## Detailed Materials and Methods

### Study design:

All animal procedures were approved and conducted in accordance with the Qingdao Agriculture University Animal Care and Use Committee. Mice were maintained under a light:dark cycle of 12:12 h, at a temperature of 23 ℃ and humidity of 50%–70%; they had free access to food (chow diet) and water.[1]

**Experiment I: Mouse small intestine microbiota collection.** Three-week-old ICR male mice were dosed with dd$H_2O$ as the control or AOS 10 mg/kg BW, or AOS 100 mg/kg BW via oral gavage (0.1 ml/mouse/d). AOS dosing solution was freshly prepared on a daily basis and delivered every morning for three weeks. There were three groups (30 mice/treatment): (1) Control (dd$H_2O$); (2) A10 (AOS 10 mg/kg BW); (3) A100 (AOS 100 mg/kg BW). After treatment, the mice were humanely euthanized to collect small intestinal luminal content (microbiota) (online supplementary figure 1).

**Experiment II: FMT.**[2,3] The small intestine luminal content (microbiota) from each group was pooled and homogenized, diluted 1:1 in 20% sterile glycerol (saline) and frozen. Before inoculation, fecal samples were diluted in sterile saline to a working concentration of 0.05 g/ml and filtered through a 70-μm cell strainer. Three-week-old ICR male mice were used in current investigation. There were five treatment groups (30 mice/treatment): (1) Control (Dosed with Saline); (2) Sa [Busulfan (a single injection 40 mg/kg BW of busulfan)[1] plus Saline]; (3) Con-FMT [Busulfan plus gut microbiota from control mice (Experiment I)]; (4) A10-FMT [Busulfan plus gut microbiota from AOS 10 mg/kg mice (Experiment I)]; (5) A100-FMT [Busulfan plus gut microbiota from AOS 100 mg/kg mice (Experiment I)]. Mice were received oral FMT inoculations (0.1 ml) once daily for two weeks. Then the mice were regularly maintained for another three weeks (eight weeks of age). Then, the mice were humanely



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euthanized to collect samples for different analyses.

**Evaluation of spermatozoa motility using a computer-assisted sperm analysis system.** Spermatozoa motility was assessed using a computer-assisted sperm assay (CASA) method according to World Health Organization guidelines[1]. After euthanasia, spermatozoa were collected from the cauda epididymis of mice and suspended in DMEM/F12 medium with 10% FBS and incubated at 37.5 ℃ for 30 min; samples were then placed in a pre-warmed counting chamber. The micropic sperm class analyzer (CASA system) was used in this investigation. It was equipped with a 20-fold objective, a camera adaptor (Eclipse E200, Nikon, Japan), and a camera (acA780-75gc, Basler, Germany), and it was operated by an SCA sperm class analyzer (MICROPTIC S.L.). The classification of sperm motility was as follows: grade A linear velocity >22 μm $s^{-1}$; grade B <22 μm $s^{-1}$ and curvilinear velocity >5 μm $s^{-1}$; grade C curvilinear velocity <5 μm $s^{-1}$; and grade D = immotile spermatozoa. The spermatozoa motility data represented only grade A + grade B since only these two grades are considered to be functional.

**Morphological observations of spermatozoa.** The extracted murine caudal epididymides were placed in RPMI medium, finely chopped, and then Eosin Y (1%) was added for staining as described previously[1]. Spermatozoon abnormalities were then viewed using an optical microscope and were classified into head or tail morphological abnormalities: two heads, two tails, blunt hooks, and short tails. The examinations were repeated three times, and 500 spermatozoa per animal were scored.

**Assessment of acrosome integrity.** After harvest, mouse spermatozoa were incubated at 37.5 ℃ for 30 min, after which a drop of sperm suspension was uniformly smeared on a clean glass slide. Smeared slides were air dried and incubated in methanol for 2 min for fixation. After fixation, the slides were washed with PBS three times.

Assessment of an intact acrosome was accomplished by staining the sperm with 0.025% Coomassie brilliant blue G-250 in 40% methanol for 20 min at room temperature (RT). The slides were then washed three times with PBS and mounted with 50% glycerol in PBS. Acrosomal integrity was determined by an intense staining on the anterior region of the sperm head under bright-field microscopy (AH3-RFCA, Olympus, Tokyo, Japan) and scored accordingly[1].

**RNA Isolation and RNA-seq analyses.**[1] Briefly, total RNA was isolated using TRIzol Reagent (Invitrogen) and purified using a Pure-Link1 RNA Mini Kit (Cat: 12183018A; Life Technologies) following the manufacturers' protocol. Total RNA samples were first treated with DNase I to degrade any possible DNA contamination. Then the mRNA was enriched using oligo(dT) magnetic beads. Mixed with the fragmentation buffer, the mRNA was broken into short fragments (about 200 bp), after which, the first strand of cDNA was synthesized using a random hexamer-primer. Buffer, dNTPs, RNase H, and DNA polymerase I were added to synthesize the second strand. The double strand cDNA was purified with magnetic beads. Subsequently, 3'-end single nucleotide A (adenine) addition was performed. Finally, sequencing adaptors were ligated to the fragments. The fragments were enriched by PCR amplification. During the QC step, an Agilent 2100 Bioanaylzer and ABI StepOnePlus Real-Time PCR System were used to qualify and quantify the sample library. The library products were prepared for sequencing in an Illumina HiSeqTM 2500. The reads were mapped to reference genes using SOAPaligner (v. 2.20) with a maximum of two nucleotide mismatches allowed at the parameters of "-m 0 -x 1000 -s 40 -l 35 -v 3 -r 2". The read number of each gene was transformed into RPKM (reads per kilo bases per million reads), and then differentially expressed genes were identified using the DEGseq package and the MARS (MA-plot-based method with random sampling model) method. The threshold

was set as FDR ≤0.001 and an absolute value of $\log_2$ ratio ≥1 to judge the significance of the difference in gene expression. Then on the data were analyzed by GO enrichment, KEGG enrichment.

**Sequencing of microbiota from small intestine digesta samples and data analysis.**[1] ***DNA Extraction*.** Total genomic DNA of small intestine digesta was isolated using an E.Z.N.A.R Stool DNA Kit (Omega Bio-tek Inc., USA) following the manufacturer's instructions. DNA quantity and quality were analyzed using NanoDrop 2000 (Thermo Scientific, USA) and 1% agarose gel. Ten samples/groups were determined. ***Library preparation and sequencing.*** The V3-V4 region of the 16S rRNA gene was amplified using the primers MPRK341F (50-ACTCCTACGGGAGGCAGCAG -30) and MPRK806R: (50-GGACTACHVGGGTWTCTAAT -30) with Barcode. The PCR reactions (total 30 μL) included 15 μL PhusionR High-Fidelity PCR Master Mix (New England Biolabs), 0.2 mM primers, and 10 ng DNA. The thermal cycle was carried out with an initial denaturation at 98 ℃, followed by 30 cycles of 98 ℃ for 10 s, 50 ℃ for 30 s, 72 ℃ for 30 s, and a final extension at 72 ℃ for 5 min. PCR products were purified using a GeneJET Gel Extraction Kit (Thermo Scientific, USA). The sequencing libraries were constructed with NEB NextR UltraTM DNA Library Prep Kit for Illumina (NEB, United States) following the manufacturer's instructions and index codes were added. Then, the library was sequenced on the Illumina HiSeq 2500 platform and 300 bp paired-end reads were generated at the Novo gene. The paired-end reads were merged using FLASH (V1.2.71). The quality of the tags was controlled in QIIME (V1.7.02), meanwhile all chimeras were removed. The "Core Set" of the Greengenes database3 was used for classification, and sequences with >97% similarity were assigned to the same operational taxonomic units (OTUs). ***Analysis of sequencing data*** Operational

taxonomic unit abundance information was normalized using a standard of sequence number corresponding to the sample with the least sequences. The alpha diversity index was calculated with QIIME (Version 1.7.0). The Unifrac distance was obtained using QIIME (Version 1.7.0), and PCoA (principal coordinate analysis) was performed using R software (Version 2.15.3). The linear discriminate analysis effect size (LEfSe) was performed to determine differences in abundance; the threshold LDA score was 4.0. GraphPad Prism7 software was used to produce the graphs.

**Plasma and testis metabolite measurements by LC-MS/MS.** Plasma samples were collected and immediately stored at -80 °C. Before LC-MS/MS analysis, the samples were thawed on ice and processed to remove proteins. Testis samples were collected and the same amount of tissue from each mouse testis was used to isolate the metabolites using CH3OH: H2O (V: V) = 4:1. Then samples were detected by ACQUITY UPLC and AB Sciex Triple TOF 5600 (LC/MS) as reported previously.[1] Fifteen samples/groups were analyzed for plasma or testis samples.

The HPLC conditions employed an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm), solvent A [aqueous solution with 0.1% (v/v) formic acid], and solvent B [acetonitrile with 0.1% (v/v) formic acid] with a gradient program:

| Time | A% | B% |
|---|---|---|
| 0 | 95 | 5 |
| 2 | 80 | 20 |
| 4 | 75 | 25 |
| 9 | 40 | 60 |
| 17 | 0 | 100 |
| 19 | 0 | 100 |
| 19.1 | 95 | 5 |
| 20.1 | 95 | 5 |

The flow rate was 0.4 mL/min and the injection volume was 5 μL.

The mass spectrometry program with ESI was:

| Parameters | Positive ion | Positive ion |
|---|---|---|
| Nebulizer Gas (GS1, PSI) | 40 | 40 |
| Auxiliary Gas (GS2, PSI) | 40 | 40 |
| Curtain Gas (CUR, PSI) | 35 | 35 |
| Ion Source Temperature (℃) | 550 | 550 |
| Ion Spray Voltage (V) | 5500 | 4500 |
| Declustering Potential (DP,V) | 100 | -100 |
| Mass Scan Range (TOF MS scan) | 70–1000 | 70–1000 |
| Collision Energy (TOF MS scan, eV) | 10 | -10 |
| Mass Scan Range (Product Ion scan) | 50–1000 | 50–1000 |
| Collision Energy (Product Ion scan,eV) | 30 | 30 |
| Interface Heater Temperature (℃) | 550 | 600 |

Progenesis QI v2.3 (Nonlinear Dynamics, Newcastle, UK) was implemented to normalize the peaks. Then the Human Metabolome Database (HMDB), Lipidmaps (v2.3), and METLIN software were used to qualify the data. Moreover, the data were processed with SIMCA software (version 14.0, Umetrics, Umeå, Sweden) following pathway enrichment analysis using the KEGG database (http://www.genome.jp/KEGG/pathway.html).

**Histopathological analysis.** Testicular tissues were fixed in 10% neutral buffered formalin, paraffin embedded, cut into 5 μm sections and subsequently stained with hematoxylin and eosin (H&E) for histopathological analysis.

**Western blotting.** Western blotting analysis of proteins was carried out as previously reported.[1] Briefly, testicular tissue samples were lysed in RIPA buffer containing the protease inhibitor cocktail from Sangong Biotech, Ltd. (Shanghai, China). Protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology, Shanghai, China). Goat anti-actin was used as a loading control. The remaining primary antibodies (Abs) were purchased from Abcam or Beijing Biosynthesis Biotechnology CO., LTD, (Beijing, China; online supplementary table 1). Secondary donkey anti-goat Ab (Cat no.: A0181) was purchased from Beyotime Institute of Biotechnology, and goat anti-rabbit (Cat no.: A24531) Abs were bought from Novex® by Life Technologies

(USA). Fifty micrograms of total protein per sample were loaded onto 10% SDS polyacrylamide electrophoresis gels. The gels were transferred to a polyvinylidene fluoride (PVDF) membrane at 300 mA for 2.5 h at 4 ℃. The membranes were then blocked with 5% bovine serum albumin (BSA) for 1 h at RT, followed by three washes with 0.1% Tween-20 in TBS (TBST). The membranes were incubated with primary Abs diluted with 1:500 in TBST with 1% BSA overnight at 4 ℃. After three washes with TBST, the blots were incubated with the HRP-labelled secondary goat anti-rabbit or donkey anti-goat Ab respectively for 1 h at RT. After three washes, the blots were imaged. The bands were quantified using Image-J software. The intensity of the specific protein band was normalized to actin first, then the data were normalized to the control. The experiment was repeated >6 times.

**Detection of protein levels and location in testis using immunofluorescence staining.** The methodology for immunofluorescence staining of testicular samples is reported in our recent publications.[1] Sections of testicular tissue (5 μm) were prepared and subjected to antigen retrieval and immunostaining as previously described. Briefly, sections were first blocked with normal goat serum in PBS, followed by incubation with primary Abs (online supplementary table 1; 1:100 in PBS-0.5% Triton X-100; Bioss Co. Ltd. Beijing, PR China) at 4 ºC overnight. After a brief wash, sections were incubated with an Alexa 546-labeled goat anti-rabbit secondary Ab (1:100 in PBS; Molecular Probes, Eugene, OR, USA) at RT for 30 min and then counterstained with 4',6-diamidino-2-phenylindole (DAPI). The stained sections were examined using a Leica Laser Scanning Confocal Microscope (LEICA TCS SP5 II, Germany). Ten animal samples from each treatment group were analysed. Positively stained cells were counted. A minimum of 1000 cells were counted for each sample of each experiment. The data were then normalized to the control.

**Statistical analysis.** Data were analyzed using SPSS statistical software (IBM Co., NY) with one-way analysis of variance (ANOVA) followed by LSD multiple comparison tests. All groups were compared with each other for every parameter. The data were shown as the mean ± SEM. Statistical significance was based on $p < 0.05$.

**Data and materials availability**:

RNA-seq raw data is deposited in NCBI's Gene Expression Omnibus under accession number GSE135431. The microbiota raw sequencing data generated in this study has been uploaded to the NCBI SRA database with the accession number PRJNA 592332.

## References

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