Elemental sulfur upregulated testicular testosterone biosynthesis by associating with altered gut microbiota in mice

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Abstract: Elemental sulfur has been used as a traditional Chinese medicine to treat the late-onset hypogonadism and impotence without a clarified mechanism for many hundreds of years. In the present study, mice were received sulfur or distilled water for 35 days by daily intragastric gavage at a dose of 250 mg/kg body weight. Then, the serum testosterone level and genes associated with testicular testosterone biosynthesis (TTB) were detected. The gut microbiota was also analyzed by 16S rRNA gene sequencing. Serum testosterone level was significantly increased by 291.1% in sulfur-treated mice. The H₂S levels in serum and feces were significantly increased. The expression of genes associated with TTB including *StAR*, *p450c17*, *3β-HSD*, and *P450scc* in testes were significantly upregulated by Sulfur and NaHS, suggesting that sulfur promotes TTB depending on H₂S. In addition, sulfur increased the diversity of gut microbiota and the abundance of several bacteria associated with sulfur metabolism, including genus *Prevotella*, which might be positively associated with serum level of testosterone in boys. Five pathways including bile secretion, carotenoid biosynthesis, lipid biosynthesis proteins, propanoate metabolism, and biosynthesis of type II polyketide products, were identified to associate with sulfur. Together, our results suggested that sulfur upregulated testicular testosterone biosynthesis via H₂S, which was associated with alteration of gut microbiota in mice. Our study highlights a mechanism for the treatment of late-onset hypogonadism and impotence by sulfur.

Introduction

Testosterone deficiency (TD), also known as hypogonadism, is characterized by decreased serum testosterone, associating with multiple metabolic diseases, coronary heart diseases, cancers and male infertility (Buvat *et al.*, 2013; Khera *et al.*, 2016; Kim and Schlegel, 2008). Bioavailable testosterone was decreased gradually with aging in adult men (Liu *et al.*, 2015; Tsujimura *et al.*, 2003), resulting in 5.6% symptomatic androgen deficiency in men aged 30–79 years old (Araujo *et al.*, 2007). In general, the production of testosterone by testicular Leydig cells is controlled by luteinizing hormone (LH), a tropic hormone released from the pituitary. LH activates adenylyl cyclase by activating G protein-coupled receptor and inducing the production of the intracellular second messenger cAMP, and then activates the PKA

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pathway and promotes the expression of key steroidogenic genes including steroidogenic acute regulatory protein (StAR) and P450scc. StAR is responsible for intracellular cholesterol transport into the mitochondria, the ratelimiting step of testosterone biosynthesis. Transcriptional upregulation of the StAR gene involves the concerted action of multiple proteins that bind directly or indirectly to the cis-elements located in its promoter region (Manna and Stocco, 2005). One of them is a cAMP-responsive elementbinding protein (CREB), which is activated by phosphorylation mediated by PKA (Clem et al., 2005; Manna and Stocco, 2005). During steroidogenesis, cholesterol is converted to pregnenolone in the mitochondria by P450scc. Pregnenolone then moves from the mitochondria into the smooth endoplasmic reticulum (ER) and is converted to testosterone by several steroidogenic enzymes, including 3β-HSD, cytochrome P450 17A1 (P450c17) and 17β-hydroxysteroid dehydrogenase (17 β -HSD). These enzymes play important roles in maintaining the testicular testosterone biosynthesis (TTB) (Wang et al., 2017; Zirkin and Papadopoulos, 2018).

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Among the factors that affect the steroidogenesis in Leydig cells, an imbalance between prooxidants and antioxidants has been widely studied. For example, the Leydig cells from aged rats produce significantly more reactive oxygen than the cells from young rats (Chen et al., 2001). High levels of reactive oxygenic species (ROS) have been linked with the repression of TTB in human and different murine models (Ge et al., 2007; Toppari et al., 2006; Wang et al., 2017). In recent years, the influence of gut microbiota on human health has been paid more and more attention. Gut microbiota is closely related to many diseases, such as aging, inflammatory bowel disease, colorectal cancer, obesity, and diabetes (De Almeida et al., 2019; Mailing et al., 2019). Interestingly, gut microbiota affects TTB, since specific pathogen-free mice have a higher serum testosterone level and levels of 3β-HSD and p450scc than in germ-free mice (Al-Asmakh et al., 2014). Early-life exposure to doxycycline will result in testosterone deficiency in mice (Hou et al., 2019). However, it remains unclear how gut microbiota affects steroidogenesis.

The testosterone replacement therapy (TRT) using exogenous testosterone reversed many of the symptoms of low testosterone. However, severe side effects were also observed (Bosland, 2014; Dimopoulou *et al.*, 2016). For example, recent studies suggest that there may be an increased risk of cardiovascular disease in older men after TRT (Finkle *et al.*, 2014; Vigen *et al.*, 2013). TRT is not recommended in men prostatic cancer and lower urinary tract symptoms caused by an enlarged prostate (McGill *et al.*, 2012). Males who desire to maintain fertility are also advised against the use of TRT because exogenous testosterone can suppress the hypothalamic-pituitarygonadal axis (HPG axis) and result in infertility (McGill *et al.*, 2012). Thus, drugs for boosting testosterone production demonstrate great potential for application.

For treating the late-onset hypogonadism (LOH) and impotence in China, Japan, and other regions of Southeast Asia, some traditional medicines are often the first choice. Some extracts that include sulfur have been reported to raise serum level of testosterone in different murine models (Chen *et al.*, 2006; Gao *et al.*, 2019; Hsia *et al.*, 2009; Leu *et al.*, 2011). Elementary sulfur has been used to treat LOH and impotence for many hundreds of years (Jia, 2008). However, its mechanism has been unknown. In the present study, we studied the effect of elementary sulfur on steroidogenesis in Leydig cells and gut biota in mice. Our results showed that sulfur significantly upregulates androgen biosynthesis and H_2S generation and increased the diversity of gut microbiota.

Materials and Methods

Elemental sulfur and animals

Elemental sulfur powder with a purity of \geq 99% was purchased from the Shanghai Chemical Reagent supply station (Shanghai, China).

Male Kunming mice aged between 6 and 7 weeks (weight range, 34–37 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd., kept under the controlled photoperiod conditions (lights on 07:00–19:00) and supplied

with food and sterilized H_2O ad libitum. Eighty mice were randomly divided into control and sulfur-treated groups, with 40 mice in each group. For control groups, mice were administered with 0.2 mL distilled water by gavage for 5 weeks, 6 days per week. For sulfur-treated groups, mice were administered with the suspension of sulfur powder in 0.2 mL distilled water (250 mg/kg mouse weight) by gavage for 5 weeks, 6 days per week, as used in our previous study (Duan *et al.*, 2015).

The study was approved by the animal ethics committees of Shanghai Institute of Planned Parenthood Research.

Measurement of serum testosterone and cytokines

Serum testosterone was determined by the enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6) were measured by cytokine bead array (CBA) Mouse cytokine kit (BD Biosciences, USA) according to the manufacturer's instructions.

Immunohistochemistry (IHC)

Testes from mice were collected and immediately fixed in 4% paraformaldehyde (PFA) for immunohistochemistry. To ensure the reproducibility of the results, samples from ≥ 3 animals were used. For IHC assay, sections (4–5 µm) were deparaffinized in xylene and rehydrated in gradient alcohols. After antigen retrieval, the blocked sections were incubated with primary antibodies overnight at 4°C. The sections were incubated with secondary antibodies for 20 min, and then developed with DAB and counterstained with hematoxylin (Zeng *et al.*, 2019). Antibodies were diluted as follows: StAR, 3β-HSD, pCREB, at 1:200 (Santa Cruz Biotechnology, USA).

Measurement of hydrogen sulfide

The methylene blue method was used to measure levels of hydrogen sulfide in plasma and fecal as previously reported (Shen et al., 2011; Zheng et al., 2012). In addition, 100 μ L of 5% zinc acetate solution (pH 5.2) was added into 100 µL of a serum sample, vortexed vigorously, then 100 µL of 5 mol/L NaOH solution was added. After centrifuged for 10 min at 4°C, 12000 \times g, the supernatant was discarded and mixed with 100 µL of 5% zinc acetate solution, 0.2% N,Ndimethyl-p-phenylenediamine sulfate solution and 20% trichloroacetic acid. For rest samples, 100 µL of 5% zinc acetate solution (pH 5.2) was added into 10 mg of fecal sample, homogenized into suspension, then mixed with 100 µL of 0.2% N,N-dimethyl-p-phenylenediamine sulfate solution and 20% trichloroacetic acid. After centrifuged for 5 min at 4°C, 12000 \times g, 200 µL of the supernatant was mixed with 20 µL of 10% ferric ammonium sulfate solution for 15 min. The absorbance of the reaction solution was measured by a spectrophotometer at 665 nm.

Measurement of oxidative stress

Malondialdehyde (MDA), a marker of lipid peroxidation, was determined with thiobarbituric acid (TBA) according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, China). Testis were weighed and immersed into 0.02 mmol/L Tris-HCl (pH 7.4) at the ratio of 1:10 (mg/mL). Tissue homogenate was centrifuged at 13000 rpm for 15 min, and precipitates were discarded. The supernatant (0.15 mL) was transferred to a testing tube. The following steps were performed as described by Liu *et al.* (2010).

For measurement of superoxide dismutase (SOD) and GST-Px activity, frozen testes were homogenized in 100 mmol/L Tris-HCl buffer and centrifuged at 10000 rpm for 20 min, and then their activity was respectively determined using assay kits (Nanjing Jiancheng Bioengineering Institute, China) and the activity was expressed as units per microgram of total protein (μ /mg). Total protein content in samples was analyzed using a bicinchoninic acid protein assay kit.

Treatment of exogenous H_2S

NaHS (Sigma, USA) was used as the donor of H_2S (Hu *et al.*, 2015). Testes were obtained from male Kunming mice aged 8–10 weeks, and cut into small pieces of 10–20 mm³, and then incubated in DMEM: F12 (1:1) medium with 10% Fetal bovine serum with gentamicin (0.02 g/L; Sigma), maintained in 24-well plates in a humidified atmosphere at 34°C with 5% CO₂. Three hours later, 100 or 200 mM NaHS in DMEM: F12 (1:1) medium was added to the plates. After treated with NaHS for 24 h, the pieces of testis were collected for extraction of the total RNA.

Quantitative real-time PCR

Total RNA was extracted from the testes of control and sulfurtreated mice with TRIzol reagent (Invitrogen, USA), followed by RNA precipitation. cDNA was synthesized with a reverse transcription kit (TaKaRa, Japan). Real-time PCR was performed using SYBR Premier EX Taq (TaKaRa, Japan). Genes were amplified with the indicated primers (Tab. 1). Relative levels of mRNAs were calculated using MX3500pro software and normalized to the levels of endogenous β -Actin in the same samples.

Statistical analysis

All statistical data were analyzed with GraphPad Prism software (version 5, GraphPad Software Inc., CA, USA). The data were presented as means \pm SEM. ANOVA was used. Statistical significance set: NS, p > 0.05; * $p \le 0.05$; * $p \le$

TABLE 1

DNA sequences of primers for qPCR

Gene	$Primer(5' \rightarrow 3')$
β-actin	F: CCTGAAGTACCCCATTGAAC
	R: TTCTCTTTGATGTCACGCAC
StAR	F: CTCTATGAAGAACTTGTGGACC
	R: CTGGTTGATGATTGTCTTCG
p450c17	F: CTTGATACTTACATACGACGGG
	R: GCTGGAGAGAGATTCACTATTG
3β-HSD	F: CTGTTGTCATCCACACTGCT
	R: TGGACTTCTCTCCCCATAAA
P450scc	F: AAGACCGAATCGTCCTAAAC
	R: CCCCAAATATAACACTGCTG

0.01. All the presented results were from at least three independent experiments.

Genomic DNA extraction, PCR amplification and 16S rRNA gene sequencing

Bacterial genomic DNA was extracted from each mouse fecal sample by QIAamp DNA Stool Mini Kit (QIAGEN). The V3-4 region of 16S rRNA genes was amplified with primers 338F and 806R (Huse *et al.*, 2007) using TransStart Fastpfu DNA Polymerase (TransGen). The thermocycling steps were as follow conditions: 95°C for 5 min, 20 cycles of at 95°C for 45 s, 55°C for 30 s, 72°C for 30 s and a final extension step at 72°C for 10 min. Three PCR amplification repeats of each sample were performed, then the amplicons were purified with AxyPrep DNA Gel Extraction kit (AXYGEN) and pooled equivalent after assessed by spectrophotometry (QuantiFluor-ST, Promega). 2×300 bp paired-end sequencing of 16S rRNA gene amplicons were performed on an Illumina MiSeq instrument.

Bioinformatics and statistical analysis

The assembling of paired FASTQ files was performed using Mothur (version 1.39.0) (Schloss et al., 2011). Raw assembled sequences were excluded if it had ambiguous bases or the length was shorter than 350 bp, the sequences were also excluded if it was identified as chimeric sequence or contaminant. The high-quality DNA sequences were grouped into OTUs (operational taxonomic units) under the threshold of 97% identity compared to the SILVA reference database (V119) (Quast et al., 2013). The minimum sample size was the criteria for data normalization. Community richness, evenness, and diversity analysis (Shannon, Simpsonenven, ACE, Chao and Good's coverage) were performed using Mothur. The Student's t-test (with 95% confidence intervals) was used to determine whether the means of evaluation indices were the statistical difference, and p < 0.05 was considered as significant standard. Taxonomy was assigned using the online software RDP classifier (Wang et al., 2007) at the default parameter (80% threshold) based on the Ribosomal Database Project (Cole et al., 2009). Genera and family abundance differences between samples were analyzed by Metastats (White et al., 2009). For taxonomy features, the significant differences were taken with q-value < 1E-5. Differences between normal and sulfur-fed samples were assessed using Analysis of Molecular Variance (AMOVA) and Parsimony in Mothur. LEfSe (Segata et al., 2011) used the Kruskal-Wallis test to detect different abundance taxa (mainly for genera, p < 0.05) among two groups and estimate linear discriminant analysis effect size (LDA score > 2.5). Through normalizing the 16S rRNA copy numbers, PICRUSt (Langille et al., 2013) was used to predict the microbiome functions from the KEGG pathways.

Accession numbers

The sequence data have been submitted to the GeneBank Sequence Read Archive (accession number PRJNA596549).

Results

Sulfur increased serum testosterone level in mice We previously reported that elemental sulfur repressed the growth of androgen receptor (AR)-negative prostate cancer 304



FIGURE 1. Effects of sulfur on testicular testosterone biosynthesis. (A) Serum level of testosterone in male mice-treated with elemental sulfur. The level of testosterone was detected by ELISA (n = 36). (B) Relative expression levels of StAR, p450scc, 3β -HSD, and p450scc genes in testis from sulfur-treated mice (n = 20), measured by qPCR. (C) Immunohistochemical analysis of StAR, 3β -HSD, and pCREB in testis from sulfur-treated mice. The area with the dashed frame is also with shown an enlargement. Photomicrographs represented one of three independent analyses with a similar result. Scale bar, 200 µm. The data are expressed as mean ± SEM. $p^{**}p < 0.01; p^{*} < 0.05.$

cells subcutaneous xeno-transplanted in nude male mice with much higher efficiency than that was observed in ARexpressing prostate cancer cells (Duan *et al.*, 2015). One simple explanation for this discrepancy is that sulfur might upregulate the androgen signaling pathway. Therefore, we first investigated the effects of sulfur on serum testosterone levels of male mice. As a result of treatment with sulfur, the serum testosterone level reached 7.66 ng/mL, while that of the control mice remained at 2.63 ng/mL, indicating that sulfur significantly increased (2.91 fold) the serum testosterone level (Fig. 1A).

The expression of the genes including StAR, p450c17, 3β -HSD, and p450scc was detected by RT-PCR (Fig. 1B). We observed all these genes significantly increased in sulfurtreat mice testis compared with the control group (Fig. 1B), with *StAR* increasing 99.8%, *p450c17* increasing 174.6%, 3β -HSD increasing 150.9%, and *p450scc* increasing 131.5%.

The effect of sulfur on TTB was further examined using the immunostaining assay. The staining of both StAR and 3β-HSD was restricted in the Leydig cells, while their expressions were absent in the seminiferous tubules. The results showed that immunostaining of both StAR and 3β-HSD was obviously stronger in Sulfur-treat mice testis compared with the control group (Fig. 1C). Phosphorylation of CREB (pCREB) binds to the StAR proximal promoter, which is indispensable for the upregulation of StAR transcription (Clem et al., 2005). The signal of pCREB was barely detected in the Leydig cells from the control mice, while a strong signal was present in Leydig cells of sulfur-treated mice (Fig. 1C), indicating that sulfur upregulated pCREB. In addition, we did not observe any significant histological effect of sulfur on seminiferous tubules. Together, these results demonstrated that treatment with sulfur raised serum testosterone levels in mice and upregulated the key genes for TTB.

Association of H_2S with effect of sulfur on TTB

 H_2S is the simplest product generated from elementary sulfur. Circulating H_2S in the human body mainly exists in three forms including free H₂S, HS⁻, S²⁻ (Shen *et al.*, 2011). To investigate the metabolism of sulfur *in vivo*, levels of the total hydrogen sulfide in serum and feces of mice were measured. The results showed that the serum level of hydrogen sulfide in the sulfur-treated group was 10.39 µmol/mL, which was higher by 29.1% than that in the control group (8.05 µmol/mL) (Fig. 2A). Total sulfide in the feces of the sulfur-treated group was 406.3 µmol/100 mg, which was 1.43 times higher than in the control group (283.5 µmol/100 mg) (Fig. 2A). The results indicated that sulfur was significantly converted into hydrogen sulfide *in vivo*.

The levels of reliable oxidative stress markers, including malondialdehyde (MDA), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px), were detected. Sulfur treatment significantly reduced the testicular level of MDA by 17.3% (Fig. 2B). In addition, the relative SOD activity and GSH-Px activity in the testis in sulfur-treated mice were increased by 26.7% and 35.6% (Fig. 2B), respectively. These results indicated that sulfur raised the antioxidative capability in testis.

In the presence of NaHS, the expression of StAR, p450c17, 3 β -HSD, and p450scc were tested. The results showed all the genes were higher in testis treated with NaHS (Fig. 2C). The highest upregulation was observed in 3 β -HSD, whose expression increased approximately by 3.63 folds in the presence of 200 μ M NaHS. These resulted indicated that hydrogen sulfide upregulated steroidogenesis in Leydig cells.

IL-6 is a key cytokine responsible for gut inflammation (Kittana *et al.*, 2018). We detected that the plasma level of IL-6 increased by 3.21 folds in the sulfur treated group than the control (Fig. 2D). However, sulfur did not significantly affect the levels of the other two cytokines, IL-2 and IL-4.

Gut bacterial populations affected by treatment with sulfur A total of 38 fecal samples were separately collected from 19 mice treated with Sulfur and from 19 controls. A total of



FIGURE 2. Effect of sulfur on levels of H₂S and MDA, the activity of SOD and GSH-Px and interleukins. (A) Level of H₂S in serum and fecal (n = 20). (B) Levels of MDA and activity of SOD and GSH-Px (n = 20). (C) Effect of H₂S on expression of *StAR*, *p450scc*, *3β-HSD*, and *p450scc*. The incubated pieces of testis were treated with different concentrations of NaHS for 24 h and then submitted for measurement of the gene expression by qPCR, as described in Materials and Methods. The data were the average of results from three independent experiments. (D) Levels of IL-2, IL-4, and IL-6 (n = 20), measured in ELISA assay. The data are expressed as mean \pm SEM. **p < 0.01; *p < 0.05.

1,1072,275 (22,628–44,021) 16S rRNA genes from the 38 samples were obtained by high-throughput DNA sequencing that met quality-filtering requirements. To normalize data to avoid statistical bias, 22,628 as normalization size for each sample was chosen for calculation of richness and diversity indices. Operational taxonomic unit (OTU), the group of 16S rRNA genes, was set at 97% similarity to estimate the diversity and richness of the bacterial community. After 38 samples were classified into two groups, a total of 17,275 OTUs (16,430 in the sulfur-treated group and 13,098 in the control) were obtained. The Good's coverage was over 99% for both sulfur-treated mice and the control group (Tab. 2), which meant the sequencing depth was sufficient for microbiota investigation of mice gut with different diets.

Core microbiota in mice gut

Phylogenetic and taxonomic assessments of the 16S rRNA V3-4 regions revealed the microbiota in the mouse gut. Using RDP analysis under a threshold of 80%, 99.6% of all 16S rRNA genes could be aligned to 17 phyla, and 93.2% could be aligned to 80 families, though only 30.8% could be aligned to 126 genera. At the phylum level, Bacteroidetes (normal 51.2%, sulfur 53.8%), Firmicutes (normal 45.0%, sulfur 42.8%) and Proteobacteria (normal 1.83%, sulfur 1.34%) were detected as the dominant phyla in the two groups of mice, which were also the common phyla in all the 38 samples.

At the family level, 10 families were major and core microbiota in both dietary groups (accounting for about 90% in both dietary groups) (Fig. 3), reflecting that microbiota

was stable at family level despite dietary differences. Among 10 families, Lachnospiraceae and Porphyromonadaceae were dominant families (>65% of all microbiome).

In 126 identified genera, 29 genera were relatively abundant of the major genera (>0.1% in at least one type of dietary group), including *Alloprevotella*, *Barnesiella*, *Bacteroides*, *Alistipes*, *Clostridium XIVa*, *Lactobacillus*, *Prevotella*, *Oscillibacter*, *Helicobacter*, and *Mucispirillum*, etc., (Tab. 3). Among major genera, there were 15 ubiquitous (core) genera that were consistently found across all analyzed samples and comprised over 20% of total microbiota.

Sulfur induced changes in the bacterial composition of mice gut Gut bacterial populations in sulfur-treated mice and the control showed variable richness and high diversity. Based on the evaluation of OTU, the total estimated bacterial richness per sample differed between the two groups. The number of OTUs in sulfur-treated mice was significantly higher than the controls (p = 0.0001, Tab. 2). Similarly, the samples collected from sulfur-treated mice contained higher species richness according to the ACE and Chao indices (p < 0.001, Tab. 2). While, for the evenness, as measured by the Simpsoneven calculator, the control samples had more evenness than the sulfur-fed samples (p = 0.026). Combined with the richness and evenness, the sulfur raised a relative diversity, which was assessed by the Shannon index (p =0.024). Effect of sulfur on microbial membership was also evidenced by Parsimony and AMOVA (pParsimony < 0.001, p_{AMOVA} < 0.001), suggesting that sulfur changed the microbiota compositions of the mice gut.

TABLE 2

The evaluated microbiota diversity of two dietary groups

Group	Sample	OTUs	Coverage	Richness		Evenness	Diversity
				Chao	ACE	Simpsoneven	Shannon
Normal	19	13,098	0.992531	15118.54	15339.03	0.008519	6.301084
Sulfur-treated	19	16,430	0.996374	16894.04	16956.73	0.008824	6.937134



FIGURE 3. Ten major families in normal and sulfur-treated mice gut. The relative abundance was shown as log (reads + 1) for families with false discovery rate. The *q*-value (corrected *p*-value) was computed by Meatstats. Five families were significantly different between the two groups.

In the ten core families, five of them showed a significant difference between the sulfur-treated group and the control (Fig. 3). Family Lachnospiraceae, Ruminococcaceae,

Rikenellaceae, and Helicobacteraceae had a relatively lower abundance in sulfur-treated group, while Lactobacillaceae had relatively higher abundance in the sulfur-fed microbiota.

TABLE 3

Relatively abundant genus of gut microbiota in different dietary Metastats analysis

Phylum	Genus	Feature	Normal	Sulfur-feed	<i>q</i> -value (corrected <i>p</i> -value)	Enriched
Bacteroidetes	Barnesiella	ubiquitous	3.58%	2.65%	<0.001	Control
Bacteroidetes	Parabacteroides	ubiquitous	0.90%	0.61%	< 0.001	Control
Bacteroidetes	Alloprevotella		6.29%	5.19%	< 0.001	Control
Bacteroidetes	Alistipes	ubiquitous	2.58%	1.85%	< 0.001	Control
Bacteroidetes	Rikenella		0.38%	0.31%	< 0.001	Control
Deferribacteres	Mucispirillum		1.05%	0.44%	< 0.001	Control
Firmicutes	Acetatifactor	ubiquitous	0.69%	0.39%	< 0.001	Control
Firmicutes	Clostridium_XlVb	ubiquitous	0.72%	0.24%	< 0.001	Control
Firmicutes	Eisenbergiella		0.11%	0.03%	< 0.001	Control
Firmicutes	Lachnospiracea_incertae_	_sedis	0.80%	0.31%	< 0.001	Control
Firmicutes	Roseburia		0.30%	0.06%	< 0.001	Control
Firmicutes	Anaerotruncus	ubiquitous	0.09%	0.04%	< 0.001	Control
Firmicutes	Butyricicoccus	ubiquitous	0.63%	0.24%	< 0.001	Control
Firmicutes	Oscillibacter	ubiquitous	1.33%	1.10%	< 0.001	Control
Firmicutes	Pseudoflavonifractor		0.24%	0.18%	< 0.001	Control
Proteobacteria	Helicobacter	ubiquitous	1.15%	0.61%	< 0.001	Control
Actinobacteria	Rhodococcus		0.01%	0.28%	< 0.001	Sulfur-treated
Firmicutes	Dorea		0.02%	0.11%	< 0.001	Sulfur-treated
Firmicutes	Intestinimonas	ubiquitous	0.09%	0.19%	< 0.001	Sulfur-treated
Bacteroidetes	Prevotella	ubiquitous	1.99%	2.78%	< 0.001	Sulfur-treated
Proteobacteria	Parasutterella		0.19%	0.25%	< 0.001	Sulfur-treated
Firmicutes	Clostridium_XlVa	ubiquitous	2.36%	2.91%	< 0.001	Sulfur-treated
Firmicutes	Lactobacillus	ubiquitous	2.22%	2.58%	< 0.001	Sulfur-treated
Tenericutes	Mycoplasma		0.00%	0.15%	< 0.001	Sulfur-treated
Bacteroidetes	Bacteroides	ubiquitous	3.56%	3.68%		
Bacteroidetes	Odoribacter		0.30%	0.31%		
Bacteroidetes	Paraprevotella		0.10%	0.09%		
Firmicutes	Flavonifractor	ubiquitous	0.46%	0.52%		
Tenericutes	Anaeroplasma		0.08%	0.10%		

*The ubiquitous meant the genus was identified in all samples.

In the control group, about 67.6% of all 16S rRNA genes were identified at two family levels, which were Lachnospiraceae (34.1%) and Porphyromonadaceae (33.6%), 86.3% of Lachnospiraceae and 85.8% but of Porphyromonadaceae were not identified at the genus level. Similar results were also present in sulfur-fed diet bacteria, 65.5% of 16S rRNA genes belonged to family Lachnospiraceae (31.5%),and Porphyromonadaceae (34.0%), while 87.1% of Lachnospiraceae and 89.5% of Porphyromonadaceae were not identified at the genus level. Among major relatively abundant genera, there were 24 genera showing a significant difference between normal and sulfur-fed dietary microbiota (Tab. 3). We also performed LEfSe tests (Segata et al., 2011) to detect different abundance taxa (Fig. 4), and found results were all covered by Metastats results (Tab. 3).

Identified bacteria associated with sulfur metabolism

Twenty-four genera showed a significant difference between control and sulfur-treated group (Tab. 3), and eight genera of which were increased, and the other were reduced in sulfur-treated mice compared with the controls. Among them, genus *Rhodococcus* was enriched in the sulfur-treated group, with a 26.5-fold increase of richness. Genus *Prevotella* increased 1.4-fold in the sulfur-treated group. Strikingly, serum levels of testosterone in boys were positively correlated with levels of *Prevotella* (Nakagawa *et al.*, 1994). Other bacteria whose richness increased after treatment with sulfur included genera *Intestinimonas*, *Dorea, Parasutterella, Clostridium* clusters XIVa, and *Lactobacillus*.

Other richness increased bacteria after sulfur-fed included genus *Parasutterella*, decreased strongly during





FIGURE 4. LDA Effect Size (LEfSe) algorithm was used to identify the most differentially abundant taxa between normal and sulfur-feed dietary groups. (A) Sulfur-enriched taxa were indicated with a positive LDA score (green) and taxa enriched in normal dietary with a negative score (red). (B) Taxonomic cladogram obtained from sulfur-enriched (green) and normal-enriched (red) taxa.

aging, and significantly reduced in patients with colorectal cancer (Van Der Lugt *et al.*, 2018). *Intestinimonas* (Klaring *et al.*, 2013) and *Clostridium* clusters XIVa (Lopetuso *et al.*, 2013) can produce butyrate in the mouse intestine. The elevated richness of *Clostridium* clusters XIVa is beneficial for the resistance to allergy and intestinal inflammation in mice (Atarashi *et al.*, 2011). *Lactobacillus*, a well-known probiotic bacteria, could produce volatile sulfur compounds (VSCs), including H₂S (Sreekumar *et al.*, 2009), and could increase serum testosterone levels and testicular size (Poutahidis *et al.*, 2014).

Functional prediction of normal and sulfur-fed microbiota

We used PICRUSt (Langille *et al.*, 2013) to predict the potential function of microbiota induced by sulfur fed. 405 KOs were

found to be significantly enriched (FDR < 0.05) between normal and sulfur-fed groups. Most of these different KOs (95.1%) were higher in sulfur-fed groups. There was no significant difference between the two dietary groups at KEGG level 2 pathways. At KEGG level 3 pathways, five pathways were identified as significantly higher (p < 0.05) in the sulfurtreated group (Fig. 5), including bile secretion, carotenoid biosynthesis, lipid biosynthesis proteins, propanoate metabolism and biosynthesis of type II polyketide products.

Discussion

Cadmium causes testicular toxicity and inhibits androgen production in adult male rats, and that concurrent



FIGURE 5. Prediction of metabolic contributions of sulfur-feed microbiota using PICRUSt.

administration of diallyl sulfide provides protection against cadmium-induced testicular toxicity (Sadik, 2008). We demonstrated that the treatment of sulfur significantly raised serum testosterone levels of the male mice approximately by 2.91-fold via upregulating testicular steroidogenesis in the present study (Figs. 1A and 1B). For the first time, our study clearly provides a mechanism by which sulfur, as a traditional Chinese medicine, has been used to clinically treat LOH and impotence (Jia, 2008). On the other hand hydrogen sulfide in excess has been suspected to be detrimental for colonic epithelium energy metabolism and DNA integrity (Beaumont et al., 2016). Keeping in line with the literature, our results suggested that sulfur caused gut inflammatory response, as indicated by a raised level of IL-6 (Fig. 2D). By contrast, a few studies have demonstrated a protective anti-inflammatory role of hydrogen sulfide in lung pathologies (Zhang et al., 2016). It still needs cautions in evaluating outcomes of the effect of sulfur and hydrogen sulfide on inflammation, given they are influenced by multiple factors, including host genetics, immune cell involvement, and cytokine profiles.

As a gaseous signaling molecule, H₂S freely diffuses across cell membranes in a receptor-independent manner and activate various cellular targets, exerting many different biological effects (from cytotoxic effects to cytoprotective actions) (Carbonero et al., 2012a; Zhang et al., 2018). Treatment of sulfur significantly raised levels of H₂S both in serum and fecal (Fig. 2A). Addition of NaHS, a well-used H₂S donor, which causes rapid H₂S release, upregulated expression of the genes involved in Leydig cell steroidogenesis (Fig. 2C). The antioxidant role of H_2S has been most extensively investigated and was thought of as the major mechanism underlying the effects of H₂S (Xie et al., 2016). Interestingly, the treatment of sulfur significantly lowered oxidative stress via raising the expression of SOD and GSH-Px and reducing the level of MDA (Fig. 2B). Given that oxidative stress is repressive to TTB (Wang et al., 2017), sulfur may upregulate TTB via the antioxidative role of H₂S. Thus, our study provided evidence supporting the hypothesis that H₂S is the key molecule that mediates sulfur-associated upregulation of TTB.

H₂S has been reported elsewhere to upregulate pCREB (Sulen *et al.*, 2016). Importantly, we also observed that sulfur significantly raised pCREB was higher in Leydig cells (Fig. 1C). pCREB upregulated StAR expression by binding its promoter and indirectly raises expression of P450c17, 3β-HSD (Kumar *et al.*, 2018). Moreover, pCREB upregulated expression of Nrf2, a master regulator of an antioxidant

gene, by binding with its promoter (Yang *et al.*, 2013; Ziady *et al.*, 2012). Nrf2 plays an important role in Leydig cell steroidogenesis (Chen *et al.*, 2015). Collectively, the treatment of sulfur can raise TTB via pCREB-targeted genes. Currently, it remains unknown how H₂S regulates steroidogenesis in Leydig cells. Considering that protein S-sulfhydration modulates a few important cellular functions, including reducing oxidative stress, as an important downstream event in H₂S signaling (Paul and Snyder, 2012), it is worth studying whether the protein posttranslational modification can alter the activity of the proteins that participate Leydig cell steroidogenesis.

H₂S plays important role in inflammatory bowel disease (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), which afflicts 0.1-0.5% of individuals in western countries (Carbonero *et al.*, 2012a). IL-6 is a key cytokine responsible for gut inflammation (Kittana *et al.*, 2018). We detected that the plasma level of IL-6 increased by 3.21-fold in the sulfur treated group compared with the control. However, sulfur did not significantly affect the levels of the other two cytokines IL-2 and IL-4. The results suggested that sulfur induced gut inflammatory response.

Treatment of sulfur extensively altered gut microbiota, highlighting the potential of the gut microbiota as a pharmaceutical target of sulfur. Accumulating evidence has revealed that gut microbiota can regulate TTB (Al-Asmakh et al., 2014; Hou et al., 2019; Tian et al., 2019). For example, early life exposure in mice to doxycycline resulted in testosterone deficiency, while supplementation of some probiotics increased serum level of testosterone (Poutahidis et al., 2014; Tian et al., 2019). However, the mechanisms underlying the results have still been elusive. Our aforementioned results strongly suggested the role of H₂S in Leydig cell steroidogenesis. Sulfur-reducing bacteria (SRB) represent the greatest source of H₂S in the body, and gutderived H₂S can produce systemic effects (Tomasova et al., 2016). Although mammals have not been reported to express the enzymes that can directly metabolize elemental sulfur, some species of SRB, which are ubiquitous members in the mammalian colon (Carbonero et al., 2012b), can grow with elemental sulfur and reduce sulfite, dithionite, thiosulfate and elemental sulfur under anaerobic conditions, eventually generating H₂S (Anantharaman et al., 2018; Teigen et al., 2019). Importantly, we detected that an SRB genus Desulfovibrio increased approximately by three folds after treatment with sulfur, consistent with the hypothesis that gut-derived H₂S makes an important contribution in raised TTB in sulfur-treated mice. Interestingly, genus

Desulfovibrio is the main SRB in the human colon (Rey et al., 2013). In addition, Lactobacillus, a well-known probiotic bacteria, can produce volatile sulfur compounds including H₂S (Sreekumar et al., 2009), and increase serum testosterone levels in mice (Poutahidis et al., 2014). Our analysis showed that treatment with sulfur also significantly raised richness of Lactobacillus (Tab. 3). The serum level of testosterone in boys was reported to be positively correlated with levels of some Prevotella species (Nakagawa et al., 1994). The abundance of Prevotella has been found to be a marker in the ecosystem of biota in the people with healthy living (Jeffery and O'toole, 2013), and was also raised by sulfur. Interestingly Prevotella can also produce a high level of H₂S (Basic et al., 2015; Ye et al., 2019). Taken together, our study suggests that gut biota can raise Leydig cell steroidogenesis via H₂S.

However, our study did not exclude other mechanisms by which sulfur upregulated steroidogenesis. Noteworthy, our analysis showed that five pathways and 405 kinds of genes were changed in sulfur-treated mice, including the raised abundance of genes encoding catalase/peroxidase, which played an antioxidative role in sulfur-treated mice. Carotenoid biosynthesis was one of the five pathways, which was highly enriched in sulfur-treated mice. For example, the abundance of Rhodococcus, which can synthesize carotenoid (Barone et al., 2018), was raised by 26.5-fold by treatment with sulfur (Tab. 3). Carotenoids are natural antioxidants, having anti-apoptotic and anti-inflammatory properties, and it is responsible for the integrity of cell membranes and activating antioxidants and phase II enzymes such as glutathione-S-transferases (Kaulmann and Bohn, 2014; Walczak-Jedrzejowska et al., 2013). In addition, the of probiotic Lactobacillus, abundance the oral administration of which improved systemic antioxidant capacity and stimulated intestinal immune response in a piglet model (Li et al., 2019), was also upregulated by sulfur. Butyrate is another antioxidative chemical, which can counterbalance the age-related microbiota dysbiosis, and exhibit anti-inflammatory (Mathew et al., 2014), and is considered to have a health-promoting role (Louis and Flint, 2009). Three butyrate-producing genera were also elevated after treatment with sulfur. They genus Dorea, which is highly abundant in the healthy human gut (Anand et al., 2016), genus Intestinimonas (Klaring et al., 2013), and Clostridium cluster XIVa (Lopetuso et al., 2013). Elderly individuals have a reduced abundance of Clostridium cluster XIVa when compared to younger individuals (Jeffery and O'toole, 2013), consistent with its antioxidative role. Collectively, these results highlight the presumable positive consequences of enrichment of these genera in the generation and maintenance of optimal antioxidative status in sulfur-treated mice.

Aging is associated with pronounced changes in gut microbiota composition (Van Der Lugt *et al.*, 2018). The relative abundance of genus *Parasutterella* was increased after sulfur-treatment (Tab. 3), whose relative abundance decreased strongly during aging, and significantly reduced in patients with colorectal cancer (Van Der Lugt *et al.*, 2018). By contrast, both *Lachnospiracea_incertae_sedis* (Vital *et al.*, 2015) and *Parabacteroides* (Claesson *et al.*, 2011) were reported to be upregulated with aging, while sulfur significantly reduced their richness. These results are not surprising if considering sulfur can raise antioxidative activity, which is favorable to healthy elderly. The above healthy role of sulfur was further revealed via its effects on age-associated microbiota.

Nevertheless, the current study still has a limitation, since the association between gut microbiota and testosterone rise caused by sulfur is still unclear. In this study, we used both control and sulfur administrated mice to compare the testicular testosterone biosynthesis. In subsequent studies, we will consider feeding germ-free mice with sulfur to see whether the testosterone level rise and validate the role of gut microbiota. Furthermore, it has not been reported whether endogenous H₂S is important for TTB. Given that aging is associated with a reduced level of H₂S in different models (Mun et al., 2019; Perridon et al., 2016; Zhan et al., 2018) and with gradually-falling plasma testosterone in male mammals, one of the tasks for the future is to approach the association of plasma H₂S levels with LOH. With clear evidence that the human colonic mucosa is persistently colonized by SRB, the beneficial vs. toxic effects of sulfur need to be delineated, in order to avoid adverse reactions caused by H₂S in some special circumstances.

In summary, we demonstrated that treatment with elemental sulfur raised levels of serum testosterone in male mice. The mechanisms include upregulated levels of H_2S and downregulated oxidative stress, which probably resulted from an extensively altered gut microbiota. Our study will probably promote a wider application of sulfur in the treatment of LOH and impotence. Our study also indicated the potential of some H_2S -releasing chemicals such as GYY4137 could be used for clinical treatment of LOH.

Availability of Data and Materials

The datasets used during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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