Detailed Materials and Methods

Study design:

All animal procedures were approved and conducted in accordance with Institute of Animal Sciences, Chinese Academy of Agricultural Sciences Animal Care and Use Committee (CAASIAS201928176R). The animals were housed in conventional facility. Mice were maintained under a light:dark cycle of 12:12 h, at a temperature of 23 °C and humidity of 50%–70%; they had free access to food (chow diet) and water [regular water and food (not sterile)].[1]

Experiment I: Mouse small intestine microbiota collection. Three-week-old ICR male mice were dosed with ddH_2O as the control or AOS 10 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 two groups (30 mice/treatment): (1) Control (ddH_2O); (2) A10 (AOS 10 mg/kg BW). After treatment, the mice were humanely euthanized to collect small intestinal luminal content (microbiota) (online supplementary figure 1).

Experiment II: Microbiota transplants (FMT) with or without busulfan treatment.[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 six treatment groups (30 mice/treatment): (1) Con-sa (Dosed with Saline); (2) Con-FMT [Gut microbiota from control mice (Experiment I)]; (3) A10-FMT [Gut microbiota from AOS 10 mg/kg mice (Experiment I)]; (4) B-sa [Busulfan (a single injection 20 mg/kg BW of busulfan)[1] plus Saline]; (5) B+ Con-FMT [Busulfan plus gut microbiota from control mice (Experiment I)]; (6) B+

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A10-FMT [Busulfan plus gut microbiota from AOS 10 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 mated with regular ICR female mice (male 1:2 female) for 4 days. Then the male mice were taken out and humanely euthanized to collect samples for different analyses. The female mice were maintained regularly. The pregnancy rate and the number of live pups/litter were determined.

Experiment III: AOS dosing with or without busulfan treatment.[2,3] This experiment was similar as Experiment II. There were three treatment groups (30 mice/treatment): (1) Con (Dosed with Saline); (2) A10 [Dosed with AOS 10 mg/kg]; (3) B+A10 [Busulfan plus AOS 10 mg/kg]. Mice were received oral AOS inoculations (0.1 ml) once daily for five weeks (eight weeks of age). Then, the mice were mated with regular ICR female mice for 4 days. Then the male mice were taken out. The female mice were maintained regularly. The pregnancy rate and the number of live pups/litter were determined.

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 °C 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 °C 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].

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

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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 $p < 0.05$.

References

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