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ORIGINAL ARTICLE

Gut microbiota and graft-versus-host disease in hematopoietic stem cell transplant patients

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ABSTRACT

Background and Objectives: Graft-versus-host disease (GvHD) frequently complicates hematopoietic stem cell transplantation (HSCT). Emerging evidence suggests a correlation between gut microbiota and GvHD risk. This study aims to elucidate the microbiota profiles in HSCT patients before and after transplantation and their association with GvHD.
Materials and Methods: This study, conducted from December 2022 to December 2023, involved the collection of 15 stool samples from HSCT patients. Bacterial content was quantified using real-time PCR, while interleukin-6 levels were assessed via ELISA.
Results: Among the 15 participants (8 male, 7 female), 9 underwent allogeneic HSCT (allo-HSCT) and 6 received autologous HSCT. In the aGvHD group, there was a significant reduction in the abundance of *Bacteroides* and *Bifidobacterium* compared to those without aGvHD. Additionally, declines were observed in *Clostridium* and *Firmicutes* populations. The genus *Blautia* also showed reduced prevalence in the aGvHD group, whereas no significant differences were noted in the uncomplicated group. ELISA analysis revealed that interleukin-6 levels remained within the normal range (30-960 pg/ml) with no significant elevation in the aGvHD group.
Conclusion: The study highlights a notable association between alterations in gut microbiota, specifically reductions in certain bacterial populations and the development of aGvHD following allo-HSCT.

Keywords: Gut microbiota; Hematopoietic stem cell transplantation; Graft-versus-host disease; Interleukin-6; Real-time polymerase chain reaction; Enzyme-linked immunosorbent assay

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INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a highly effective immunotherapeutic modality that is widely acknowledged for its efficacy in treating a range of both malignant and non-malignant hematological conditions (1). Despite recent advances as a treatment option, patients are severely immunocompromised after HSCT. Meanwhile, These patients remain vulnerable to viral infections as well as graft-versus-host disease (GvHD), which constitutes a significant factor contributing to mortality (2).

GvHD is an important and common complication of allogeneic HSCT and can cause complications in 30-70% of allogeneic HSCT recipients and 15% post-transplant mortality (3, 4). GvHD can be classified into two disease groups: acute (symptoms appearing before 100 days after allo-HSCT) or chronic (symptoms appearing > 100 days after allo-HSCT) (5). GvHD affects multiple organs in the body, particularly the gastrointestinal tract, liver, skin, eyes, and lungs. This condition is characterized by the damage to host tissues and organs, primarily mediated by immune cells, especially donor T cells. These T cells recognize and respond to major and minor histocompatibility alloantigens presented by antigen-presenting cells (3, 6). Research involving both animal models and humans has demonstrated a significant correlation between gut microbiota and the risk of GvHD; however, this association remains ambiguous (3). Modifying gut microbiota offers a promising strategy for preventing or treating this common condition.

The gut microbiota comprises various microorganisms that inhabit the gastrointestinal (GI) tract, and this microbial community in an adult human consists of 10^{13} to 10^{14} CFU/ml microorganisms (7, 8). The human intestinal microbiota is composed of a wide variety of microorganisms, including bacteria, archaea, fungi, protozoa, and viruses, with bacteria representing the predominant group among these organisms (9). In general, four phyla of bacteria including *Firmicutes* and *Bacteroidetes* (approximately 90) and *Proteobacteria* and *Actinomycetes* (with lower abundance) dominate the human gut microbiota. Recent studies indicate a correlation between specific arrangements of microbial communities and various health outcomes, including mortality rates, the recurrence of diseases, susceptibility to infections, and the incidence of GVHD (10, 11). On the other hand, the microbiota may directly influence the risk of infection and shape or disrupt the microbial communities in the patients.

The release of inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6), during the conditioning regimen plays a critical role in T cell activation and substantially contributes to the development of graft-versus-host disease (GvHD) following transplantation (12). This study aims to investigate the microbiota in HSCT patients before and after transplantation and its association with individuals experiencing GvHD.

MATERIALS AND METHODS

Patients and specimens. This research was conducted from December 2022 to December 2023. 15 stool samples of HSCT patients were collected from the Bone Marrow Transplant Center of Imam Reza Hospital, Kermanshah, Iran. The specimens were appropriately labeled and preserved at -70°C until the process of DNA extraction. Also, blood samples were taken from patients one week after transplantation and their serum was stored at -70°C. Stool samples were collected from these patients on three occasions (before transplantation, one week, and one month after transplantation). The reason for choosing one week and one month of sampling after transplantation was the initiation of the inflammatory phase, which typically occurs between three to seven days post-allo-HSCT transplantation, as well as the subsequent emergence of GvHD complications (13). The age range of patients with HSCT was between 21 to 65 years.

The inclusion and exclusion criteria of this study are allo-HSCT and auto-HSCT patients, respectively, because GvHD occurred only in patients who underwent allo-HSCT.

DNA extraction. DNA was extracted from stool samples using the FavorGen extraction kit (Pintung, Taiwan) according to the manufacturer's instructions and stored at -20°C. The evaluation of DNA quality was performed using an ultraviolet spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE, USA), measuring at wavelengths of 260 and 280 nanometers.

Real-time PCR. The Light Cycler system (Roche)

was utilized to perform the real-time PCR assay. Amplification reactions were performed in a volume of 20 µl containing 5 µl of DNA (20 ng/µl) template, 0.5 µL each for forward and reverse primers (10 pmol/µl), 10 µl of SYBR Green PCR Master Mix (Ampliqon UK) and 4 µl of sterile double distilled water. The primer sequences in this study were developed using Oligo 7 software, (14) (Molecular Biology Insights, Inc., Cascade, CO, USA) and the OligoAnalyzer online server (https://www.idtdna.com/pages/tools/oligoanalyzer). These primers were subsequently synthesized by Metabion in Germany. Furthermore, the alignment of the primers was conducted through multiple sequence alignment (MSA) methods using Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/). The sequences of the designed primers are detailed in Table 1. The real-time PCR temperature program is outlined in Table 2. The real-time PCR assay was performed in duplicate to ensure accuracy.

Enzyme-linked immunosorbent assay (ELISA). Enzyme-linked immunosorbent assay (ELISA) is widely a significant method for the serodiagnosis of infectious diseases (15). To measure Interleukin 6 (IL-6) of patients after transplantation, their serum samples, which were centrifuged after 10 minutes at 4°C and 3000 r/min, were used to perform the ELISA test. In this study, a kit (Zellbio, Germany) was used to detect IL-6 in serum samples. Briefly, combinations (40 µl samples + 10 µl anti-IL-6) and 50 µl standard with 50 µl streptavidin-HRP in 96-well flat plates, were incubated at 37°C for 60 minutes. Then the plate was washed five times with 300 µl of diluted wash buffer. 100 µl of chromogen solution was added and incubated at room temperature for 10 to 20 minutes. Subsequently, 50 µl of stop solution was added and its OD was measured at wavelength 450 nm.

Statistical analysis. The data were analyzed by IBM SPSS statistics® Version 25 (IBM Corp, Armonk, NY, USA). Friedman tests were used to estimate the significant difference between group comparisons. P value ≤ 0.05 was considered statistically significant in comparative data.

Table 1. Primers were designed in this study.

Gene	Primer sequence (5' to 3')		Product length	Reference
	Forward	Reverse	(bp)	
Firmicutes	CTGATGGAGCAACGCCGCGT	ACACCTAGTACTCATCGTTT	429	This study
Bacteroides	GAGAGGAAGGTCCCCCAC	CGCTACTTGGCTGGTTCAG	106	This study
Bifidobacterium	TCGCGTCYGGTGTGAAAG	CCACATCCAGCRTCCAC	243	This study
Clostridium	GCACAAGCAGTGGAGT	CTTCCTCCGTTTTGTCAA	239	This study
Escherichia coli	GTTAATACCTTTGCTCATTGA	ACCAGGGTATCTAATCCTGTT	320	This study
Actinobacteria	GCGTCCTATCAGCTTGTT	CCGCCTACGAGCTCTTTACGC	330	This study
Fusobacterium	GATCCAGCAATTCTGTGTGC	CGAATTTCACCTCTACACTTGT	292	This study
Blautia	GTGAAGGAAGAAGTATCTCGG	TTGGTAAGGTTCTTCGCGTT	412	This study
Lactobacillus	CGATGAGTGCTAGGTGTTGGA	CAAGATGTCAAGACCTGGTAAG	235	This study

Table 2. Real-time PCR temperature program in this study.

Gene	Preliminary step (min)	Denaturation	Annealing	Cycles
Actinobacteria	95°C for 7 min		60°C for 55s	40
Bacteroides	95°C for 5 min		63°C for 55s	40
Bifidobacterium	95°C for 7 min		58°C for 55s	40
Blautia	95°C for 5 min		55°C for 45s	40
Clostridium	95°C for 5 min	95°C for 10s	55°C for 60s	40
Escherichia coli	95°C for 5 min		57°C for 55s	40
Firmicutes	95°C for 7 min		64°C for 55s	40
Fusobacterium	95°C for 7 min		55°C for 60s	40
Lactobacillus	95°C for 5 min		63°C for 45s	40

Table 3. Comparison of the mean number of bacteria in the group without GvHD.

Bacterial species	Mean copy number Before transplantation	Mean copy number 1 week after transplantation	Mean copy number 1 month after transplantation	P value Before transplantation-1 week after transplantation	P value Before transplantation-1 month after transplantation	P value 1 month after transplantation-1 week after transplantation
Bacteroides	1.6E10 ± 3.5E11	4.2E9 ± 8.8E10	1.6E10 ± 2.2E11	Statistically equal	Statistically equal	Statistically equal
Bifidobacterium	6.6E8 ± 32E9	4.3E8 ± 8.7E8	4.2E9 ± 8.7E10	Statistically equal	Statistically equal	Statistically equal
Closteridium	4.2E8 ± 3.6E9	2.5E7 ± 4.1E8	1.4E8 ± 3E9	0.027	0.011	0.752
Enterobacterial	1.4E10 ± 3.1E11	2.4E7 ± 4.2E8	3.2E10 ± 2.5E11	0.635	0.058	0.018
Actinobacteria	2E6 ± 2.7E7	4.4E5 ± 2E6	2E8 ± 2E9	0.114	0.114	0.002
Fusobacterium	4.3E7 ± 5E8	9E5 ± 6E6	1.2E10 ± 1.5E11	0.114	0.114	0.002
Blautia	1.9E6 ± 2.2E7	7.8E5 ± 7.2E6	1.1E6 ± 4.7E6	Statistically equal	Statistically equal	Statistically equal
Lactobacillus	4.3E7 ± 8.7E8	1.4E6 ± 1E7	1E8 ± 2.2E9	0.058	0.343	0.004
Firmicutes	1.8E10 ± 1.6E11	2.5E6 ± 2.1E7	2.2E10 ± 1.3E11	0.040	0.527	0.007

RESULTS

From a total of 15 (8 male and 7 female) HSCT patients, 9 and 6 allo-HSCT and Auto-HSCT patients were included in our study, respectively. Among allo-HSCT patients, 4 patients were full-matched and 5 patients were Haplo-HSCT. On the other hand, out of a total of 15 patients, 40%, 13.33%, and 46.67% had multiple myeloma, aplastic anemia and acute myeloid leukemia (AML), respectively. GvHD occurred only in patients who underwent allo-HSCT. In other words, out of 9 allo-HSCT patients, 4 (44.44%) patients became GvHD.

Evaluation of ELISA test. The purpose of the ELISA test was to determine whether interleukin-6 could be useful as a prognostic factor for GvHD. The results of this study showed that all our samples were reported within the normal range (30-960 pg/ml) and no significant increase in interleukin-6 levels was observed in GvHD in HSCT patients.

Prevalence of bacteria in allo-HSCT patients. The alterations in microbiota diversity were investigated in both the GvHD and non-GvHD groups (Tables 3 and 4; Figs. 1 and 2). In the evaluation of the genus *Bacteroides* in the GvHD group, the patients experienced a reduction in the bacterial count between the before the transplant and the intervals of one week and one month following the transplant. (P-value≤0.05). On the other hand, in the non-GvHD group, no significant difference was observed before and after transplantation.

In *Bifidobacterium* in the GvHD group, the number of bacteria decreased one week and one month after transplantation compared to patients before transplantation (P-value=0.013). In the non-GvHD group, no significant difference was observed before and after transplantation.

Our research indicated a significant difference in the quantity of *Clostridium* bacteria in the GvHD group between before transplantation and one month after transplantation (P-value=≤0.05) and the number of bacteria decreased in this group. Also, in the group without GvHD, there was a decrease in the number of bacteria between one week and one month after transplantation, and there was a statistically significant difference (P-value≤0.05).

While no notable correlation was observed regarding *Enterobacterials* in the GvHD group before and after transplantation, the group without GvHD exhibited a significant increase in bacterial levels one-month post-transplantation when compared to one-week post-transplantation (P-value=0.018).

Actinobacteria increased in the group without GvHD between one month and one week after transplantation, but a significant difference (P-value=0.022) was observed in the group with GvHD.

Fusobacterium was significantly different in both GvHD and non-GvHD groups between one week and one month after transplantation. In addition, *Blautia* in the group without GvHD, there was no significant difference in *Blautia* levels observed between the pre-transplant and post-transplant stages, but in the GvHD group, it increased between before and one week after transplantation (P-value=0.034).

According to (Tables 3 and 4), there was a significant difference in the levels of *Lactobacillus* and *Firmicutes* in the group that did not experience GvHD, with

Table 4. Comparison of the mean number of bacteria in the GvHD group.

Bacterial species	Mean copy number Before transplantation	Mean copy number 1 week after transplantation	Mean copy number 1 month after transplantation	P value Before transplantation-1 week after transplantation	P value Before transplantation-1 month after transplantation	P value 1 month after transplantation-1 week after transplantation
Bacteroides	4.2E8 ± 2.8E9	2.2E6 ± 2.5E7	5.7E6 ± 2.8E7	0.005	0.157	0.157
Bifidobacterium	4.2E8 ± 4E9	2.5E7 ± 4.9E8	0	0.480	0.013	0.077
Clostridium	1.2E8 ± 2E9	35E5 ± 1E6	1.5E5 ± 1E6	0.157	0.005	0.157
Enterobacterial	1.7E8 ± 2E9	2E8 ± 4E9	2.2E10 ± 2.1E11	Statistically equal	Statistically equal	Statistically equal
Actinobacteria	2E7 ± 4E8	8E5 ± 9E6	1E8 ± 2E9	0.112	0.289	0.008
Fusobacterium	4E6 ± 2.4E7	7.5E5 ± 1.7E6	3.5E10 ± 2.3E11	0.157	0.157	0.005
Blautia	6E5 ± 2.4E6	0	0	0.034	0.034	1.000
Lactobacillus	3.2E6 ± 2E7	7E5 ± 3.1E6	2.6E7 ± 4.9E8	0.034	1.000	0.034
Firmicutes	3.2E10 ± 9.5E10	1.1E6 ± 5.5E6	2.5E10 ± 1E11	0.022	0.724	0.052

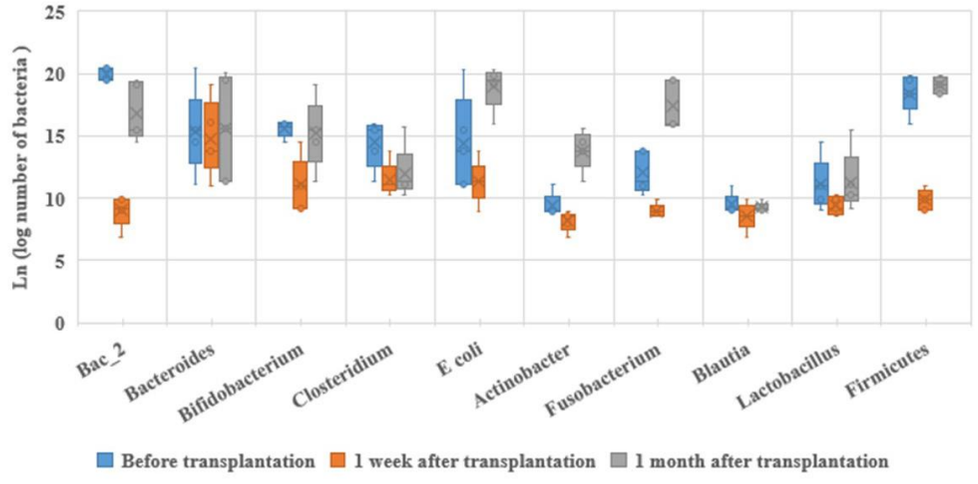

Fig. 1. Comparison of the log number of bacteria in the group without GvHD.

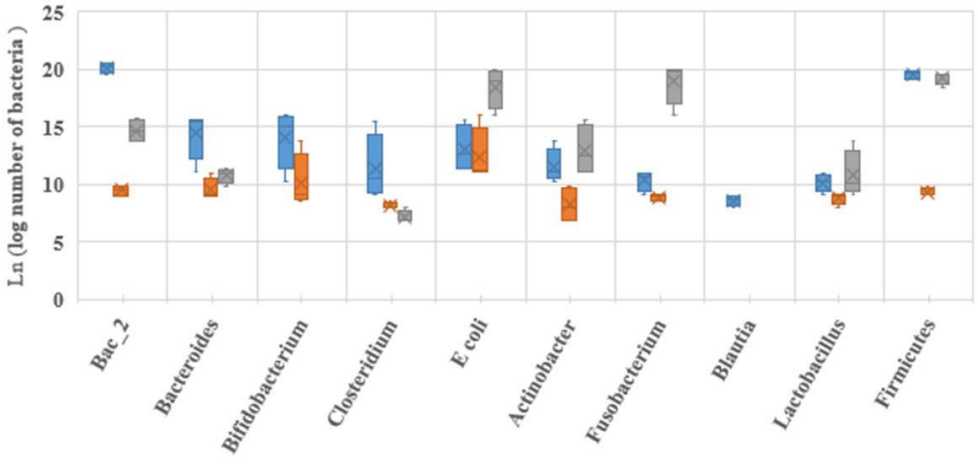

Fig. 2. Comparison of the log number of bacteria in the GvHD group.

a marked increase in bacterial counts observed one-month post-transplantation compared to one week after the procedure. (Lactobacillus; P-value=0.004 and *Firmicutes*; P-value=0.007). In GvHD patients, these bacteria decreased between before and one week after transplantation (*Lactobacillus*; P-value=0.034 and *Firmicutes*; P-value=0.022).

DISCUSSION

Studies investigating the microbiota composition in patients before and after allo-HSCT have shown a decrease in bacterial diversity following treatment. This reduction in diversity was especially marked in patients with intestinal GvHD (10). Our findings from HSCT patients revealed significant alterations in the intestinal bacterial ecosystem. Compared with the pre-transplant microbiota, the gut microbiota following HSCT exhibited a reduction in bacterial diversity which is associated with an increased risk of infection, GvHD, and mortality following allo-HSCT. In the aGvHD group, a decrease in the number of *Bacteroides* and *Bifidobacterium* bacteria was evident compared to the group without aGvHD. A decrease in the number of *Clostridium* and *Firmicutes* bacteria was also reported in aGvHD group. Another notable result was the genus *Blautia*, which was present and reduced in the aGvHD group, but no significant difference in the number of bacteria was observed in the uncomplicated group. In addition, the number of *Actinobacteria*, *Lactobacillus* and *Fusobacterium* increased in the aGvHD group.

Consistent with our study, Doki et al analyzed stool samples from approximately two weeks before the conditioning phase in recipients of allogeneic hematopoietic stem cell transplantation (allo-HSCT). Their results indicated that patients with aGvHD exhibited a markedly increased prevalence of *Firmicutes* while demonstrating a reduced occurrence of *Bacteroides* in comparison to patients without aGvHD (16). In another study, Li et al. analyzed the stool samples of 11 patients to study the changes in intestinal microbiota following allo-HSCT. The results indicated that the diversity of intestinal microbiota in patients with aGvHD was significantly reduced compared to those without aGvHD, both before and after to the transplantation (17). Similar to the mentioned study, we also saw a decrease in the number and diversity of intestinal microbiota using specific primers. In another study, Kouidhi et al investigated changes in gut microbiota composition in stool samples using Next-generation sequencing (NGS). Their results were associated with the abundance of *Actinobacteria*, *Firmicutes*, and *Bacteroides* after allo-HSCT (18). However, we observed an increase in *Actinobacteria* and a decrease in *Firmicutes*, and *Bacteroides* between one week and one month after transplantation.

In addition, our results showed that a reduction in microbial diversity within the intestinal tract, specifically the genus *Blautia* following hematopoietic stem cell transplantation (HSCT), correlated with increased lethality from graft-versus-host disease (GvHD), which was similar to the study by Jenq et al. (19). Consistent with our results, other studies have indicated that the bacterial community experienced a reduction in *Clostridia* following allogeneic hematopoietic stem cell transplantation (allo-HSCT) (18, 20). Studies have shown that a conditioning regimen including radioactive sources may result in dysbiosis and alterations in the microbiota (21).

Evidence indicates that both pro-inflammatory and anti-inflammatory cytokines play a significant role in influencing the risk of acute graft-versus-host disease (aGvHD) so IL6 can be associated with several inflammatory diseases (22). Interleukin-6 (IL-6) has the potential to provide benefits for individuals at increased risk of developing acute graft-versus-host disease (aGvHD) (23). In other words, this cytokine is crucial for implementing preventive strategies aimed at enhancing the quality of life during the initial phase following transplantation and the outcomes of allo-HSCT. It should be noted that the main limitation of this study was the absence of next-generation sequencing (NGS) equipment.

CONCLUSION

Our results demonstrated that the presence of a specific bacterial group within the gut microbiota of transplant patients is associated with an increased incidence of transplant-related diseases among individuals undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT). The study investigated changes in the diversity of gut microbiota in both groups with GvHD and those without it. In the aGvHD group, a decrease in the number of *Bacteroides*, and *Bifidobacterium* bacteria was evident compared to the group without aGvHD. Also, a decrease in the number of *Clostridium* and *Firmicutes* bacteria was seen, which was not significant in the

other group. Another significant result was the genus *Blautia*, which was present and reduced in the aGvHD group, but no significant difference in bacterial counts was observed in another group. In addition, an increase in *Actinobacteria* and *Fusobacterium* genera was observed in the aGvHD group. It is important to highlight that a major limitation of this research was the absence of next-generation sequencing (NGS) technology.

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