Rescue of male fertility following faecal microbiota transplantation from alginate oligosaccharide-dosed mice

A very recent publication in *Gut* highlights that faecal microbiota transplantation (FMT) from alginate oligosaccharide (AOS)-dosed animals improves mouse sperm quality and spermatogenesis after busulfan treatment.[1] The results suggest the potential of FMT for the improvement of infertility,[1] since worldwide 10%–15% of couples are infertile and many of them have failed spermatogenesis.[1 2] In addition, many investigations have found that gut microbiota may affect male or female reproduction.[3 4] Although the improvement of male infertility is an

emerging novel area of interest and many investigations have attempted to ameliorate spermatogenesis by various methods, little progress has been achieved.[5 6] In the study done by Zhang *et al*,[1] FMT from AOS-dosed animals increased spermatozoa quality and the process of spermatogenesis; however, that gut microbiota from AOS-dosed animals can actually increase fertility rate is as yet unknown.

To confirm the beneficial advantages of FMT from AOS dosed animals, we set out to explore the fertility rate (pregnancy rate and number of live pups/litter) following FMT from AOS-dosed animals to busulfan-treated mice (online supplemental file 1 and online supplemental figure 1). We found that B+A10 FMT (busulfan plus gut microbiota from AOS 10 mg/kg mice) significantly increased pregnancy rate (10-fold) and number of live pups/litter (twofold) compared with busulfan (B-sa; figure 1A,B). Notably, the number of live pups/litter was almost the same for B+A10 FMT and control (Con-sa; blank control) which suggested that A10-FMT had a strong potential for rescuing male fertility. However, B+Con FMT (busulfan plus gut microbiota from control mice) did not significantly increase the pregnancy rate or number of live pups/litter compared with busulfan (figure 1A,B). At the same time, we compared the beneficial advantages of AOS 10 mg/kg (A10) and A10-FMT after busulfan treatment. A10 and A10-FMT produced a similar improvement on the pregnancy rate and number of live pups/litter (figure 1A,B). In our earlier studies,[1 5] we discovered that AOS 10 mg/kg improves the gut microbiota to, in turn, improve spermatogenesis and semen quality. Furthermore, A10-FMT similarly benefited gut microbiota[1] through an increase in the 'beneficial' bacteria[7] *Bacteroidales*, *Bifidobacteria*, *Sphingomonadales* and *Campylobacterales* which have beneficial effects such as protecting the intestinal barrier,[8] production of antioxidant compounds[9] and the possession of reduction enzymes.[10] It is also interesting that the microbes from A10-FMT showed a good correlation with sperm concentration/motility, blood metabolome and testis metabolome.[1] It is even more profoundly important that the microbiota from A10-dosed mice and A10-FMT-treated mice were well correlated.[1 5] Moreover, A10, A10-FMT and Con-FMT did not affect the fertility rate of control mice (without busulfan; figure 1C,D) which indicated that these treatments did not pose a disadvantage for male animal reproduction. In the current investigation, spermatogenesis was significantly improved by A10-FMT as shown by the germ cell marker VASA (figure 2).

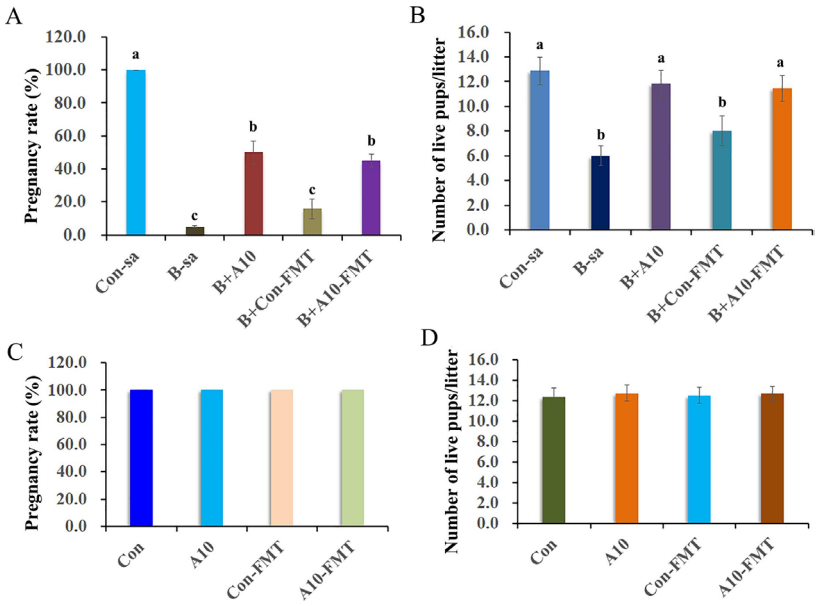

Figure 1 Mouse pregnancy rate and the number of live pups/litter. (A) Mouse pregnancy rate (number of pregnant mice/total mice in each group: Con-sa (experiment II), B-sa (experiment II), B+A10 (experiment III), B+A10 FMT (experiment II) and B+Con FMT (experiment II); n=60/group). [a,b,c] Means not sharing a common superscript are different ($p<0.05$). (B) The number of live pups/litter. The average number of live pups/litter in each group: Con-sa (experiment II), B-sa (experiment II), B+A10 (experiment III), B+A10 FMT (experiment II) and B+Con FMT (experiment II); (n=60/group). [a,b,c] Means not sharing a common superscript are different ($p<0.05$). (C) Mouse pregnancy rate (number of pregnant mice/total mice in each group: Con (experiment II), A10 (experiment III), A10-FMT (experiment II) and Con-FMT (experiment II); n=60/group). [a,b,c] Means not sharing a common superscript are different ($p<0.05$). (D) The number of live pups/litter. The average number of live pups/litter in each group: Con (experiment II), A10 (experiment III), A10-FMT (experiment II) and Con-FMT (experiment II); (n=60/group). [a,b,c] Means not sharing a common superscript are different ($p<0.05$). Note: (1) Con-sa (dosed with saline); (2) B-sa (busulfan (a single injection 20 mg/kg body weight of busulfan)[1 4] plus saline); (3) B+A10 (busulfan plus AOS 10 mg/kg); (4) B+Con FMT (busulfan plus gut microbiota from regular mice); (5) A10-FMT (busulfan plus gut microbiota from AOS 10 mg/kg dosed mice); (6) Con (dosed with saline); (7) A10 (dosed with AOS 10 mg/kg); (8) Con-FMT (dosed with gut microbiota from regular mice); (9) A10-FMT (dosed with gut microbiota from AOS 10 mg/kg dosed mice). See more detailed information in online supplemental file 1). AOS, alginate oligosaccharide; FMT, faecal microbiota transplantation.

There were almost no VASA-positive cells in the busulfan group (B-sa) and a very small number in B+Con FMT group; however, a significant number of VASA-positive cells were found in the B+A10 FMT and B+A10 groups (figure 2), which suggested that spermatogenesis was improved by A10-FMT, since busulfan mainly disrupted germ cells.[2 5 6] At the same time, protein levels of the sperm cell marker PGK2 were determined by immunofluorescence staining.[1 2 5] There were almost no PGK2-positive cells in the B+Sa and B+Con FMT groups (figure 2). However, a similar number of PGK2-positive cells were found in the B+A10, B+A10 FMT and control (Con-sa) groups (figure 2). The data further revealed that A10-FMT rescued busulfan disrupted spermatogenesis, while Con-FMT did not. The data in this investigation confirmed that gut microbiota from AOS-dosed mice had the potential to improve spermatogenesis and then to increase male fertility rate.

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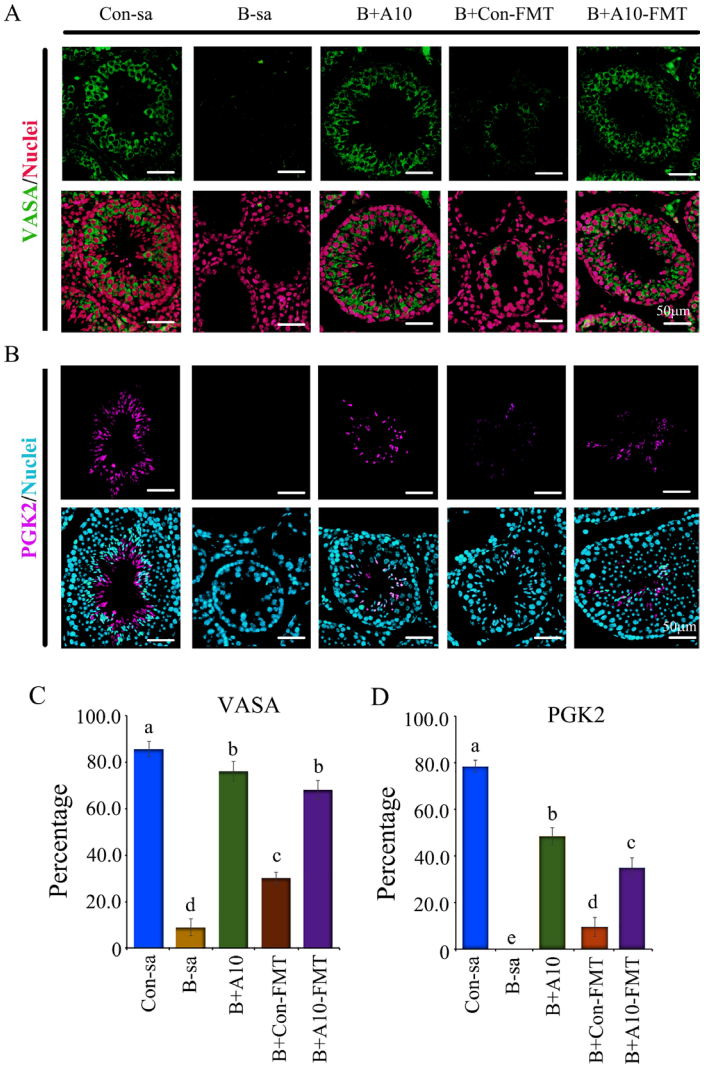

Figure 2 VASA and PGK2 staining of mouse testicular samples. (A) Germ cell marker VASA staining of mouse testicular samples. (B) Sperm cell marker PGK2 staining of mouse testicular samples. (C) Quantification data for VASA staining. (D) Quantification data for PGK2 staining. Note: (1) Con-sa (dosed with saline); (2) B-sa (busulfan (a single injection 20 mg/kg body weight of busulfan)[1 4] plus saline); (3) B+A10 (busulfan plus AOS 10 mg/kg); (4) B+Con FMT (busulfan plus gut microbiota from regular mice); (5) A10-FMT (busulfan plus gut microbiota from AOS 10 mg/kg dosed mice). For more details, see information in online supplemental file 1). The letters a, b, c, d and e indicate a significant difference among different treatments ($p<0.05$). AOS, alginate oligosaccharide; FMT, faecal microbiota transplantation.

YZ designed and supervised the study. ZS, QS, HZ, WS and YZ wrote the manuscript. All the authors edited the manuscript and approved the final manuscript.

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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 ℃ 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+

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 ℃ 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].

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 $p < 0.05$.

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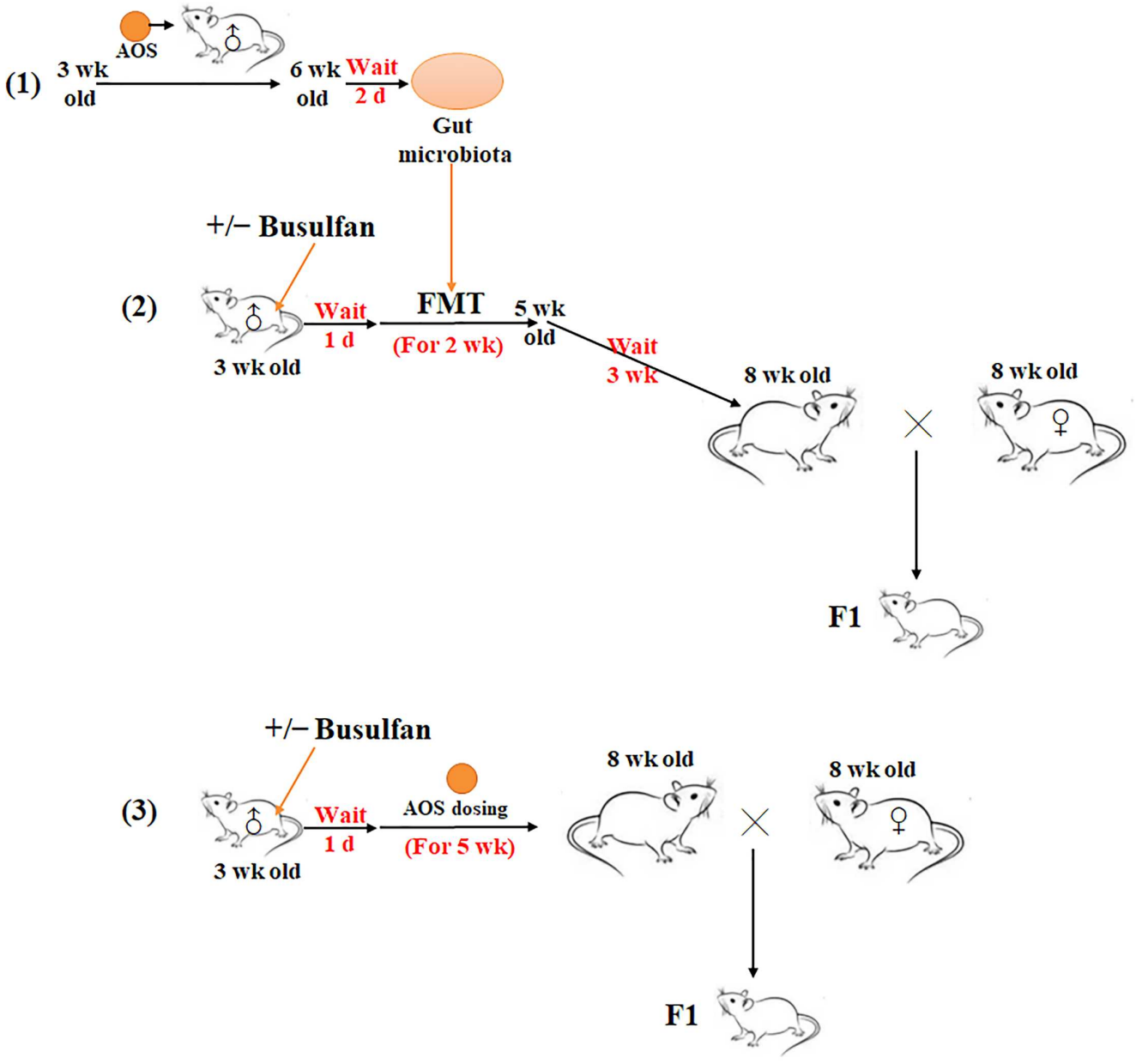
Supplementary Materials

Supplementary File 1 Detailed Materials and Methods

Supplementary Figure 1 Study design.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3 wk old
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Wait 2 d
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