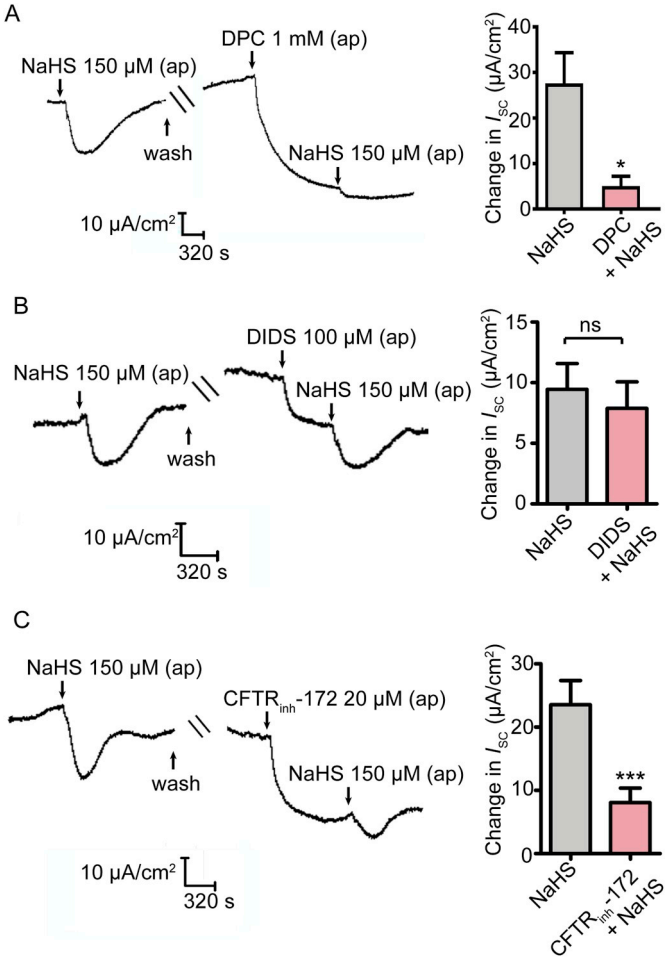

Fig. 5. H_2S-induced I_{sc} response was attenuated by $CFTR_{inh}$-172. (A) Left, representative trace of the I_{SC} responses induced by the apical application of H_2S donor NaHS (150 μM) in the apical absence or presence of DPC (1 mM). The tissue resistances at the beginning and the end of the experiment were 24.8 and 25.8 Ω cm^2. Right, statistical analysis showing the effect of DPC on the NaHS-stimulated I_{SC} responses (n = 5). *$P < 0.05$ compared with the control (paired t-test). (B) Left, representative trace of the I_{SC} responses induced by the apical application of H_2S donor NaHS (150 μM) in the apical absence or presence of DIDS (100 μM). The tissue resistances at the beginning and the end of the experiment were 17.4 and 15 Ω cm^2. Right, statistical analysis showing the effect of DIDS on the NaHS-stimulated I_{SC} responses (n = 4). ns, not significant (paired t-test). (C) Left, representative trace of the I_{SC} responses induced by the apical application of NaHS (150 μM) in the apical absence or presence of $CFTR_{inh}$-172 (20 μM). The tissue resistances at the beginning and the end of the experiment were 19.8 and 23.9 Ω cm^2. Right, statistical analysis showing the effect of $CFTR_{inh}$-172 on the NaHS-stimulated I_{SC} responses (n = 9). ***$P < 0.001$ compared with the control (paired t-test). Symbols and bars indicated the means ± SEM. All data were from at least three independent experiments.

3.7. H_2S did not affect the intracellular cAMP level in mouse endometrial epithelial cells

As a cyclic adenosine monophosphate (cAMP)-dependent channel, CFTR can be suppressed by reducing intracellular cAMP concentration. A depressed intracellular cAMP level can be attributed to the inhibition of adenylyl cyclases (ACs) that synthesize cAMP, or/and the activation of cyclic nucleotide phosphodiesterases (PDE) that degrade cAMP. Given that H_2S can reduce the intracellular cAMP level by inhibiting AC activity in a variety of cells and tissues [45], we then asked whether H_2S inhibited CFTR via decreasing the cAMP level in MEECs. As illustrated in Supplementary Fig. 4, 150 μM NaHS failed to decrease the basal

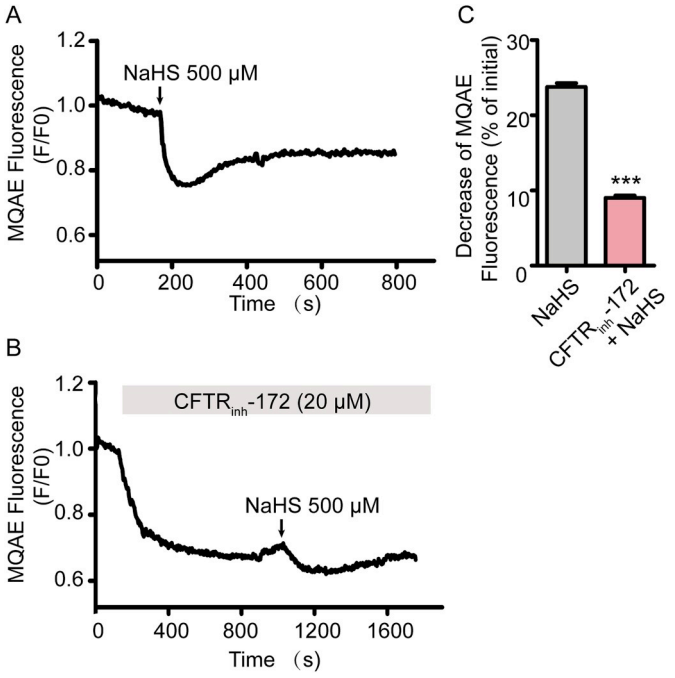

Fig. 6. H_2S-induced increase in $[Cl^-]_i$ of mouse endometrial epithelial cells was attenuated by $CFTR_{inh}$-172. (A–B) Representative trace of the NaHS-induced change in $[Cl^-]_i$ in the absence or presence of $CFTR_{inh}$-172 (20 μM) indicated by MQAE fluorescence intensity, reduction of which reflected the increase of $[Cl^-]_i$. (C) Statistical analysis showing the effect of $CFTR_{inh}$-172 on the NaHS-induced change in $[Cl^-]_i$ of primary cultured mouse endometrial epithelial cells (n = 110–146 cells from four experiments). ***$P < 0.001$ compared with the NaHS group (unpaired t-test). Symbols and bars indicated the means ± SEM.

cAMP level of MEECs, while 10 μM forskolin, an activator of ACs as the positive control, remarkably increased the cAMP level in MEECs as expected. These results suggested that H_2S blocked CFTR in a cAMP-independent way.

3.8. Interference with H_2S synthesis in vivo partially impaired embryo implantation

It has been well documented that the ion transport activity of endometrial epithelium contributes to embryo implantation by regulating endometrial receptivity and uterine fluid volume and composition [3]. In view of the antisecretory effect of H_2S on preimplantation endometrial epithelium, we then tested whether the endogenous H_2S was implicated in embryo implantation. Given that CBS and CTH were located in the endometrial epithelium and myometrium, both intraperitoneal and intrauterine injections of AOAA (1 mM) and PAG (10 mM) were performed to suppress enzymatic activity on mouse uteri from day 3 of pregnancy. At day 7, pregnant mice were killed and the implantation sites on both sides of uteri were counted. As shown in Fig. 7, administration of AOAA and PAG reduced the number of implantation sites compared to the control group, suggesting the dysregulation of H_2S synthesis in preimplantation uterus partially impaired embryo implantation.

4. Discussion

In the present study, we examined the modulatory role of H_2S in ion transport in endometrial epithelium for the first time. The results revealed that H_2S inhibits the transepithelial anion secretion of early pregnant mouse endometrial epithelium mainly via blockade of CFTR, dysregulation of which in preimplantation uterus impairs embryo implantation.

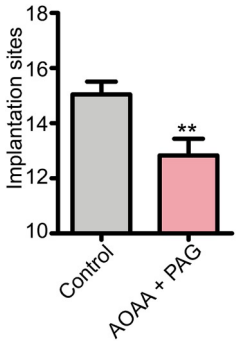

Fig. 7. Interference with H_2S synthesis partially impaired embryo implantation. The numbers of implantation sites in mouse uteri on day 7 of pregnancy after intraperitoneal and intrauterine injection of saline with or without AOAA (1 mM) and PAG (10 mM) on day 3 (n = 22–24). **$P < 0.01$ compared with the control group (unpaired t-test). Symbols and bars indicated the means ± SEM. All data were from at least three independent experiments.

For the detection of endogenous H_2S, the methylene blue method is widely used in numerous studies on biological samples [17,25,36,46,47]. In our study, using this method, the H_2S production rates were reported as 52.59 ± 5.66 nmoL/g protein per min on day 3 group under the incubation of L-Cys. However, this value does not represent the physiological concentration of H_2S in mouse uterus tissue, even though it can be translated to a H_2S concentration according to the method raised by Filipovic et al. [46]. This can be attributed to the relatively high concentration of L-Cys, the limitation of detection methods, and interference from biological samples. Nevertheless, it will not obstruct the conclusion that the amount of H_2S-generating enzymes and H_2S production presented an increasing tendency in pre-implantation as illustrated in Figs. 1 and 2.

In our study, PAG was used as a selective inhibitor of CTH and AOAA as an inhibitor of CBS. The strong selectivity of PAG in inhibiting CTH versus CBS was confirmed by *in vitro* and *in vivo* studies [36], whereas AOAA has been widely used as a CBS inhibitor to investigate the H_2S signaling pathway and yet exhibited an inhibitory effect on CTH in a recent study [36,48,49]. This may provide a possible explanation of non-additive effect in the joint use of AOAA and PAG on H_2S production (Fig. 2B), since the abundance of CTH is higher than that of CBS in uterus [45]. Despite its limitation, AOAA is an undoubtedly useful and effective inhibitor of CBS *in vitro* and *in vivo* [36,50] and is still recommended to be used as a CBS inhibitor (or as a combined CBS/CTH inhibitor), given that no alternative selective pharmacological CBS inhibitor is available at present [36,51].

Most studies on endometrial epithelium used the cell monolayer as a model and some researchers described endometrial epithelium as "tight" epithelium based on the high resistance ($600-3000\,\Omega\,cm^2$) [52–54]. In our study, the electrical resistance of the stripped endometrial mucosal layer was 18.19 ± 1.10 $\Omega\,cm^2$ (n = 98). We have also observed that the electrical resistance of the intact mouse uterus was 45.79 ± 5.59 $\Omega\,cm^2$ (n = 11). Although no published study has indicated the electrical resistance of intact or stripped mouse endometrial epithelial tissue, these values are much lower than those in "tight" epithelium and closer to those in "leaky" epithelium [55]. Hence, the mouse endometrial epithelium is more likely to be a "leaky" epithelium, though the ratio of the conductances of the extracellular and cellular pathways for ions across epithelial tissues (g_{shunt}/g_{cell}) should be further determined.

NaHS or Na_2S has been employed as an inorganic exogenous H_2S donor at a wide range of concentrations (from nM to mM) to reveal the roles of H_2S in multiple physiological processes in many studies [18,56–59]. In the present study, 150 μM NaHS applied in physiological solution (pH 7.4, 37 °C) in most experiments released approximately 27 μM free H_2S [60], close to the reported "physiological"

concentration in some studies but higher than those in others [61]. However, the physiological concentrations of H_2S in tissues are hard to determine and remain controversial [61,62]. The limited sensitivity of techniques also confines the lower limit of the working concentration of H_2S donors. Despite all these, we believe that the modulatory role of H_2S in micromolar concentration revealed in our study is significant for understanding the physiological function of this gaseous mediator.

Numerous and diverse types of ion transport proteins regulated by H_2S have been well documented, including Cl^- channels [22]. Interestingly, the modulatory effects of H_2S on Cl^- channels vary in different cell types and physiological processes. Growing evidence based on the response to the known Cl^- channels antagonists have demonstrated that H_2S activates Cl^- channels in different manners in various systems [17,56,63–66]. For instance, H_2S activated CFTR to elicit a sustained increase in I_{SC} as a consequence of Cl^- secretion in airway epithelial cells [18]. Conversely, some studies have shown that H_2S might inhibit the activity of Cl^- channels. Using a bilayer lipid membrane fused with Cl^- channels derived from rat heart lysosomal vesicles, H_2S was found to inhibit Cl^- channel currents by directly decreasing the channel open probability [67]. A study in a diarrhea mouse model suggested that H_2S reduced the accumulation of intestinal fluid volume and extracellular Cl^- caused by the excessive opening of CFTR and secretion of Cl^- due to cholera toxin treatment [68]. In our present study, H_2S revealed an inhibitory effect of CFTR and anion secretion in mouse endometrial epithelium. These discrepant effects of H_2S on CFTR reflect the complexity of the regulatory mechanism.

As an important Cl^- channel apically located in mouse endometrial epithelial cell, CFTR can be activated by accumulation of intracellular cAMP and subsequent activation of protein kinase A (PKA), primarily mediating the Cl^- flux in response to multiple extracellular signals and intracellular regulators. An increase in cAMP level can either be the result of the activation of ACs or inhibition of PDEs and vice versa. H_2S has been indicated to either stimulate [68,69] or inhibit AC activity [70,71], leading to the elevation or reduction of intracellular cAMP concentration, respectively. In addition, H_2S was reported to directly inhibit PDEs in a cell-free system [72]. In accordance with this, a recent study claimed that H_2S inhibited endogenous PDEs, probably resulting in an accumulation of intracellular cAMP and downstream activation of human CFTR that heterologously expressed in the *Xenopus* oocyte [73]. In support with this study, an increase in the cAMP concentration in *Xenopus* oocytes in response to H_2S has been reported earlier [69]. Interestingly, H_2S has no effect on the cAMP formation and PKA activation in the human lung adenocarcinoma cell line H441 [38]. In our study, H_2S failed to affect the cAMP level in MEECs (Supplementary Fig. 4), excluding the involvement of cAMP in the inhibitory effect of H_2S on CFTR. Similarly, H_2S was demonstrated to inhibit CFTR in an AC-cAMP-PKA independent manner in mouse intestine [68]. These diverse regulatory effects of H_2S on AC-cAMP-PKA pathway might be ascribed to the difference in species, cell types or involvement of other multiple regulators *in vivo*, providing a possible explanation for the discrepancy between the present study and previous reports. The precise underlying mechanisms by which H_2S might modulate CFTR activity remain to be elucidated.

Although CFTR primarily acts as a Cl^- channel, it also mediates HCO_3^- secretion in mouse endometrial epithelial cells [41]. Consistent with this, NaHS-induced I_{SC} responses diminished in HCO_3^- free K–H solution, and the response in both Cl^-/HCO_3^- free K–H solution was almost abolished (Fig. 4). These observations inferred that impaired basal transepithelial HCO_3^- secretion might contribute to NaHS-induced ΔI_{SC}, though more evidence is needed to elucidate whether HCO_3^- flux was involved in NaHS-induced effect. Besides, the involvement of other Cl^- channels can not be excluded, given that $CFTR_{inh}$-172 failed to completely prevent the H_2S effect (Figs. 5C and 6C), while the non-selective blocker of Cl^- channels DPC almost abolished the H_2S-induced ΔI_{SC} (Fig. 5A). Despite this, the potent inhibitory effect of $CFTR_{inh}$-172 supported the conclusion that CFTR is the main channel involved in H_2S effect.

During the preimplantation period, up-regulation of ENaC and down-regulation of CFTR lead to maximal fluid absorption and minimal fluid secretion, together resulting in a uterine fluid reduction for uterine luminal closure and embryo implantation [3]. Evidence from whole-cell patch clamp excluded the direct inhibitory effect on ENaC by H_2S on airway epithelial cells and ENaC-expressing *Xenopus* oocytes, but H_2S could decrease amiloride-sensitive Na^+ transport across airway epithelial monolayer indirectly by inhibition of basolateral K^+ channels and consequent impairment of Na^+/K^+-ATPase activity [74]. Conversely, the ENaC blocker amiloride failed to inhibit the modulatory effect of H_2S on the ion transport activity in rat vaginal epithelium [17]. In our present study, we found that amiloride failed to attenuate H_2S-induced ΔI_{SC}, indicating H_2S did not inhibit ENaC to decrease I_{SC} in mouse endometrial epithelium (Supplementary Fig. 2). It shall also be noticed that apically administration of amiloride did not change the basal I_{SC} in mouse endometrial epithelium on day 3 of pregnancy (Supplementary Fig. 2). This observation indicated that Na^+ absorption contributed little to the basal current of mouse endometrial epithelium on day 3 of pregnancy, which is in contrast with the findings from endometrial epithelial monolayers [39,75]. This discrepancy might be attributed to different types of preparations (stripped mucosal layer vs. cultured cell monolayer). Indeed, even in the monolayer, the culture condition (eg. the presence of Matrigel) would affect the contribution of amiloride-sensitive current to the basal current [76]. Given that ENaC expression was reportedly enhanced in mouse uterus on day 3 of pregnancy [40] and played an essential role in embryo implantation when activated by embryo-released serine protease [14], the absence of amiloride-sensitive response might be attributed to the lack of certain stimulations *in vitro* or the difference of cell population in cultured monolayer and tissue preparations.

Previous studies have suggested that mucosal CFTR mediated Cl^- secretion contributed little to basal I_{SC} in mouse endometrial epithelial monolayer [39,77]. In our study, however, the apical administration of DIDS and $CFTR_{inh}$-172 decreased the basal I_{SC} (Fig. 5). The basal currents in Cl^- free K–H, HCO_3^- free K–H, and anion-free K–H solution were $14.46 \pm 5.91\,\mu A/cm^2$ ($n = 3$), $10.46 \pm 2.80\,\mu A/cm^2$ ($n = 4$), $13.11 \pm 2.72\,\mu A/cm^2$ ($n = 4$), respectively, markedly lower than that in normal K–H solution. These results suggested that Cl^- channels mediated anion secretion was still fairly active in mouse endometrial epithelial preparations from day 3 of pregnancy. Since excessive expression or activation of CFTR will cause implantation failure [13], we speculated that blockade of CFTR by H_2S in pre-implantation might help to down-regulate the function of CFTR for preparation of implantation. Reduction of implantation sites when using AOAA and PAG to interfere with H_2S synthesis *in vivo* confirmed our speculation (Fig. 7), though the limited reduction percentage suggested that compensatory H_2S generating sources or signaling pathways regulating CFTR might exist in uterus. Indeed, AOAA and PAG failed to completely inhibit the H_2S production rate (Fig. 2B), indicating that there are alternative sources of H_2S in pregnant mouse uterus. In view of the fact that the third H_2S-generating enzyme 3-MST was identified in human uterus [78], it might play a role in H_2S-generation in mouse uterus. Nevertheless, the lack of selective commercial 3-MST inhibitor confined further study [51]. Similar problems occurred in other H_2S-forming enzymes. In contrast to the μM IC_{50} values reported with purified enzymes, mM concentrations of AOAA and PAG were usually employed to inhibit CBS and CTH in functional studies [51,79], probably due to the limited cell membrane permeability [51]. However, it also raised an inevitable problem that they might have a non-specific inhibitory effect on other pyridoxal-5′-phosphate-dependent enzymes at high concentrations [36,79]. More efforts are needed to address the gap in selective targeting of H_2S-generating enzymes. Although a previous study has showed that there was no significant change in the number of the implantation site in *Cbs* knockout mice [80], improved RNA interference techniques *in vivo* or conditional *Cth/Cbs/3-Mst* triple-knockout

genetic models shall be applied to completely block the enzymatic synthesis of H_2S in further studies and clarify the physiological role of H_2S in implantation. In addition, we applied GYY4137 as a slow-releasing H_2S donor [81] *in vivo* to investigate whether the detrimental effect of impaired H_2S synthesis can be rescued by supplementing exogenous H_2S. However, it appeared that this compound had a detrimental effect on its own, resulting in few or even no embryo implantation sites after single administration of GYY4137 or combined administration of H_2S-generating enzyme inhibitors and GYY4137 (data not shown). Despite those results, interference with the normal metabolism of H_2S pathway using AOAA and PAG in our study still provides valuable insights into the physiological role of endogenous H_2S in uterus.

5. Conclusion

In conclusion, we demonstrated that H_2S inhibits the transepithelial anion secretion of early pregnant mouse endometrial epithelium via blockade of CFTR, participating in preparation for embryo implantation. Our study expanded the physiological function of H_2S in the female reproductive system and provided insights into the regulatory effect of H_2S on Cl^- channels for a thorough understanding of H_2S biology.

Author contributions

J.W.X. conceptualized the study, designed and carried out experiments, analyzed the results, and wrote the manuscript. D.D.G. performed the experimental work, processed figures, and revised the manuscript. L.P., Z.E.Q. and L.J.K. performed the experimental work. Y.X.Z. facilitated the study and manuscript preparation. Y.L.Z and W.L.Z obtained fundings, conceptualized and supervised the study, and revised the manuscript.

Declarations of interest

None.

Acknowledgment

This study was supported by the National Natural Science Foundation of China [grant numbers 31771286, 81571427] and the Natural Science Foundation of Guangdong Province [grant number 2018A030310074]. We would like to take this chance to thank Dr. Wulin Zuo (Department of Genetic Medicine, Weill Cornell Medical College, New York, USA) and Dr. Pengxiao Chen (College of Life Science and Technology, Jinan University, Guangzhou, China) for their critical reading of this manuscript and suggestions. We are also grateful to Dr. Zheng Gao (The Third Affiliated Hospital, Guangzhou Medical University, Guangzhou, China) and Dr. Shuangbo Kong (Reproductive Medical Center, The First Affiliated Hospital of Xiamen University, Xiamen, China) for providing technical support and reagents.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.niox.2019.05.011.

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