The effect of gut dysbiosis on bladder function of rats with spinal cord injury

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The effect of gut dysbiosis on bladder function of rats with spinal cord injury

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ABSTRACT

The effect of gut dysbiosis on bladder function of rats with spnial cord injury

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Objectives: Gut dysbiosis occurs after spinal cord injury (SCI). Neurogenic bladder is a major complication of SCI and gut microbiota (GM) has been known to be related with voiding related diseases. We hypothesized that gut dysbiosis after SCI would affect bladder function. To verify this, the fecal microbiota transplantation (FMT) from SCI-rat was performed on antibiotic induced microbiota depletion (AMD)-rats and their bladder function was evaluated.

Methods: The experiment was composed of 3 parts and conducted in 7-week old female Sprague-Dawley rats. First, when gut dysbiosis occurred in SCI-rat was assessed with 16S-rRNA sequencing. Then the effect of GM on bladder function was analyzed in sham AMD-rats. AMD was induced with 4weeks of cocktail of antibiotics in drinking water. They were divided into 3 groups; one group with FMT from sham-rats, other group with FMT from SCI-rats and the other group without FMT, respectively. Their bladder function was evaluated with cystometry after 4 weeks along with bladder tissue analysis. Finally, it was analyzed in the SCI AMD-rats with same grouping and same intervention as the second parts.

Results: Gut dysbiosis was maximized on the 3rd day after SCI. Inter-contraction interval (ICI) at cystometry was highly increased and non-voiding contraction (NVC) developed after SCI. In sham AMD-rats, NVC and voiding efficiency were significantly different between FMT from sham group and FMT from SCI group. In SCI AMD-rats, NVC was observed more frequently than control SCI rats. After FMT from sham rat, NVC was significantly decreased. After FMT from SCI-rat, ICI and NVC was significantly increased. Voiding efficiency were also significantly different between FMT from sham group and FMT from SCI group.

Conclusions: AMD affects the incidence of NVC and decrease the voiding efficiency. FMT from SCI-rat is considered to be involved in the increase in ICI and the occurrence of NVC. I suggest that FMT changes the composition of gut microbiota and decrease the development of NVC in AMD-SCI rat model.

Key words : microbiota; fecal microbiota transplantation; spinal cord injuries; urodynamics; neurogenic bladder

The effect of gut dysbiosis on bladder function of rats with spinal cord injury

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I. INTRODUCTION

Recently, the role of gut microbiota (GM) has been increasingly emphasized in various diseases. Gut dysbiosis has been found in many diseases, and it is considered to be a direct cause of a certain disease or a target for treatment.[1,2] This is also true for neurological disorders. It is well known that GM affect mood in depressive disorder by changing neuropeptide, or affect nerve function in multiple sclerosis or Parkinson's disease.[3,4] Accordingly, there is also a possibility that these diseases can be treated by improving the dysbiosis of GM in the future.

In spinal cord injury (SCI), one of the major acquired central nervous system disease, several researches about GM have been conducted. They have shown that gut dysbiosis occurs immediately after the occurrence of SCI.[5,6] It has been repeatedly reported that the distribution of butyrate producing bacteria necessary for maintaining homeostasis of intestinal epithelial cells is significantly reduced. Recently, gut dysbiosis found in SCI is not recognized merely as a sequelae of SCI, and dysbiosis itself is presumed to exacerbate other complications of SCI.[6]

We focused on the neurogenic bladder, a major complication that reduces the lifespan and quality of life of patients with SCI despite known treatment methods such as anticholinergic drugs and clean intermittent catheterization.[7] Recently, gut dysbiosis has been observed

in some voiding related diseases. Okamoto et al. reported that GM is associated with overactive bladder in cross-sectional study.[8] Okuyama et al. reported that GM is also associated with a risk for the progression of overactive bladder symptoms in other longitudinal study.[9]

Based on this flow, we hypothesized that gut dysbiosis found in SCI-rat would affect bladder function. In order to verify this hypothesis, the fecal microbiota from SCI-rat and sham rat were transplanted into antibiotic induced microbiota depletion (AMD)-rat model, respectively and their bladder function was evaluated.

II. MATERIALS AND METHODS

1. Animal models and treatments

This study protocol was approved by Institutional Animal Care and Use Committee of our institution. (Approval No: 2020-0150) All experiment was conducted in 7-week old female Sprague-Dawley rats (body weight: 200-250g) underwent 1 week of acclimatization period. For SCI-rat model development, the spinal cord was transected at the level of T8-9.[10] The bladder was then emptied by abdominal compression twice daily until voiding restored. AMD-rat model was developed with 4 weeks use of cocktail of five antibiotics according to the Kelly et al.'s protocol.[3] In detail, rats were given a mixture of ampicillin and metronidazole (all at 1 g/L), ciprofloxacin HCl (200 mg/L), vancomycin (500 mg/L), and imipenem (250 mg/L) once daily for 4 weeks in drinking water. Then they were stabilized with sterilized water intake for 3 days.

2. Experimental design

This research is composed of three parts. First, when GD occurred in the SCI rats was analyzed to prepare proper materials for following fecal microbiota transplantation (FMT) in following parts (Figure 1A). Sham operation was performed in 6 rats and SCI was developed in other 6 rats. Fecal microbiota was analyzed with feces sampled at before

operation and 1, 3, 7, and 28 days after the operation. Cystometry was performed at 28th day from operation. These 12 rats were used as sham-control group and SCI-control group at following parts. Their feces were also used for donation material at following parts.

Then, the effect of GM on bladder function was analyzed in the sham AMD-rats (Figure 1B). They were divided into 3 groups (6 rats each); one group with FMT from sham rats, other group with FMT from SCI-rats and the other group without FMT (control), respectively. Sham operation was performed at day 0 and FMT was done 3 times at day 1, day 2, and day 3. Fecal microbiota was analyzed immediately after the 3rd FMT on day 3 and followed at day 4, day 6, day 10 and day 28. Cystometry was performed at day 28.

Finally, the effect of GM on bladder function was analyzed in the SCI AMD-rats (Figure 1C). They were also divided into 3 groups (6 rats each); one group with FMT from sham rats, other group with FMT from SCI-rats and the other group without FMT (control), respectively. SCI was developed at day 0 and FMT was done 3 times at day 1, day 2, and day 3. Cystometry was performed at day 28.

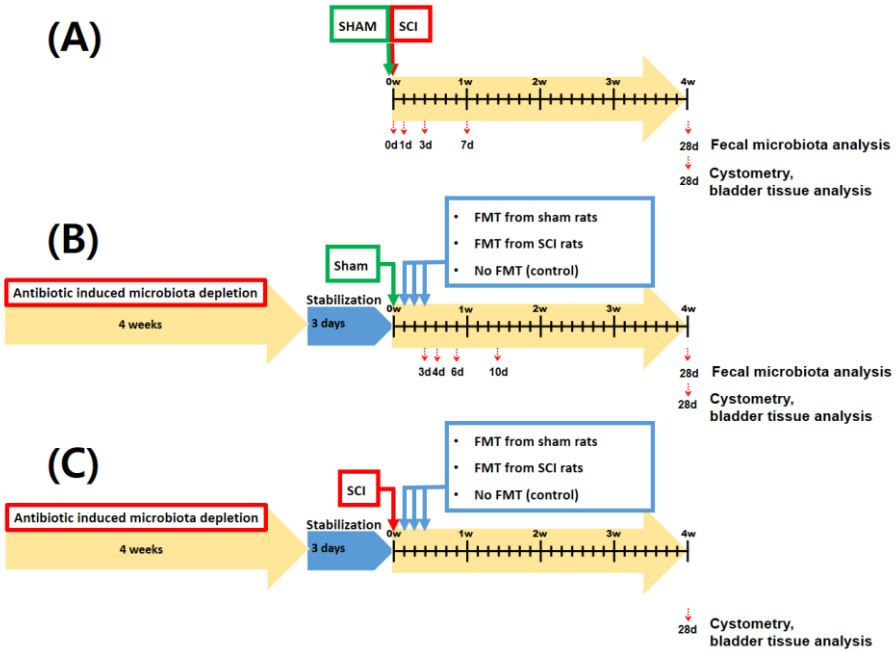


Figure 1. Experimental design. (A) When gut dysbiosis occurred in the spinal cord injury (SCI) rats was analyzed. (B) The effect of gut microbiota (GM) on bladder function was analyzed in sham antibiotic induced microbiota depletion (AMD)-rats. They were divided into 3 groups; one group with fecal microbiota transplantation (FMT) from sham rats, other group with FMT from SCI-rats and the other group without FMT (control), respectively. (C) The effect of GM on bladder function was analyzed in the SCI AMD-rats. They were also divided into 3 groups; one group with FMT from sham rats, other group with FMT from SCI-rats and the other group without FMT (control), respectively.

3. Fecal microbiota transplantation

Donor microbiota was acquired from feces of SCI-rat model at 3 days after development of SCI and sham rat, respectively. One gram of fecal material was homogenized with 2 ml of Phosphate-buffered saline. The fecal residue was removed using a 100 μm filter. Rats were colonized by once a daily oral gavage of 300 μL donor microbiota for 3 consecutive days.[3]

4. Fecal microbiota analysis

A. Fecal sampling

Feces were sampled for 16S rRNA sequencing of microbiota. In SCI-rats, to obtain sufficient feces, defecation was induced by massaging the abdomen of rats. One pellet of collected feces is stored at -80℃ until used for analysis.

B. DNA extraction and quantification

DNA was extracted using a DNeasyPowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In brief, 0.25 g of fecal material was placed in a powerbead tube and vortex at the maximum speed for 10 minutes. After centrifugation for 30 seconds, the supernatant was transferred to a collecting tube and cell lysis was performed. Then, DNA was bound using MB spin column, and DNA elusion was done with DNA-free PCR-grade water. The extracted DNA was quantified using Quant-IT PicoGreen

(Invitrogen, Waltham, MA).

C. Library construction and sequencing

The sequencing libraries is prepared according to the Illumina 16S Metagenomic Sequencing Library protocols to amplify the V3 and V4 region. The input gDNA 2 ng was amplified by polymerase chain reaction (PCR) with 5x reaction buffer, 1 mM of dNTP mix, 500 nM each of the universal F/R PCR primer, and Herculase II fusion DNA polymerase (Agilent Technologies, Santa Clara, CA). The cycle condition for 1st PCR was 3 min at 95℃ for heat activation, and 25 cycles of 30 sec at 95℃, 30 sec at 55℃ and 30 sec at 72℃, followed by a 5-min final extension at 72℃. The universal primer pair with Illumina adapter overhang sequences used for the first amplifications were as follows: V3-F: 5`-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3`, V4-R: 5`-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3`. The 1st PCR product was purified with AMPure beads (Agencourt Bioscience, Beverly, MA). Following purification, the 2ul of 1st PCR product was PCR amplified for final library construction containing the index using NexteraXT Indexed Primer. The cycle condition for 2nd PCR was same as the 1st PCR condition except for 10 cycles. The PCR product was purified with AMPure beads. The final purified product is then quantified using qPCR according to the qPCR Quantification Protocol Guide (KAPA Library Quantification kits for Illumina Sequencing platforms) and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies, Waldbronn, Germany). The paired-end (2×300 bp) sequencing was performed by the Macrogen using the MiSeq™ platform (Illumina, San Diego, CA)

D. Operational taxonomic unit analysis

The raw data generated by Illumina MiSeq platform were demultiplexed with index sequences. Adapter sequences were trimmed and errors in where the two reads overlapped were corrected using fastp program.[11] The target sequences were assembled with

FLASH (v1.2.11) by merging paired-end sequences created by sequencing of both directions of library.[12] Among the assembled reads, only reads which were > 400 bp and < 500 bp were filtered by length. Sequences with low quality which considered as sequencing errors, with ambiguous bases and chimeric regions were removed using CD-HIT-OUT that based on cd-hit-est.[13] Then, OTUs of the remained reads were created by cluster cutoff value of 97%.

Taxonomic classification was performed with NCBI 16s Microbial DB using BLAST+ (v2.9.0). Each OTUs were defined as the taxon of the best hit subjects from blast results. When Query coverage was < 85% and Identity of matched region was < 85%, the OTUs were regarded as unassigned.

To identify phylogeny, multiple alignment of reads were conducted with MUSCLE (v7.475) program. Phylogenetic tree was constructed using FastTreeMP (v2.1.10). Community diversity was analyzed using QIIME (v1.9.0). In order to check the diversity and evenness in the microbial community, Shannon, Gini-simpson and Chao1index were calculated. Also, we check that the rarefaction curves were saturated in all samples to confirm that sequencing depths were satisfied to catch the real diversity. Beta diversity, which compares among samples within the group, was calculated based on weighted and unweighted UniFrac distance. Genetic relationships among samples were visualized to PCoA and UPGMA tree.

5. Cystometry

Cystometry was performed according to the Kang et al.'s method.[14] In brief, a lower midline abdominal incision was made under isoflurane anesthesia. A PE-50 tube (Clay-Adams, Parsippany, NJ) was inserted and secured at the dome of bladder. Then incision was closed. After recovery from anesthesia, the rats were gently restrained in a cage and the cystostomy tube was connected to a syringe pump and pressure transducer. Cystometry was performed with PowerLab® / LabChart7 instrument (ADInstruments, Bella Vista, Australia) by infusing physiological saline into the bladders of rats with an infusion rate of

0.04 ml/min to induce repetitive voiding for 2.5-4 hours. At least five reproducible micturition cycles were analyzed. Inter-contraction intervals (ICIs) were defined as the intervals between spontaneous bladder contractions developing micturition. Threshold pressure (TP) was defined as the bladder pressure at the start of micturition contraction. Maximal micturition pressure (MP) was defined as the maximum bladder pressure during micturition. Basal pressure (BP) was as the lowest bladder pressure during filling phase. Non-voiding contraction (NVC) was defined as more than 8 cmH_2O increase in intravesical pressure above the BP.[10] The number of NVC at each voiding cycle was measured and used in the analysis. Voiding efficiency was also calculated (voided volume/infused volume x 100).

6. Bladder tissue analysis

A. Tissue sampling

After cystometric evaluation, the whole bladder each rat was dissected out under anesthesia. The removed bladder was sliced into several pieces. These specimens were immediately frozen in liquid nitrogen and stored at -80℃ until used for analysis.

B. Hematoxylin and eosin stain

For Hematoxylin and eosin stain, bladder specimen was fixed with 4% paraformaldehyde overnight at 4℃. Fixed tissues were dehydrated using a series of ethanol dilutions and then embedded paraffin. Embedded tissues cut into 5 ㎛ section and stained with Hematoxylin and Eosin.

C. Enzyme-linked immunosorbent assay (ELISA)

Cold PBS solution (pH 7.4) was added to a portion of the collected bladder tissue and the tissue homogenized using a grinder. After ultra-high speed centrifugation, the supernatant was taken. The concentration of Galectin-3 was measured using ELISA kit (Novus, Co, USA). The optical density determined at 450nm.

7. Statistical analysis

The quantitative values were compared using Mann-Whitney U test and Kruskal Wallis test, whereas qualitative variables were compared using chi-square or Fisher's exact tests. To compare taxa abundances in phylum, genus, and species level between groups, Kruskal Wallis test was conducted. False discovery rate of Benjamini-Hochberg method were obtained to address multiple testing. Abundances of the phylum, genus, and species level were averaged to show taxonomic composition of each group with bar graph. Statistical analyses were performed using the Statistical Package for Social Science for Windows, version 26.0 (IBM, NY, USA). P value of <0.05 was considered significant.

III. RESULTS

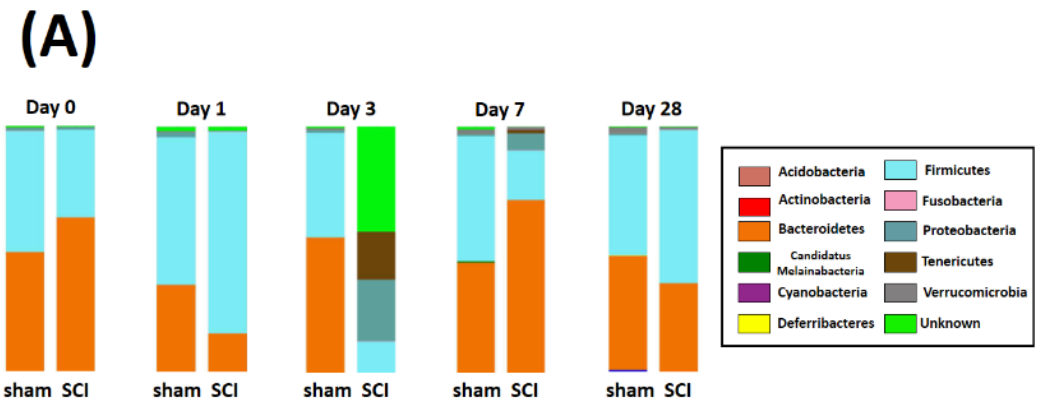
1. The development of gut dysbiosis after SCI

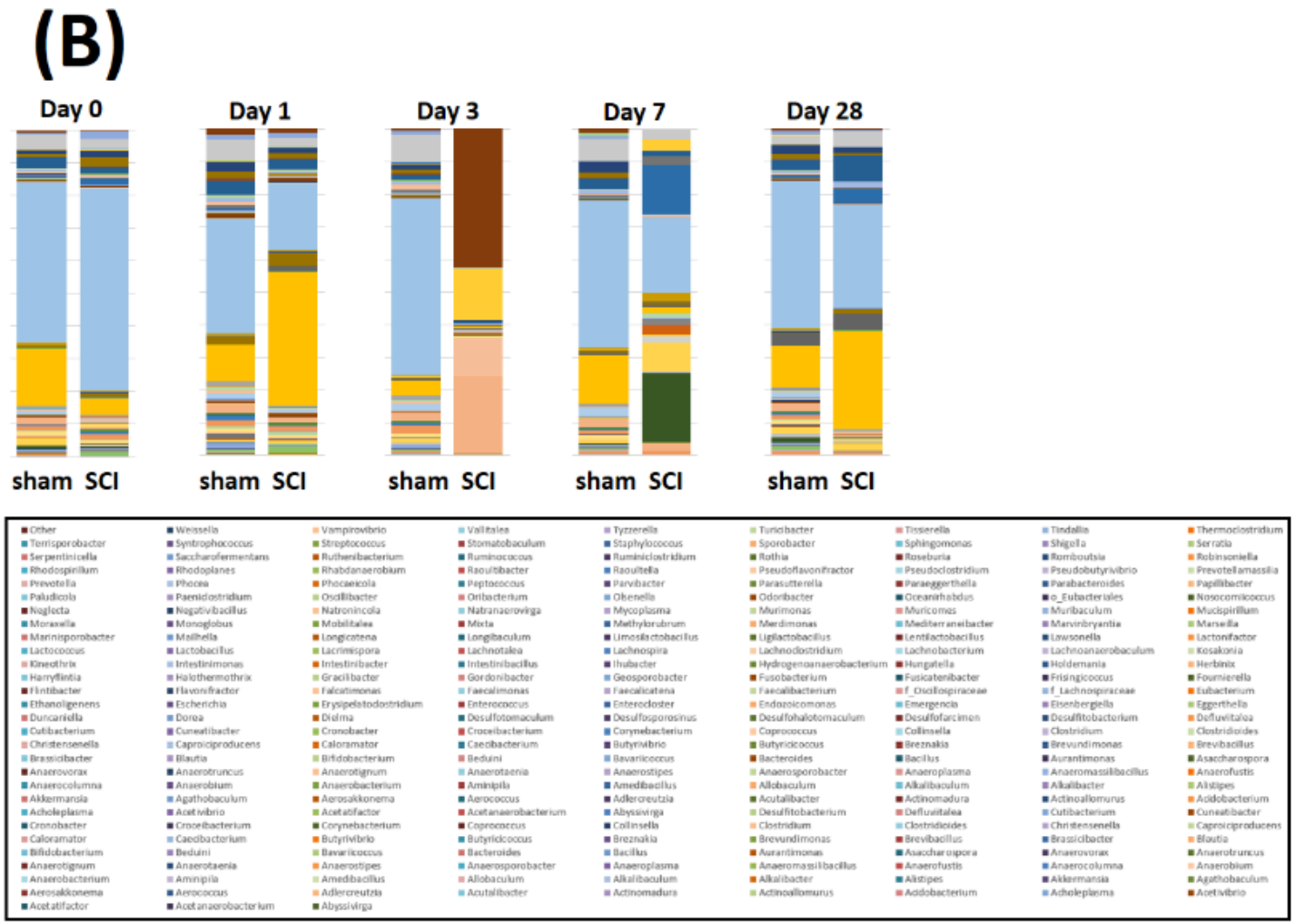
After SCI was developed, gut dysbiosis appeared at day 1 (Figure 2A). The relative portion of Bacteroidetes phylum was decreased at day 1 and almost disappeared at day 3. The relative abundance of Muribaculum genera of SCI group was 61.78%, 20.40%, 0.04%, 23.10%, and 31.70% at day 0, 1, 3, 7, and 28, respectively. The abundance of Firmicutes phylum, the main butyrate producing bacteria, was significantly reduced at day 3. The relative abundance of Proteobacteria was significantly increased at day 3 especially for Shigella genera (15.7% of relative abundance). The relative abundance of Shigella genera decreased to 3.51% at day 7 and to 0.15% at day 28. Total number of observed species was maintained at day 1 (Figure 2B). It showed marked decrease at day 3 and 7, and partly recovered at day 28.

On cystometry (Figure 3, 4, and Table 1), median ICI, BP, MP, and the development of NVC per cycle of SCI-control group was significantly different when compared to that of sham-control group (p-value = 0.001, <0.001, <0.001 and <0.001, respectively). Median voiding efficiency of SCI-control group was 65.2% (IQR: 63.6-72.4) while that of sham-

control group was 94.8% (IQR: 90.8-95.3) (p-value = 0.009).

In the bladder tissue of SCI-control group, both the detrusor muscle and total bladder were thickened compared to sham-control group. (Figure 5). Expression of Galectin-3 at bladder tissue on ELISA was markedly increased in SCI-control group compared with that of sham-control group (Figure 6).





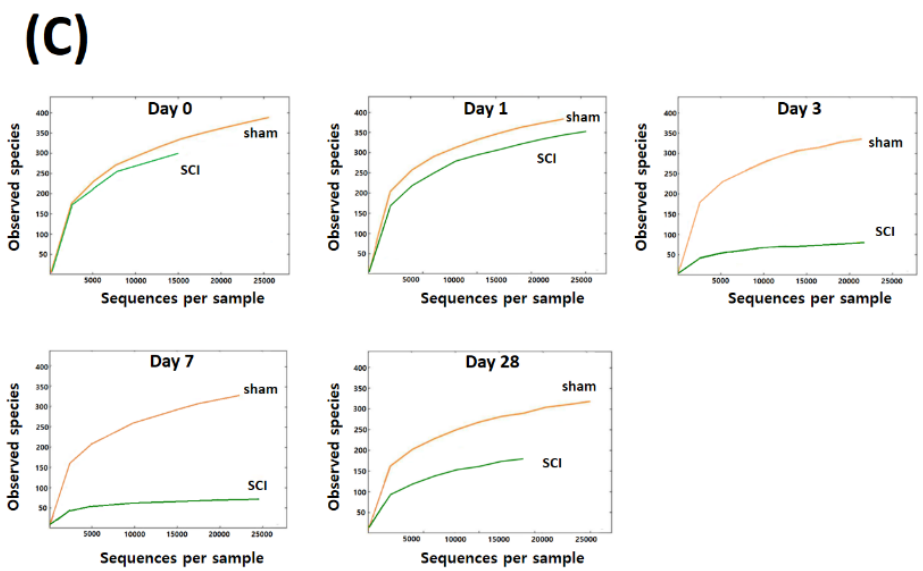


Figure 2. The change of the composition of gut microbiota after spinal cord injury (SCI). (A) After SCI, gut dysbiosis appeared at day 1. The relative abundance of Proteobacteria was significantly increased at day 3. (B) The relative abundance of Muribaculum genera of SCI group was 61.78%, 20.40%, 0.04%, 23.10%, and 31.70% at day 0, 1, 3, 7, and 28, respectively. The relative abundance of Shigella genera was 15.7% at day 3 and decreased to 3.51% at day 7 and to 0.15% at day 28. (C) Total number of observed species showed marked decrease at day 3 and 7. It showed partial recovery at day 28.

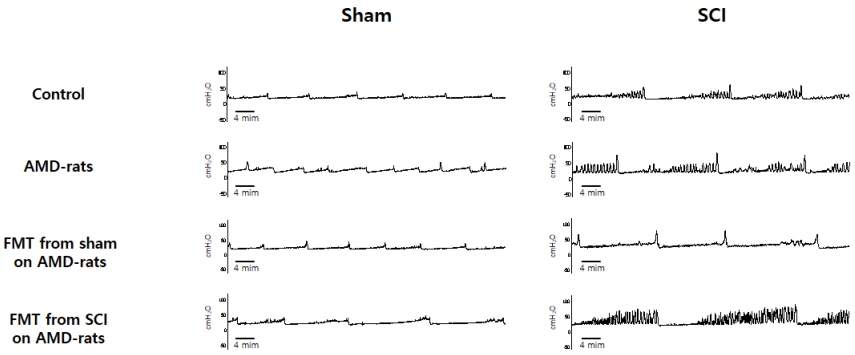


Figure 3. Cystometric patterns of each group. The development of non-voiding contraction (NVC) per cycle of SCI-control group was increased when compared to that of sham-control group. NVC per cycle was also increased in sham-AMD group compared with sham-control group. In FMT from SCI group, inter-contraction interval (ICI) was increased when compared with sham-AMD group. In AMD-rats with SCI, NVC was observed more frequently when compared to that of SCI-control's. After FMT from sham-rat, NVC was markedly decreased. ICI and NVC was significantly increased after FMT from SCI-rat. SCI, spinal cord injury; AMD, antibiotic induced microbiota depletion; FMT, fecal microbiota transplantation.

Table 1. Cystometric parameters

Group		ICI (sec)	BP (cmH_2O)	TP (cmH_2O)	MP (cmH_2O)	NVC (n)	Voiding efficiency (%)
Sham	Control	460.0 (300.0-540.0)	23.0 (22.1-23.7)	25.7 (23.3-27.8)	33.6 (31.1-37.2)	0.0 (0.0-1.0)	94.8 (90.8-95.3)
	AMD-no FMT	454.0 (333.0-517.0)	20.9 (18.0-22.8)	26.6 (23.2-30.2)	40.0 (35.2-48.9)	1.0 (0.0-3.3)	75.1 (73.8-80.5)
	AMD-FMT from sham	432.0 (347.0-587.0)	21.0 (19.7-22.4)	26.9 (25.4-29.2)	39.2 (36.4-41.1)	0.0 (0.0-2.0)	82.8 (69.1-90.5)
	AMD-FMT from SCI	550.0 (416.0-651.0)	20.9 (19.3-24.0)	28.8 (24.1-32.5)	42.7 (33.9-45.8)	2.0 (0.0-3.0)	44.0 (36.1-47.0)
SCI	Control	742.0 (454.0-954.0)	21.1 (18.9-22.3)	25.0 (22.1-26.2)	65.9 (56.7-73.2)	9.0 (8.0-11.0)	65.2 (63.6-72.4)
	AMD-no FMT	603.0 (219.0-762.0)	28.9 (18.0-33.7)	32.8 (23.0-40.3)	52.6 (46.8-67.1)	13.0 (12.0-15.0)	46.8 (31.2-56.4)
	AMD-FMT from sham	544.0 (336.0-746.0)	26.2 (20.8-30.1)	28.7 (24.5-37.4)	79.9 (69.4-89.8)	7.0 (3.0-8.0)	54.0 (49.9-58.0)
	AMD-FMT from SCI	784.0 (502.0-1040.0)	21.6 (19.6-24.6)	25.3 (22.0-28.6)	64.7 (51.5-71.4)	15.0 (13.0-18.0)	42.5 (27.5-46.9)

ICI, inter-contraction interval; BP, basal pressure; TP, threshold pressure; MP, micturition pressure; NVC, non-voiding contraction; AMD, antibiotic induced microbiota depletion; FMT, fecal microbiota transplantation; SCI, spinal cord injury

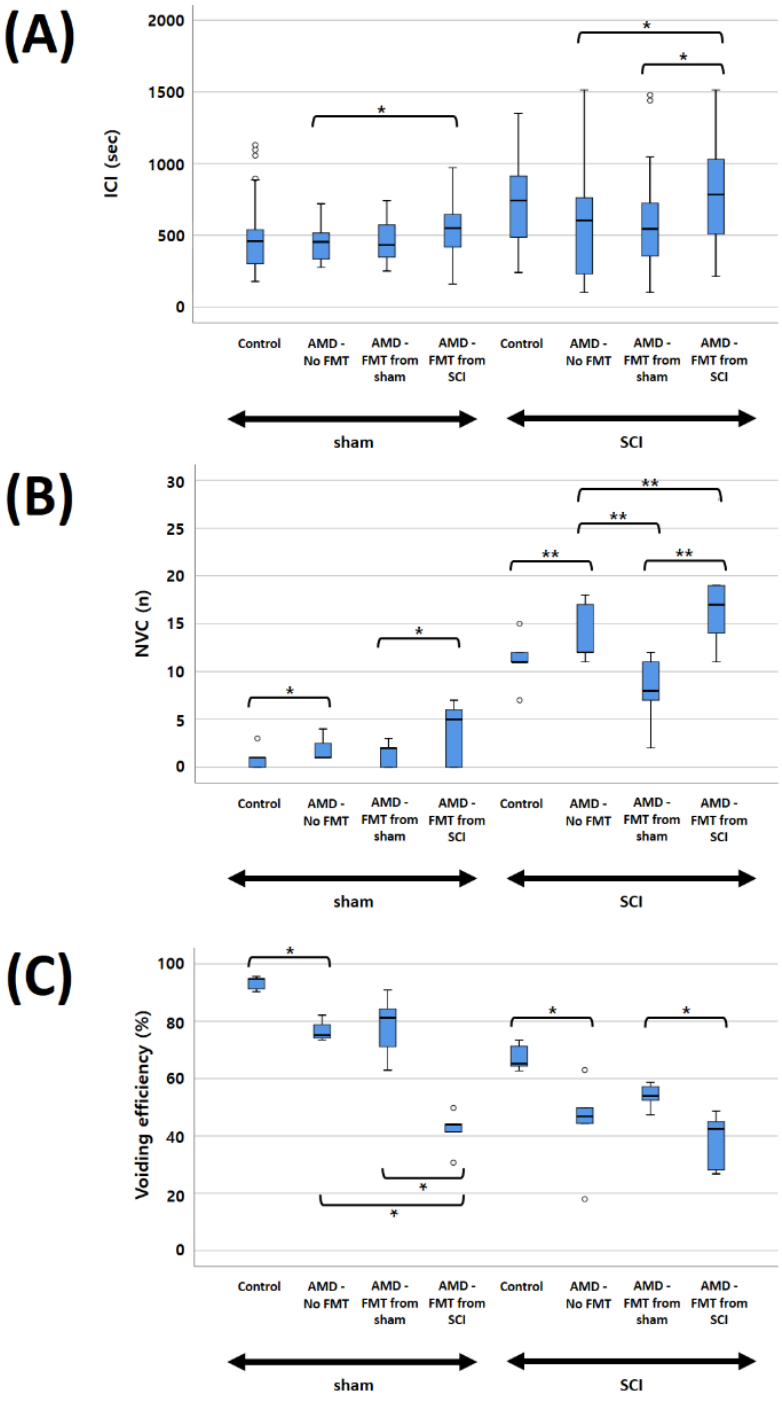


Figure 4. Cystometric parameters including inter-contraction interval (A), non-voiding contraction (B), and voiding efficiency (C). Median voiding efficiency of SCI-control group was significantly lower than that of sham-control group. NVC per cycle was increased and voiding efficiency was decreased in sham-AMD group compared with sham-control group. In FMT from SCI group, ICI was increased and voiding efficiency was decreased when compared with sham-AMD group. NVC and voiding efficiency were significantly different between FMT from sham group and FMT from SCI group. In AMD-rats with SCI, NVC was observed more frequently and voiding efficiency was decreased when compared to that of SCI-control's. After FMT from sham-rat, NVC was markedly decreased. ICI and NVC was significantly increased after FMT from SCI-rat. SCI, spinal cord injury; AMD, antibiotic induced microbiota depletion; FMT, fecal microbiota transplantation; ICI, inter-contraction interval; NVC, non-voiding contraction; * when p-value <0.05; ** when p-value <0.001.

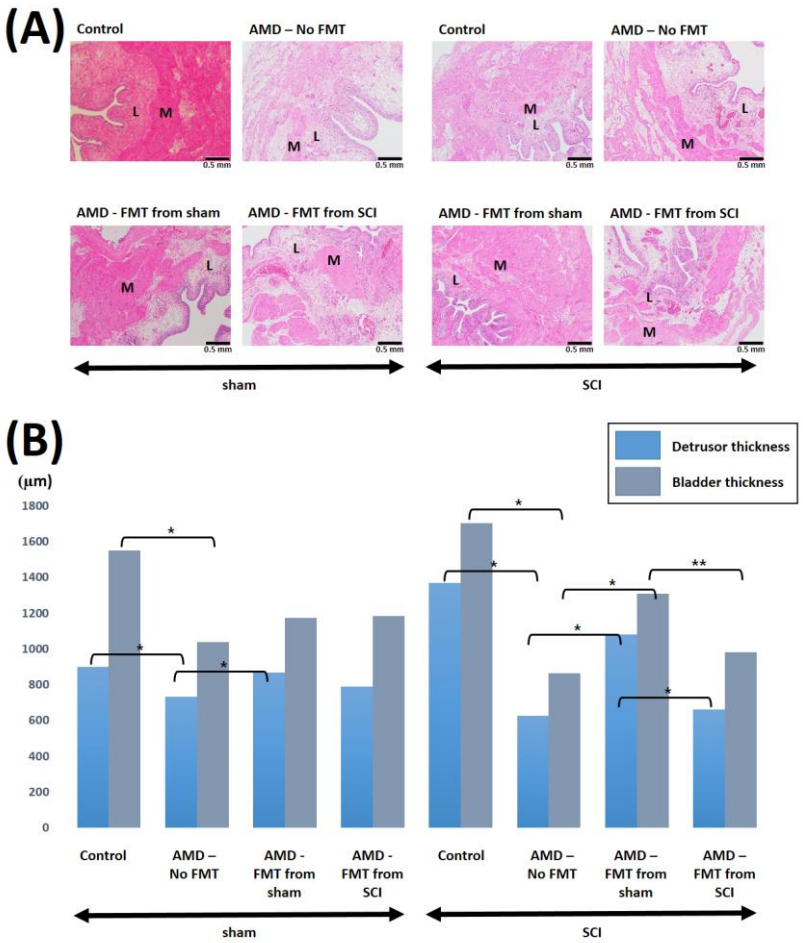


Figure 5. Hematoxylin and eosin stain of bladder tissue (10x magnification) (A) and bladder thickness (B). In the bladder tissue of SCI-control group, both the detrusor muscle and total bladder were thickened compared to sham-control group. In sham AMD group, the detrusor muscle layer and lamina propria layer were thinner than control group. In the sham AMD-FMT from sham group, compared with the sham AMD-No FMT group, angiogenesis was reduced and detrusor muscle thickening was observed. In the bladder tissue of SCI AMD group, the detrusor muscle layer was much thinner than control group (Figure 5). In the SCI FMT from sham group, compared with the SCI AMD group, both the detrusor muscle and bladder thickening was clearly observed. M, detrusor muscle; L, lamina propria; * when p-value <0.05; ** when p-value <0.001.

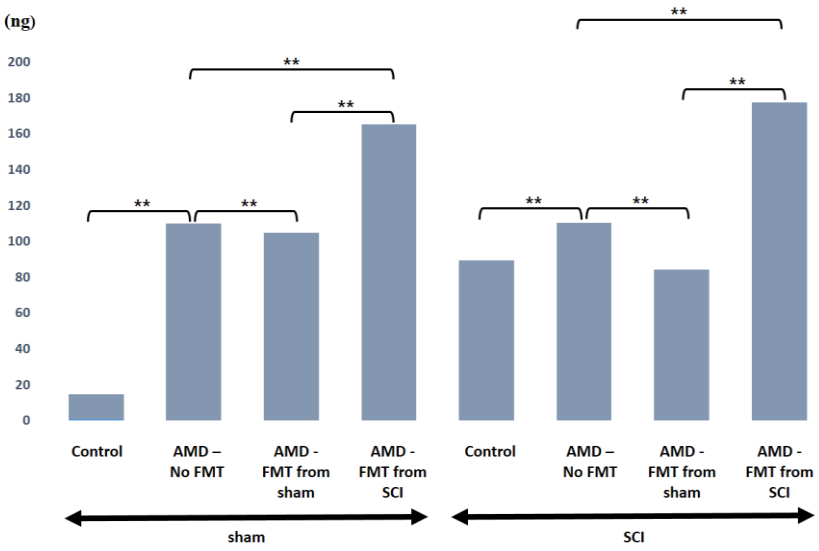


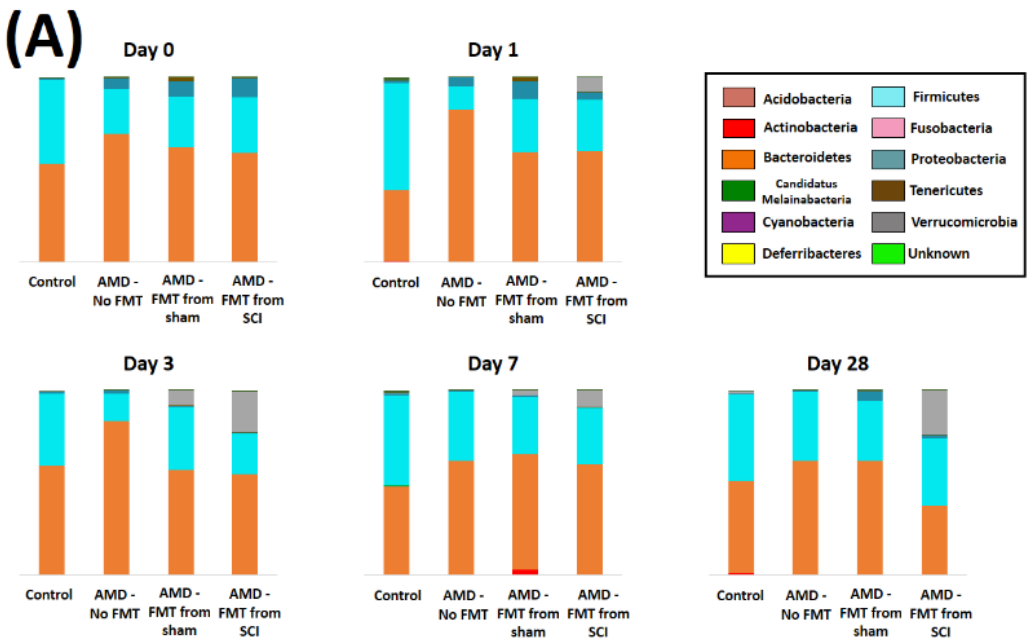
Figure 6. Expression of Galectin-3 per gram of bladder tissue on ELISA. Expression of Galectin-3 in bladder tissue on ELISA was markedly increased in SCI-control group compared with that of sham-control group. It is also significantly increased in sham AMD group compared with sham control group. In sham FMT from SCI group, the level of galectin-3 expression was markedly increased. It was also significantly increased in SCI AMD-rats, and a slight decrease and a significant increase was observed after FMT from sham-rat and SCI-rat, respectively. AMD, antibiotic induced microbiota depletion; FMT, fecal microbiota transplantation; SCI, spinal cord injury; * when p-value <0.05; ** when p-value <0.001.

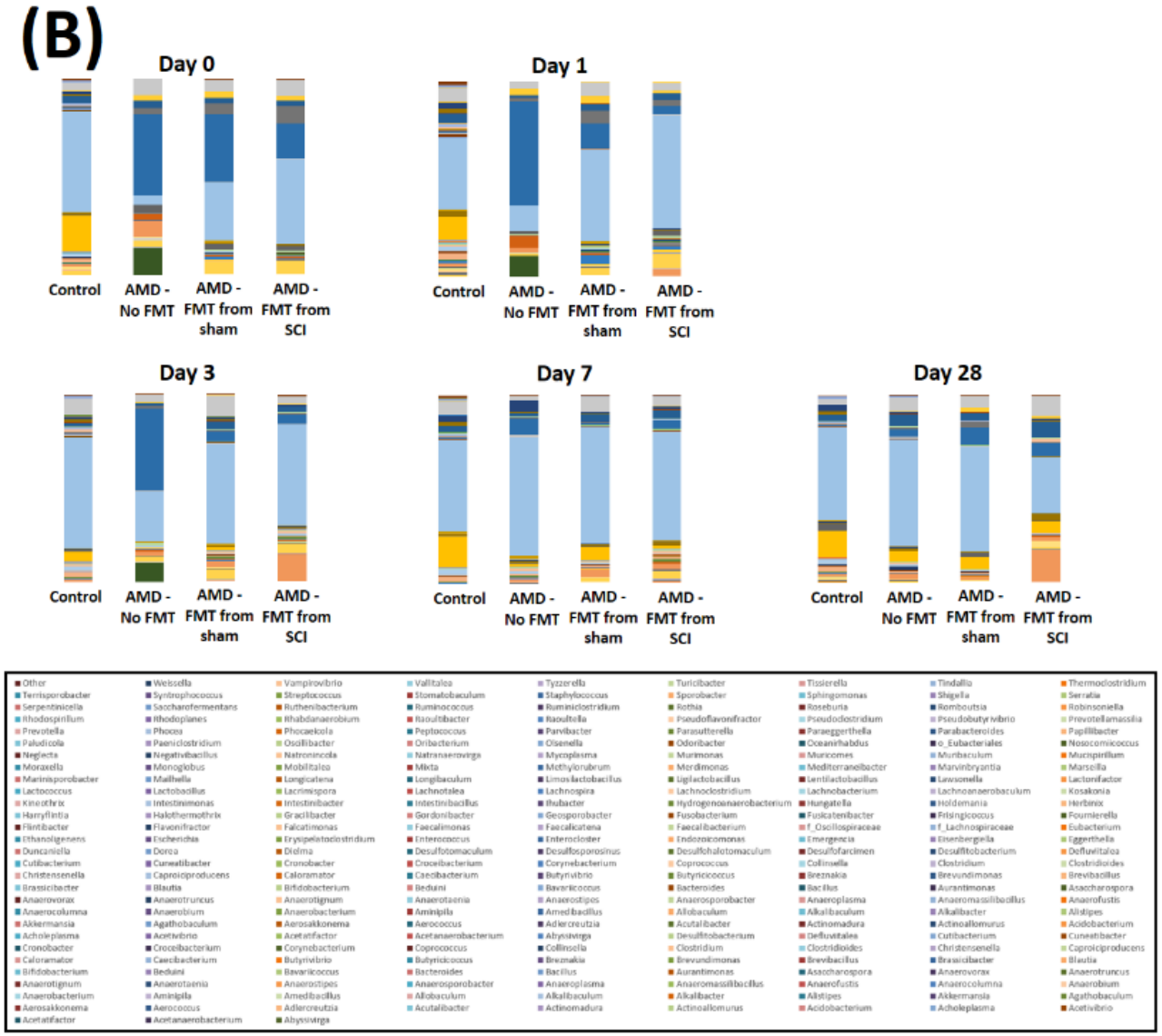
2. The bladder function in sham AMD-rats

After AMD was induced with 4 weeks use of antibiotics, the abundance of Firmicutes was decreased than control (Figure 7A). Parabacteroides genera was significantly increased and Muribaculum genera was decreased in AMD groups (Figure 7B). The fundamental difference between groups was the difference in the absolute number of species rather than the composition ratio of each microbiota (Figure 7C). On day 0 and day 1, the number of species in the AMD group was significantly decreased when compared with sham-control group. This increases with time and partly recovers on day 28.

On cystometry, NVC per cycle was increased and voiding efficiency was decreased in sham-AMD group compared with sham-control group (p-value = 0.004 and 0.014, respectively). (Figure 3, 4, and Table 1) In FMT from sham group, ICI, NVC and voiding efficiency was not significantly different from those of sham-AMD group. In FMT from SCI group, ICI was increased and voiding efficiency was decreased when compared with sham-AMD group (p-value = 0.039 and 0.014, respectively). NVC and voiding efficiency were significantly different between FMT from sham group and FMT from SCI group (p-value = 0.007, and 0.009, respectively).

In the bladder tissue of AMD group, the detrusor muscle layer and lamina propria layer were thinner than control group (Figure 5). In the FMT from sham group, compared with the AMD group, angiogenesis was reduced and detrusor muscle thickening was observed. Expression of galectin-3 at bladder tissue was significantly increased in AMD group compared with control group (Figure 6). It showed no difference with FMT from sham group. In FMT from SCI group, the level of galectin-3 expression was markedly increased.





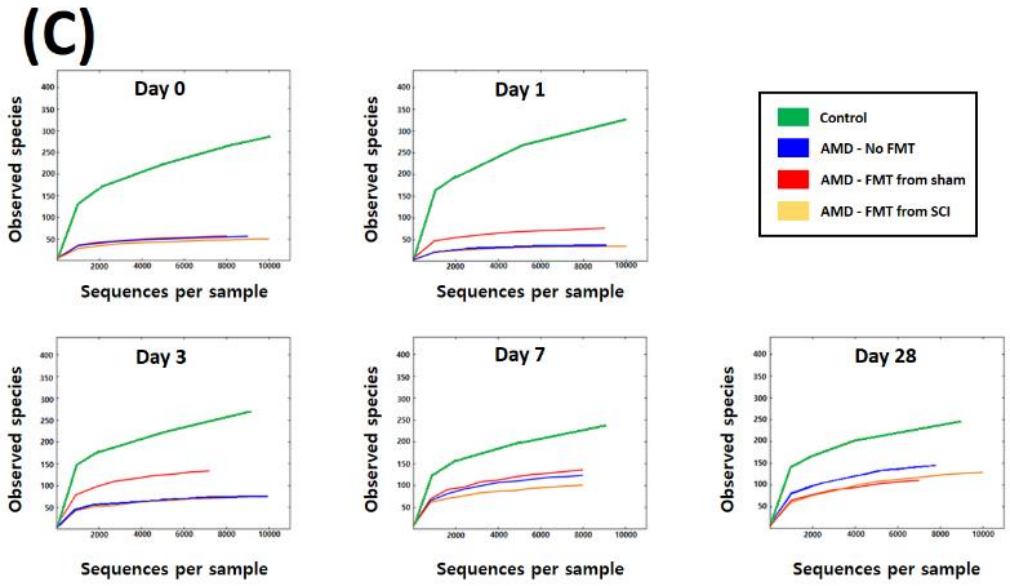


Figure 7. Gut microbiota change after antibiotic induced microbiota depletion (AMD). After AMD was induced, the abundance of Firmicutes was decreased than control (A). Parabacteroides genera was significantly increased and Muribaculum genera was decreased in AMD groups (B). The fundamental difference between groups was the difference in the absolute number of species rather than the composition ratio of each microbiota (C). On day 0 and day 1, the number of species in the AMD group was significantly decreased when compared with sham-control group. This increases with time and partly recovers on day 28.

3. The bladder function in SCI AMD-rats

In AMD-rats with SCI, median ICI showed no significant statistical difference with that of SCI-control's (Figure 3, 4, and Table 1). But NVC was observed more frequently (p-value <0.001) and voiding efficiency was decreased (p-value = 0.016). After FMT from sham-rat, NVC was markedly decreased (p-value <0.001). Meanwhile, ICI and NVC was significantly increased after FMT from SCI-rat (p-value = 0.028 and <0.001, respectively). In the bladder tissue of AMD group, the detrusor muscle layer and lamina propria layer

were much thinner than control group (Figure 5). In the FMT from sham group, compared with the AMD group, both the detrusor muscle and bladder thickening was clearly observed. In the FMT from SCI group, angiogenesis was increased without thickening of detrusor muscle. Galectin-3 was significantly increased in AMD-rats, and a slight decrease and a significant increase was observed after FMT from sham-rat and SCI-rat, respectively (Figure 6).

IV. DISCUSSION

To determine whether gut dysbiosis after SCI affects bladder function, AMD status was induced to minimize the effect of the pre-existing GM. Afterwards, FMT from sham-rat and SCI-rat showed different bladder function. In particular, AMD is related with the decrease of voiding efficiency and the fecal microbiota received from SCI-rat 3 days after the onset of SCI is considered to play a role in increasing ICI and inducing NVC. Then, several aspects should be considered as to how gut dysbiosis after SCI affects bladder function. First, it is possible that the GM will secrete a substance that directly affects bladder function. GM secretes short-chain fatty acids, which regulate the release of neurotransmitters. It is already known that about 90% of serotonin, which plays a pivotal role in various CNS diseases, is produced by GM.[15] In addition, it is known that acetylcholine and nitric oxide (NO), which are involved in the contraction and relaxation of the detrusor muscle, are also secreted by GM.[16] NO has been known to be associated with relaxation of the smooth muscles of the urethra and bladder.[17] Yu et al. reported that NO was effective in modulating bladder hyperactivity developed in pathological conditions in rats.[18] Sobko et al. reported that Lactobacilli belonging to the Firmicutes phylum produced abundant NO from nitrate in the intestine, and reported that Escherichia coli, Bacteroides thetaiotaomicron, and Clostridium difficile did not produce NO.[19] In particular, they reported that NO generation was observed in conventional rats, but not in germ-free rats. Our results are consistent with these studies in that they showed that NVC,

suggesting bladder hyperactivity, increased when NO-producing bacteria decreased in pathological conditions such as SCI or AMD.

As a next mechanism, it is possible that GM were involved in the neural crosstalk of the colon-to-bladder through the dorsal root ganglion. Majima et al. revealed the crosstalk of colon and bladder occurs in microglia of dorsal root ganglion at the S1 and L6 levels of rats.[20] Gut dysbiosis alters intestinal immunity and permeability and it affects the dorsal root ganglion via the colonic afferent nerve,[21] which may affect the bladder. For this, it may be helpful to study pathologic c-fiber expression in dorsal root ganglion after FMT through follow-up studies.

There is another possibility that GM colonized the bladder by a direct ascending route. It is known that normal flora exists in the bladder, and Firmicutes and Bacteroidetes are predominant as in the gut, but the relative abundance of Proteobacteria is known to be much higher than that in the gut. After transplanting of sham-rat's microbiota, it is possible that favorable bacteria such as Firmicutes colonized the bladder more than before and played a role in stabilizing the bladder. Similarly, after transplantation of SCI-rat microbiota, bladder dysbiosis or urinary tract infection may have occurred as the bladder abundance of pathologic bacteria increased. In order to know this clearly, it would be good to directly correlate urinary microbiota with GM, but it is not easy to prove it experimentally in rats. This is because the distribution of microbiota in the bladder is much less than that in the gut. Even when analyzing the urine of neurogenic bladder patients, there is a recent study that the analysis was available only in 70% of enrolled patients due to the insufficient number of urinary microbiota.[22] It is judged that it will be difficult to analyze this with a rat experiment with a small amount of urine.

Another mechanism is the involvement of GM in histological changes in the bladder. In our experiment, a decrease in the detrusor muscle was observed after AMD. In particular, the detrusor thickness was significantly thinner in SCI-AMD than in SCI-control. These findings are consistent with the findings of Roje et al.'s report.[23] They observed that the bladder weight decreased by 25-50% in both germ-free mice and AMD mice compared to

the control group. They suggested this was not a problem of development, but was judged to be an adaptive bladder change in the situation where GM is deficient. The absence of thickening of the detrusor muscle in AMD or SCI does not mean that the likelihood of fibrosis is reduced. In our experiment, Galectin-3 increased in both AMD and SCI. Galectin-3 is a member of the lectin family and is involved in cell apoptosis, adehesion, migration, angiogenesis, and inflammatory responses.[24] In particular, it is known that it can cause epithelial mesenchymal transition (EMT), scar formation and structural destruction of tissues, and it is known that it plays an important role in fibrosis of liver, blood vessels, kidneys, heart and lungs. Recently, it has been actively recognized as a biomarker related to myocardial fibrosis.[25] In our preliminary experiments conducted by SCI-rat, it was confirmed that Galectin-3 was closely related to bladder fibrosis through AKT and ERK pathways. In both SHAM and SCI, when Galectin-3 level was significantly increased when SCI FMT was performed, it means that GM of SCI has a role related to bladder fibrosis. However, there is an experimental limit to this, so caution is needed in the interpretation. In animal experiments to analyze the effects of donor microbiota by FMT, it is necessary to exclude the effects of existing GM of recipient. For this purpose, it is effective to use a germ-free animal model. However, in germ-free mice, the maturation of the central nervous system does not occur properly.[3] As the central nervous system has close relationship with bladder function, we use the microbiota depletion rat model induced by antibiotics instead. However, the possibility that long-term antibiotic use had a toxic effect on bladder tissue cannot be excluded. Therefore, it is difficult to clearly distinguish whether the histological changes occurring in our AMD model are due to gut dysbiosis or a side effect of antibiotic use. However, in the study of Roje, the same histologic changes occurred in the bladder either in AMD or germ-free model.[23] Also in our research, the difference in donor microbiota in the same AMD condition showed differences in bladder histology after 4 weeks. Considering these two aspects, it seems clear that GM are involved in histologic change of bladder.

It is well known that gut dysbiosis occurs in various diseases and conditions. In this study,

gut dysbiosis was clearly found in both AMD and SCI. However, there were differences in the appearance patterns. In AMD, the relative abundance of major bacteria was maintained, but the number of observed species decreased significantly. On the other hand, in SCI, the relative abundance of Firmicutes decreased significantly and Proteobacteria increased on the 3rd day. It is difficult to find a clear answer as to whether favorable bacteria have a good effect on the bladder or pathologic bacteria have a bad effect on the bladder. In AMD, even though the relative abundance or amount of pathologic bacteria did not increase, NVC was more observed than control. This suggests that the presence of sufficient favorable bacteria is helpful for normal contraction and stabilization of the bladder. On the other hand, when FMT from SCI-rat was performed, NVC occurred more, and ICI increased. At this time, the donor material was fecal microbiota on the 3rd day after SCI, and the relative abundance of Firmicutes or Bacteroidetes in GM was remarkably low, while that of Proteobacteria were significantly increased. Therefore, the change in cystometry after FMT from SCI-rat is judged to be due to an increase in the abundance of pathologic bacteria, rather than a slight increase in favorable bacteria. Considering these two aspects, the decrease in favorable bacteria or the increase in pathologic bacteria can both adversely affect bladder function.

Proteobacteria are well-known unfavorable phylum. Litvak et al. suggested that increased Proteobacteria could be a marker of epithelial dysfunction.[26] Intestinal inflammation increases epithelial oxygenation in the intestinal epithelium, which in turn decreases obligate anaerobes and may instead increase Proteobacteria. Therefore, Proteobacteria is recognized as a target for prevention or treatment of various diseases. An increase in the abundance of Proteobacteria is known to have an adverse effect also on the bladder. Magruder et al. reported that the hazard ratio of the future development of Escherichia bacteriuria was 2.8 when the relative gut abundance of Escherichia, a representative genera of Proteobacteria, increased by 1%.[27] It is also known that the relative abundance of urinary Proteobacteria is high in pathologic bladder conditions. The normally most abundant urinary microbiota phylum is Firmicutes,[28] Lane et al. reported that

Proteobacteria were the most abundant in neurogenic bladder.[22] Karstens et al. reported that the abundance of Proteobacteria was higher in women with urgency urinary incontinence than in controls.[29]

FMT is attracting attention as a new treatment method of various diseases by improving dysbiosis. In our study, FMT from sham was effectively decreased the development of NVC, but it did not increase the voiding efficiency. To increase the efficiency of FMT, the proper methods of FMT should be specified.

First of all, it is important at what point after the occurrence of SCI that FMT should be performed. In our study, gut dysbiosis began to appear 1 day after SCI, with the most severe findings on day 3. After 4 weeks, some recovery was observed. We performed FMT for 3 consecutive days starting 1 day after SCI. It showed significant change of cystometry after 4 weeks. However, our experiment could not predict the result if FMT was performed 4 weeks after SCI when GM has recovered to some extent. If further studies on this aspect are conducted, it may be helpful for future clinical application. Also, in our research, 3 consecutive days of FMT was performed without additional booster transplantation thereafter. On the other hand, Kelly et al. performed booster inoculation twice a week to maintain the effect after FMT in rats.[3] A follow-up study is needed to determine whether additional FMT booster can be helpful for bladder function improvement and if so, at what point in time booster should be administered.

To my knowledge, this is the first research to study gut dysbiosis and bladder function. This result suggest the possibility of treating voiding dysfunction by improving GM modification. Nevertheless, our study has some limitations. In this study, to make FMT effective, AMD-rat was made. However, actual SCI patients are neither germ free nor AMD at the time of SCI. Therefore, rather than performing FMT in AMD-rat as in our experiment, a follow-up study to make SCI-rat without AMD condition and confirm the effect of FMT is mandatory. Also, it is necessary to study whether the bladder function deteriorates when FMT from SCI-rat are performed to rats with normal GM conditions. Moreover, mode of action should be evaluated to confirm the mechanism how GM affects bladder function.

V. CONCLUSIONS

I suggest that the gut dysbiosis after SCI affects bladder function. AMD affects the incidence of NVC and decrease the voiding efficiency. FMT from SCI-rat are considered to be involved in the increase in ICI and the occurrence of NVC. FMT changes the composition of GM, and decrease the development of NVC in AMD-SCI rat model.

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ABSTRACT(IN KOREAN)

척수손상 백서에서 장내미생물의 불균형이 방광기능에 미치는 영향

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목적: 척수손상 후에는 장내미생물의 불균형이 발생한다. 신경인성방광은 척수손상의 주요 후유증이며, 장내미생물의 불균형은 배뇨장애와도 연관이 있다고 알려져 있다. 이에 척수손상 후 발생하는 장내미생물의 불균형이 방광기능에 영향을 미친다는 가설을 세웠다. 이를 검증하기 위해 척수손상 백서의 분변미생물을 항생제유도미생물고갈 백서에 이식한 후 방광기능을 평가하였다.

방법: 7주령 암컷 Sprague-Dawley백서를 실험에 이용하였다. 실험은 세 부분으로 나누어 진행되었다. 우선 척수손상백서에서 장내미생물의 불균형이 발생하는 시점을 16S-rRNA 분석을 통해 조사하였으며, 이 결과는 이후 실험에서의 분변미생물이식에 이용되었다. 두번째로는 항생제유도미생물고갈 백서에서 샴수술 후 장내미생물이 방광기능에 미치는 영향을 분석하였다. 항생제유도미생물고갈 백서는 항생제조합을 4주간 투여하여 유도하였다. 이들은 세그룹으로 나누어졌다; 한 그룹은 장내미생물이식을 시행하지 않았으며, 다른 한 그룹은 샴수술을 받은 백서의 장내미생물을 이식받았고, 남은 한 그룹은 척수손상을 받은 백서의 장내미생물을 이식받았다. 수술 4주 후 요역동학검사를 통하여 방광기능을 분석하였으며, 방광조직을 분석하였다. 세번째로는 항생제유도미생물고갈 백서에서 척수손상 후 장내미생물이

방광기능에 미치는 영향을 분석하였다. 이는 두번째 부분과 동일한 그룹화와 방법으로 진행되었다.

결과: 척수손상 후 장내미생물의 불균형은 손상 후 3일째에 가장 심했다. 요역동학검사에서 수축사이간격 (inter-contraction interval)은 크게 증가하였으며, 비배뇨수축 (non-voiding contraction)이 발생하였다. 항생제유도미생물고갈 백서에서 정상 백서로부터 분변미생물이식을 받은 경우와 척수손상 백서로부터 분변미생물이식을 받은 경우는 비배뇨수축과 배뇨효율이 유의하게 차이를 보였다. 세번째로 항생제유도미생물고갈 백서에서 척수손상 후에는 대조군 척수손상 백서에서보다 비배뇨수축이 빈번하게 관찰되었다. 정상 백서로부터 분변미생물이식술을 받은 후에는 비비뇨수축이 감소하였다. 정상 백서로부터 분변미생물이식을 받은 경우와 척수손상 백서로부터 분변미생물이식을 받은 경우는 역시 비배뇨수축과 배뇨효율이 유의하게 차이를 보였다.

결론: 항생제유도미생물고갈은 비배뇨수축의 발생과 배뇨효율의 감소에 영향을 준다. 척수손상 백서로부터의 장내미생물이식은 수축사이간격을 증가시키고 비배뇨수축을 일으키는 것으로 판단된다. 척수손상을 일으킨 항생제유도미생물고갈 백서에서 정상 백서의 장내미생물을 이식한 경우 비배뇨수축을 감소시킨다.

핵심되는 말 : 미생물; 분변미생물이식술; 척수손상; 요역동학검사; 신경인성방광

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