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Short Report

Anti-inflammatory Properties of Skeletal Muscle Protects Against Muscle Wasting in Colitis of Dextran Sulfate Sodium Model Mice

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Key words: DSS induced colitis, muscle wasting, LPS binding protein, tight junction, non-fiber diet

Abstract

This study aimed to clarify the influence of DSS-induced colitis on muscle in mice with a non-fiber diet (NFD). Eight-week-old male C57BL/6J mice were provided 0 to 1% of DSS in their drinking water to induce colitis. Disease activity index, plasma LPS-binding protein and mRNA expression of inflammatory cytokines in the colon increased in the 1% DSS group. However, within its own group, no changes in weight and mRNA expression of inflammatory cytokines and proteolysis markers were observed in gastrocnemius. By contrast, an increase in the mRNA expression of *Il*-10 and *Il*-10 / *Il*-6 ratios in muscle was noted. These results suggest that the 1% DSS administered in NFD-fed mice did not induce muscle wasting associated with colitis through the compensatory elevation of IL-10.

1. Introduction

Inflammatory bowel disease (IBD) is a chronic incurable condition of unknown etiology, and numerous studies are underway to elucidate its causes and determine its treatment. In particular, an understanding of muscle wasting with colitis is crucial as sarcopenia associated with IBD is associated with a poor prognosis[1-3]. Sarcopenia associated with IBD is induced by intestinal inflammation, which presents with symptoms such as abdominal pain, diarrhea, rectal bleeding, malabsorption of nutrients, inadequate dietary intake, and decrease in physical activity secondary to these symptoms[4,5]. Altogether, it is important to prevent sarcopenia associated with IBD and suppress intestinal inflammation and relapse. Recently, it has been postulated that IBD onset results from an exaggerated immune response in addition to pathogenic microorganisms and environmental factors that induce an altered gut microbiota, including lifestyle factors, such as dietary (ex. low fiber diet, high fat diet and so on) and/or exercise habits, in genetically susceptible hosts[6]. Similarly, the impact of gut dysbiosis on the development of muscle failure in patients with IBD has been reported[7].

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However, the prevention of muscle wasting associated with colitis and the impact of gut microbiota has not been investigated, and this experimental model has not yet been reported. One of the typical experimental animal colitis models is the administration of dextran sodium sulfate (DSS)[8]. This method is useful and simple as mice are fed with DSS polymers in drinking water for several days.

Therefore, this study aimed to clarify the influence of DSS-induced colitis on muscle in mice with a non-fiber diet (NFD), which induces gut dysbiosis and intestinal inflammation[9].

2. Materials and Methods

2.1 Animals

Eight-week-old male C57BL/6J mice (n = 24, CLEA Japan, Inc., Tokyo, Japan) were housed under a controlled environment (22 ° C ± 1 ° C, 12:12-h light-dark cycle) with four mice in each cage. These mice were allowed to acclimatize to the laboratory environment with free access to a control diet (MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water for a week. The experiment was approved by the Institutional Animal Care and Use Committee of Kawasaki University of Medical Welfare (no. 21-003).

2.2 Experimental design

The mice were randomly divided into three groups: 0DSS, 0.5DSS, and 1.0DSS. After 3 weeks of NFD (containing 10% fat, 20% protein, 70% carbohydrates, and 0 g dietary fiber; Dl2450Jpx1, Research Diets, New Brunswick, NJ) prefeeding, mice in those groups were provided 0%, 0.5% and 1% of DSS (molecular weight; 36,000, 50,000; MP Biomedicals, Solon, OH) in the drinking water to induce colitis for 5 days, followed by 5 days of sterile distilled water administration. This cycle was repeated three times[10]. The mice were monitored daily for body weight, rectal bleeding, and stool consistency to evaluate the disease activity index (DAI, Table 1[11,12]). The food intake and DSS solution were also monitored throughout the experiments to confirm that there was no difference among the experimental groups (data not shown).

To evaluate fecal pH before and after DSS treatment for the three instances, fresh fecal materials were collected from the mice. At the end of the experiment, blood was collected from the orbital vein under isoflurane anesthesia to measure the LPS-binding protein (LBP) in plasma, and then the mice were euthanized by cervical dislocation. Gastrocnemius muscle, epididymal fat, and cecum were collected. Colons were dissected, and their length was measured. Colon tissues were subjected to real-time quantitative polymerase chain reaction (RT-qPCR), and the myeloperoxidase (MPO) activity was analyzed.

Table 1 The criteria of disease activity index

Score	Weight loss	Stool consistency	Rectal bleeding
0	no loss	normal	no blood
1	1-5%	pasty and not adherent to the anus	
2	5-10%	pasty and mildly adherent to the anus	obvious blood in stool
3	10-15%	pasty and adherent to the anus	
4	>15%	watery	gross bleeding

2.3 Measurements of fecal pH, plasma LBP, and MPO activity in the colon

The feces were diluted five-fold (w/v) in distilled water and homogenized by the homogenizer pestle. After calibrating the pH meter (LAQUA twin-ph-11B, Horiba Ltd, Kyoto, Japan), the pH of these diluted

samples was measured. Plasma LBP concentration was measured using enzyme-linked immunoassay (ELISA) with commercially available kits (LBP, Mouse, ELISA Kit, Hycult Biotech Inc., Wayne, PA). MPO activity was measured in the homogenized colon with commercially available kits (OxiSelect™ Myeloperoxidase Chlorination Activity Assay Kit, Colorimetric, Cell Biolabs Inc., San Diego, CA).

2.4 RT-qPCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) and the RNeasy Mini Kit (Qiagen, Valencia, CA) and assessed for purity using the NanoDrop One (Thermo Fisher Scientific, Inc., Waltham, MA). RT-qPCR was performed using the reverse transcription kit (High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific, Inc., Waltham, MA) and the step one plus real-time PCR system (Thermo Fisher Scientific, Inc., Waltham, MA) with Fast SYBR Green PCR master Mix Kits (Thermo Fisher Scientific, Inc., Waltham, MA). The following amplification procedure was applied: initial denaturation for 10 min at 95 °C, followed by 40 cycles of denaturation for 3 s at 95 °C and annealing for 15 s at 60 °C. Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as the housekeeping gene for the internal control, and all data are represented relative to its expression (i.e., using standard curve methods). The specific PCR primer pair for the studied genes is shown in Table 2.

Table 2 Primer sequence in this sutdy

Primer		Sequence (5' - 3')	Primer		Sequence (5' - 3')
Gapdh	F	TGAAGCAGGCATCTGAGGG	*Il-6*	F	TAGTCCTTCCTACCCCAATTTCC
	R	CGAAGGTGGAAGAGTGGGAG		R	TTGGTCCTTAGCCACTCCTTC
Zo-1	F	CGCGGAGAGAGACAAGATGT	*Il-10*	F	CAGAGCCACATGCTCCTAGA
	R	AGCGTCACTGTGTGCTGTTC		R	TGTCCAGCTGGTCCTTTGTT
Occludin	F	TGGGAGGTTTCACAGAGGAC	*Foxo3*	F	CCTATGCCGACCTGATCACC
	R	CCAGGTAAGGGTACAGCAAG		R	ATTCTGAACGCGCATGAAGC
Claudin-3	F	GCAAGCAGACTGTGTGTCGT	*Atrogin-1*	F	AGTGAGGACCGGCTACTGTG
	R	TACCGTCACCACTACCAGCA		R	GATCAAACGCTTGCGAATCT
Claudin-4	F	GGCGTCTATGGGACTACAGG	*Murf-1*	F	TGACATCTACAAGCAGGAGTGC
	R	GAGCGCACAACTCAGGATG		R	TCGTCTTCGTGTTCCTTGC
Claudin-7	F	GCTAAGAAGCCCAACACCAG	*Lc3a*	F	AGCGCTACAAGGGTGAGAAG
	R	TGCAAAATGTACGACTCGGT		R	TCTTGGGAGGCGTAGACCAT
Muc2	F	ACAAGCTGGCAGTGGTGAA	*MyoD*	F	CCCAATGCGATTTATCAGGT
	R	TGTCCAGCTCCACCATGAG		R	GGAGTGCCTACGGTGGTG
Tnf-a	F	CCTCCCTCTCATCAGTTCTA	*Myogenin*	F	TTTGCTGCTCAGGCCAAT
	R	ACTTGGTGGTTTGCTACGAC		R	AGCTCCGAGCGATCTCCT
Il-1b	F	AAAAAAGCCTCGTGCTGTCG	*Pgc1-a*	F	GCGAACCTTAAGTGTGGAACTC
	R	GTCGTTGCTTGGTTCTCCTTG		R	GCCTTGAAAGGGTTATCTTGG

2.5 Statistical analysis

Statistical analyses were performed using free software R (R Development Core Team, 2021, version 4. 1. 2). First, the data were analyzed using the Shapiro-Wilk test to test for normality and Levene's test to evaluate for equal variance. Second, for comparisons of mean values between each group, normality and equal variance data were analyzed using one-way analysis of variance (ANOVA) followed by Bonferroni's

post hoc test. Non-normality and/or unequal variance data were analyzed using the Kruskal-Wallis test, and a post hoc test was performed using the Dunn test. Data of DAI and fecal pH were analyzed using repeated measures, ANOVA followed by Bonferroni's post hoc test. The correlation was analyzed using Spearman's rank correlation coefficient. P-values of <0.05 indicate statistical significance. The data of DAI and fecal pH are shown as the mean ± SEM, and other data are expressed by superimposing box plots on a bee swarm plot.

3. Results and discussion

3.1 Effect of DSS administration on the gut environment and body composition in mice

The DAI, calculated via the evaluation of weight loss, stool consistency, and presence of fecal blood, in the 1.0DSS group was significantly higher than that in the 0DSS group in the days 5-18 and 22-28 after DSS administration (Fig. 1a). In addition, colon length, commonly used as a morphological marker of the degree of inflammation[13], was also shorter in 1.0DSS mice compared to 0DSS mice, i.e., the effect of DSS administration was observed (Fig. 1b). However, no DSS-induced changes were observed in cecum content weight[14](Fig. 1c), which is an indirect indicator of changes in the intestinal microbiota, or in fecal pH (Fig. 1d), an index of changes in organic acids in the intestinal tract[15]. Administration of 1% DSS induced colitis without influencing the gut environment. At the end of the experiment, no differences were found in terms of body weight, epididymal fat mass, and gastrocnemius mass among the three groups (Fig. 1e-g). 1% DSS administration-induced colitis may not be associated with muscle wasting. Therefore, we first investigated the possibility of bacterial translocation via intestinal barrier damage secondary to DSS administration.

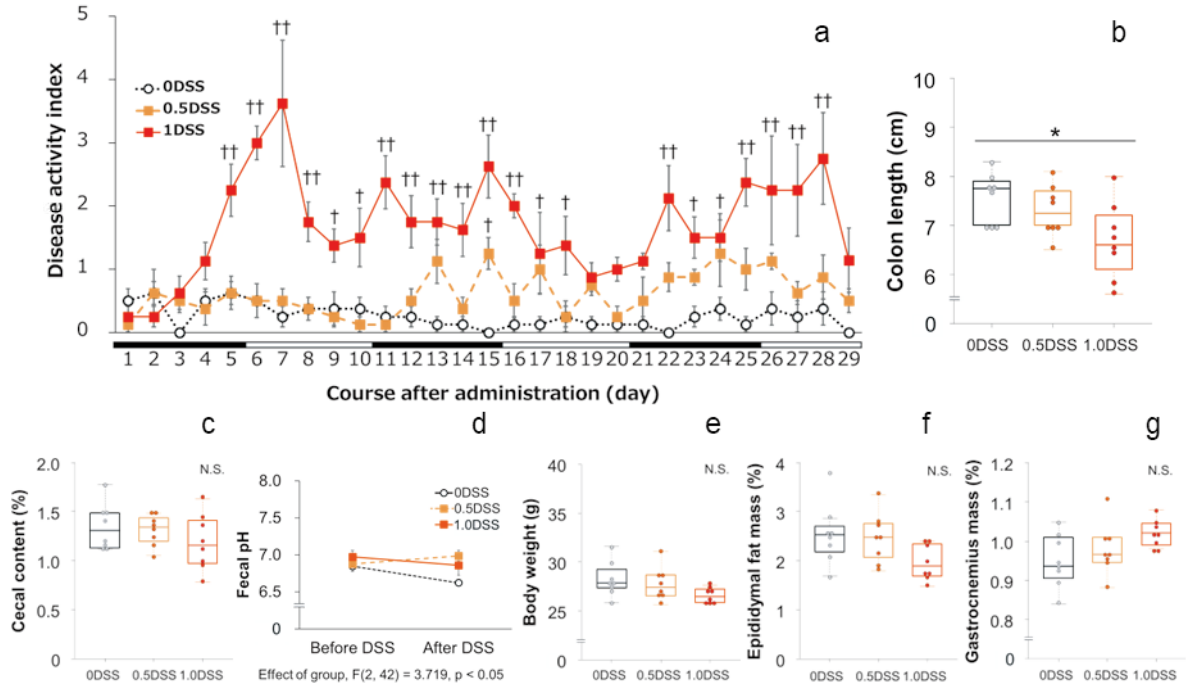

Figure 1 The effect of DSS on gut environment and body composition in mice

(a) change in DAI, (b) colon length, (c) cecal content weight and (d) change of fecal pH with the repeated DSS administration were evaluated as the effect of DSS treatment on gut environment. (e) body weight, (f) epididymal fat mass, and (g) gastrocnemius mass were shown as the body composition. The DAI was calculated as the sum of scores for weight loss, stool consistency and fecal blood presence[11,12](Table 1). The data were expressed as the mean ± S.E.M in (a) and (d), and others were expressed by superimposing box plots on a bee swarm plot. N.S.: no significant, †: p<0.05 vs. 0DSS and ††: p<0.01 vs. 0DSS, *p<0.05 and **p<0.01

3.2. Effect of DSS administration on intestinal barrier function

The plasma LBP, which is used as a major transporter of proinflammatory LPS[16], increased in the 1.0DSS group (Fig. 2). DSS-induced impairment of intestinal barrier function may increase the translocation of LPS to the circulatory system. Although, in this study, the MPO activity, as an index of neutrophil accumulation, was not increased by DSS administration, the mRNA expression of inflammatory cytokines (*Tnf-α*, *Il-1β*, and *Il-6*) in the colon was increased by DSS in a dose-dependent manner (Fig. 3b-d). Moreover, a decrease mRNA expression of tight junction molecules[17] (*Zo-1*, *occludin*, *claudin-3*, and *claudin-7*) and *Muc2* were observed, which are the structural components of the mucus layer[18] (Fig. 3g-j, l). These results indicate that 1% DSS administration induced intestinal inflammation and barrier function defects. In this study, there was an increase in the expression of the anti-inflammatory cytokine *Il-10*, but *the Il-10 / Il-6* ratio was not altered. (Fig. 3e, f). To investigate the involvement of these kinds of inflammation in skeletal muscle wasting, since colitis caused by DSS is associated with systemic inflammation, we assessed the gene expressions of each skeletal muscle inflammation and protein proteolysis using real-time qPCR.

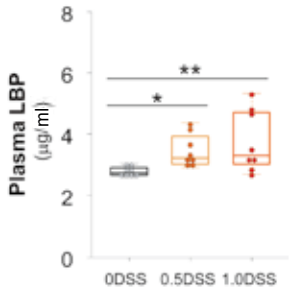

Figure 2 The effect of DSS on plasma LBP concentration

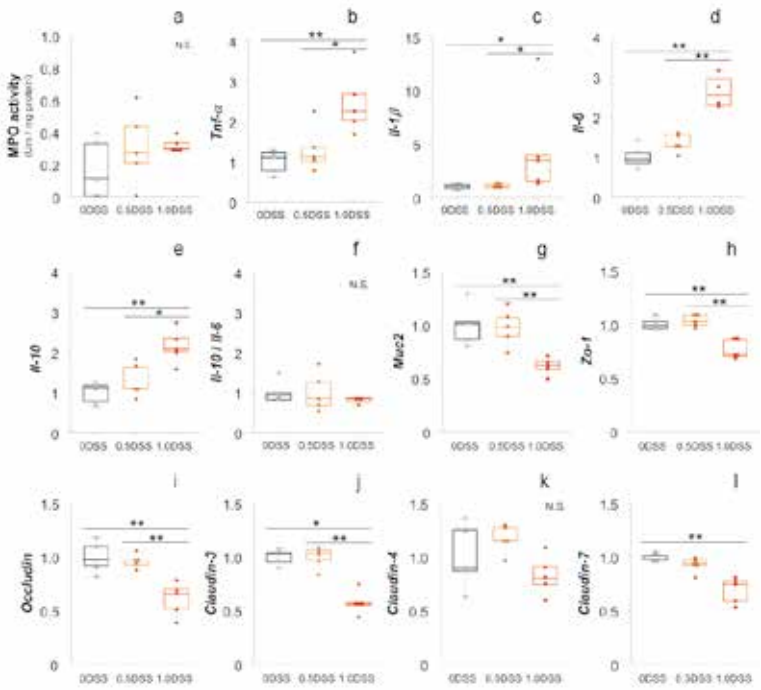

Figure 3 The effect of DSS on the mRNA expression and MPO activity in the colon

(a) MPO activity, (b) *Tnf-α*, (c) *Il-1β*, (d) *Il-6*, (e) *Il-10*, (f) *Il-10 / Il-6* ratio, (g) *Muc2*, (h) *Zo-1*, (i) *Occludin*, (j) *Claudin-3*, (k) *Claudin-4*, (l) *Claudin-7*. The values of (b-l) were expressed as relative values, with 0DSS being 1, *Gapdh* was used as the internal control. The data were expressed by superimposing box plots on a bee swarm plot. N.S.: not significant, *p<0.05 and **p<0.01

3.3 Anti-inflammation of skeletal muscle in DSS-induced colitis in mice

The 1% DSS-induced mild colitis did not change the gastrocnemius mass, including the mRNA expression of inflammatory cytokines (*Tnf-α* and *Il-6*), protein proteolysis markers[19] (*Atrogin-1*, *Murf-1*, *Lc3a*, and *Foxo3*) and transcription factors involved in muscle synthesis (*Pgc-1α*, *MyoD*, and *myogenin*)in the gastrocnemius (Fig. 1g, 4a, b, e-k). However, only the mRNA expression of *Il-10* and *Il-10 / Il-6* ratios were increased (Fig. 4c, d). Moreover, the mRNA expression of *Il-10* had a positive correlation with Gastrocnemius mass (Fig. 5, $p<0.05$). IL-10 is well known for its inhibitory function against proinflammatory cytokines, such as IFN-γ, IL-1β, TNF-α, and IL-6. For example, IL-1β is especially known to suppress IGF-1-induced myogenin expression and to cause muscle inflammation. Therefore, the present results suggest that IL-1β is downregulated by IL-10[20]. Since IL-10 is key to guiding the switch between M1 and M2 macrophages[21], a high *Il-10* expression induces anti-inflammatory, proregenerative, and protective effects against muscle wasting; however, the proinflammatory status is dominant in older people with muscle wasting such as sarcopenia[22]. These results indicate that the compensatory elevation of IL-10 protected the muscle against the proinflammatory status induced by mild colitis and increased plasma LBP.

In conclusion, our findings suggest that the administration of 1% DSS every 5 days with 3 cycles in NFD-fed mice may suppress the development of muscle wasting associated with colitis through the compensatory elevation of IL-10, although mild colitis was induced.

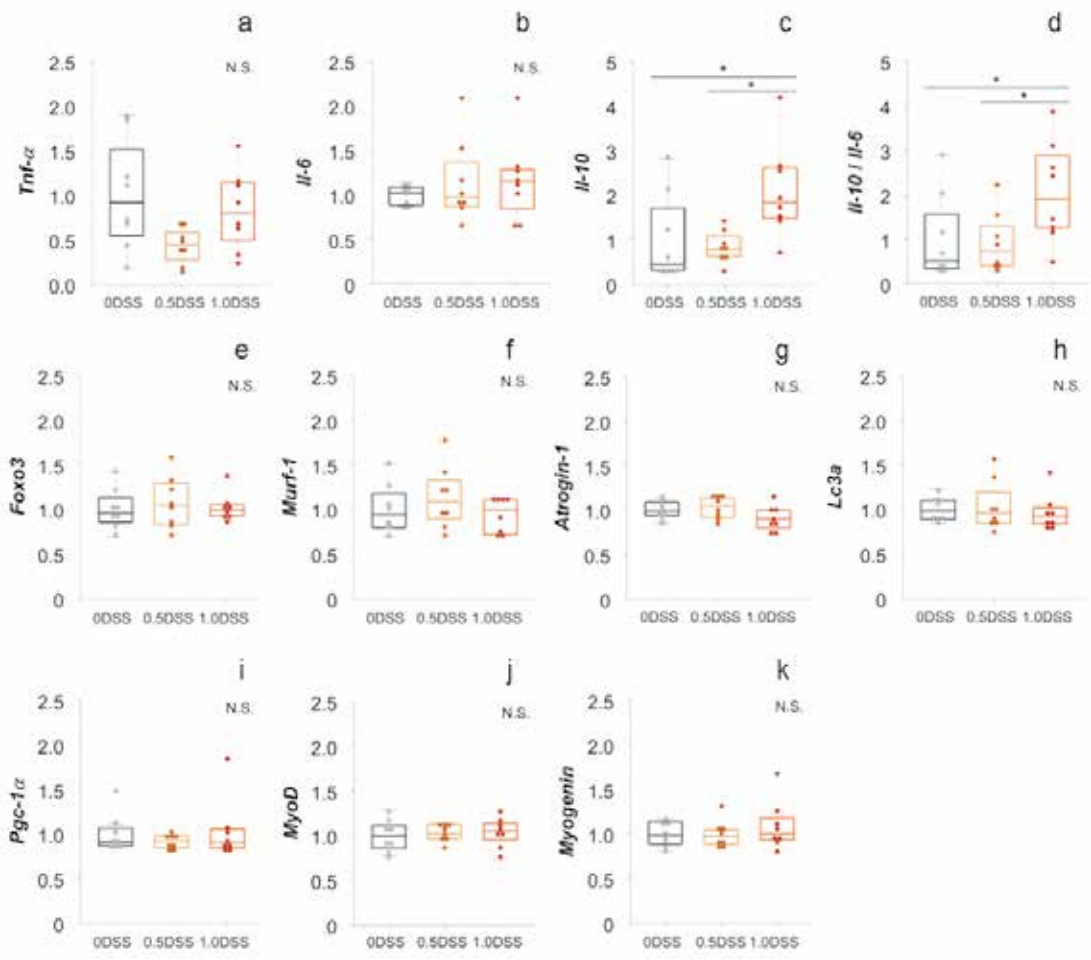

Figure 4 The effect of DSS on the mRNA expression in the gastrocnemius

(a) *Tnf-α*, (b) *Il-6*, (c) *Il-10*, (d) *Il-10 / Il-6* ratio, (e) *Foxo3*, (f) *Murf-1*, (g) *Atrogin-1*, (h) *Lc3a*, (i) *Pgc-1α*, (j) *MyoD*, (k) *Myogenin*. The values were expressed as relative values, with 0DSS being 1, *Gapdh* was used as the internal control. The data were expressed by superimposing box plots on a bee swarm plot. N.S.: not significant, *$p<0.05$

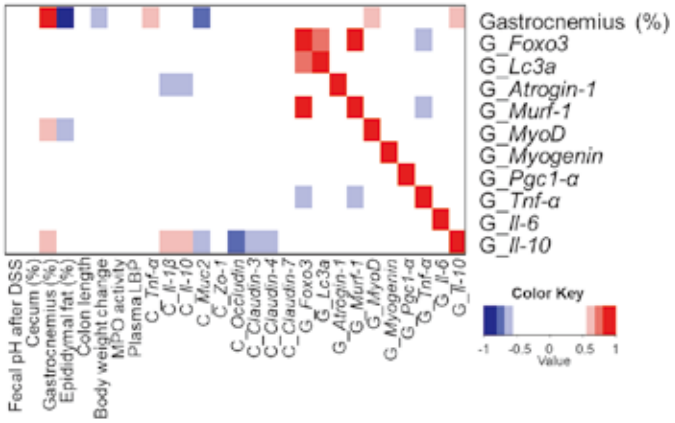

Figure 5 The heat map of correlation between gastrocnemius parameters and colitis related parameters in NFD-fed mice after DSS.

Darker red indicates a strongly positive correlation (towards r = 1), while darker blue indicates a strongly negative correlation (towards r = −1). The following ranges (represented by the white region) are not significant at p>0.05. G_: Gastrocnemius muscle, C_: Colon.

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Conflicts of interest

The authors have no conflicts of interest to declare associated with the present study.

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