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Single-Cell Analysis of the Microbiota-Gut-Brain Axis Mechanism: A Proposal in Exploring Diet Intervention in Fecal Microbiome Transplantation Mice

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Abstract. Three main problems would be investigated in the following experiments proposed. The first problem would be whether the microbiota exposure during pregnancy impact ASD-like symptom. Mice embryos are proposed to be exposed to ASD patients' fecal bacteria, and then impact is measured through macroscopic (social behavior tests) and microscopic perspectives (PCR and 16s RNA sequencing to test microbiota abundance, and tissue specific transcriptomics and flow cyometry to assist single-cell analysis). The second problem would be: whether causal relationship exists between gut microbiota and development of ASD-LIKE symptoms. The third problem would be: whether ketogenic diet (KD) is helpful to intervention of ASD-LIKE symptoms.

Keywords: Autism spectrum disorder, Fecal microbiome transplantation, Single-cell analysis, Diet intervention

1. Introduction

Autism Spectrum Disorder is a complex neuro-developmental disorder defined by core behavior impairments [1]. Its prevalence continues to increase worldwide [2].

ASD is commonly linked with certain gastrointestinal (GI) co-morbidities, and functional GI disorders have been frequently reported in ASD patients [3, 4]. Taking gut-brain axis, the bidirectional pathway made up of neurons, hormones, immune cells, and microbial molecules between gut and brain, into account, studies suggested a potential causal relationship between gut microbiota and ASD like symptoms [5]. Previous studies have suggested that altered composition of the intestinal and gut microbiota is related to altered expression of ASD-related genes and is related to development of ASD like symptoms in hosts [6, 7].

Fecal microbiome transplantation (FMT) has been considered an adequate treatment to modify the microbial ecosystem in GI tract [8]. Specifically, it's been considered useful for pediatric patients, as children's gut microbial ecosystems can be easily intervened. For instance, clinical trials of FMT treatment on ASD children have been carried out and the results were positive [9]. Further regular and long-term analysis of gut microbiota change which occur in pediatric patients with FMT

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is necessary. Also, immunological and metabolic responses are necessary for a better understanding in the potential impact of FMT in a growing child.

Studies then shine light on the intervention of ASD, bringing both behavioral and molecular improvements [10, 11]. For instance, the ketogenic diet is an eating pattern that contains high fat (75%), low carbohydrate (5%), and moderate protein (15%). KD can change the energy consumption by forcing body consume ketone bodies instead of using glycogen. This way, KD can protect the function of various tissues and organs [9]. KD also reduces the host bacteria abundance and increases the production of neurotransmitters like dopamine to improve patients symptoms [12]. More specifically, KD stimulates the release of neurotransmitters like dopamine and 5-hydroxytryptamine, which are beneficial for mood adjustment for patients [11].

Nevertheless, few recent studies have explored the underlying molecular mechanisms on gut-brain axis and ASD [13]. However, either they focus merely on social behaviors, ignoring crucial index like the spine density of neurons, or they were established on animal models with certain gene knockout, which couldn't represent ASD as a whole [14, 15]. Thus, further microscopic exploration is urgently needed to delineate the molecular and cellular pathology of mice with interfered gut microbiota. Taken together, this study would provide a wealth of single-cell transcriptomic data characterizing the neurological and intestinal phenotypes associated with ASD, establishes our understanding of causal relationship between ASD related gut microbiota interference and development of ASD symptoms, and expands our knowledge of the effect of diet intervention on ASD mice.

2. Hypothesis

In this study, it's expected that gut microbiota from ASD patients drives autism-like behaviors through the gut-brain axis. Specifically, ASD-associated microbiota modulates the transcriptomics of intestinal epithelial and immune cells. These cells then transmit signals—via microbial metabolites and immune mediators— to the brain, where they alter the gene expression and functional states of distinct neuronal sub-types, ultimately leading to behavioral abnormalities. It is expected that KD can change the percentage of host bacteria in order to lighten the symptoms of ASD like repetitive behavior and anxiety levels.

3. Experimental design

3.1. Mice breeding, grouping and mating

According to Figure1, C57BL/6 germ free (GF) mice should be used. Group A should be male GF mice, group B should be female mice that are injected with healthy-children's gut microbiota, and group C should be female mice that are injected with ASD children's microbiota.

For the control group, mice A mate with mice B to develop an embryo (F_c). For the first experimental group (F_{E1}), mice A mate with mice C to develop an embryo. For the second experimental group (F_{E2}), mice A should be mated with mice C to develop an embryo. Then, the embyo should be transplanted to mice B's ovary to ensure a GF environment. F_c, F_{E1}, and F_{E2} mice of 3 developmental stages (embryo, post-natal and adult) should be tested by single cell analysis, social behavior test, microbiome analysis and histology.

The mice should be fed with chow diet. Then, gut microbiota should be extracted from normal and ASD children and transplanted to female mice separately via FMT.

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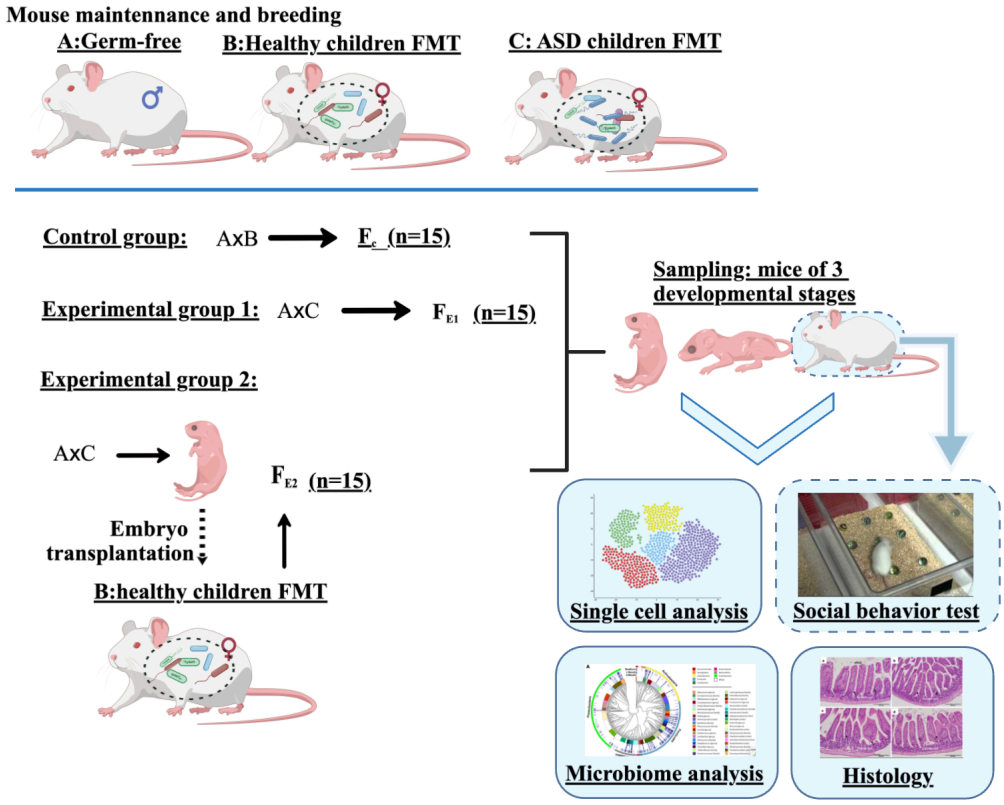


Figure 1. Experimental design (created with BioGDP.com) [16]

3.2. Transplantation of fecal micro-biome to mice

Fecal samples should be collected from healthy children and children with autism spectrum disorders separately. Samples should be stored at -80℃. Aliquots of donor samples should be used for colonization into GF female mice. To that end, frozen aliquots should be thawed in an anaerobic chamber and resuspended in two volumes of reduced sodium bicarbonate solution. Subsequently, samples should be vigorously vortexed and spun down. Supernatants should then be used to colonize GF mice by a single oral gavage. Colonized mice should then be allowed to rest for 3 weeks, and should subsequently be mated according to experimental needs [4].

3.3. Dietary treatment and ASD

The experiment should use the mice that injected with ASD bacteria and divide them into three different groups in order to test the influence of KD in the treatment of ASD. The first group should be the normal mice and they are given chow diet during the procedure, the second group should be the ASD mice and they are provided with KD every day, the third one should be the ASD mice and they are provided with chow diet during the experiment. The first group should be used as negative control group. By measuring the changes and difference between the second and third group's abundance of gut bacteria through the PCR technology, whether the ketogenic diet can adjust the balance of each bacteria type should be shown by the result. Finally, the changes after the diet therapy should be viewed through behavioral test like open field test [14].

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3.4. Macroscopic measurements

3.4.1. Social behavior test: open field test

Mice should be placed individually in a open-field area and allowed to explore freely for 10 min. By comparing the time that the mice spend on the central area and side area, the level of anxiety can be identified. The longer the time that the mice spend in the central area, the lower the anxiety level they have [17].

3.4.2. Social behavior test: restricted repetitive behavior test

The test will be set in standard mouse cages filled with unscented bedding. Twenty glass marbles should be arranged on the bedding surface.

Mice should be individually transferred to the testing room at least 30 minutes to the test to accommodate the light and sound. Each mouse should be gently placed into the center of the test cage and allowed to explore freely for 30 minutes. The entire session should be recorded by video under and minimize human distribution.

At the end of the 30-minute session, the mice should be removed, and the number of marbles buried was counted. A marble is defined as "buried" if its surface was covered by bedding by more than two-thirds [15]. The bedding and marbles are replaced completely between subjects, with marbles cleaned using 70% ethanol and air-dried to eliminate olfactory influence from previous subjects [15].

3.5. Microscopic measurements

3.5.1. Using PCR and 16s RNA sequencing to test gut microbiota abundance

Firstly, the fresh feces should be collected from mice in each group. Then, the samples should be divided into two parts, one of the samples should be used for bacterial separation, and the other group will store at −80°C for subsequent microbial DNA extraction. Secondly, the intestinal content should be diluted and then inoculated onto selective anaerobic culture media. Anaerobic incubation should be put under suitable environment that the temperature stay unchanged. Different bacterial strains should be divided according to colony morphology and biological characteristics in order to count the number of colonies, and calculate the concentration of various bacteria. Then, total microbial DNA was extracted from frozen fecal samples using a fecal microbial DNA extraction kit. By analysing the purity and concentration of DNA, these DNA samples will be used for PCR amplification [18].

The purpose of this process is to test the abundance of gut microbiota in order to compare the changes after using the diet intervention. To be specific, the abundance of host bacteria should be categorized with other microbiota in the comparison. In this way, how the diet intervention treat ASD like symptoms could be detected.

3.5.2. Tissue specific transcriptomics

This measure is carried out before scRNA-seq to prepare samples for the scRNA-seq. Mice of the three different developmental stages should be anesthetized and dissected under a microscope. Cells from the tissue should be separated into independent single cells through separate immersions in PBS, papain solution, and DNase I solution. scRNA-seq profiles the transcriptomics. The

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10×genomics platform should be utilized since its advanced microfluidic technology enables high-throughput analysis of the 3′ end of single-cell RNA with high efficiency. This high-throughput approach allows for the analysis of rare cell types in biological environments. Note that when the scRNA-seq is to be carried out, cellular activity of samples shouldn't be less than 80%.

3.5.3. Flow cyometry

Cells of both the brain and small intestines from three developmental stages of mice F_c, F_{E1}, and F_{E2} should be taken. Then, the cells should go through flow cyometry. Exact changes in single cells could be observed, and therefore verify the quantitative changes in immunal cells, glial cells, nerve cells and other types of cells brought by FMT. This step is an orthogonal validation of scRNA-seq by verifying changes in cells and corresponding body systems from perspectives other than gene expression.

3.5.4. Sampling for microscopy

Mice should be taken evenly from different timepoints, including E14.5, post-natal and adulthood. Out of ethical consideration of experimental animals, mice should be deeply anesthetized before dissection.

Then, firstly, samples of intestine should be prepared. The abdominal cavities of mice should be opened immediately, and the full small intestine should be carefully dissected. Samples of the distal ileum should be selected as the primary sampling site for all mice. This is because the distal ileum has high microbial density and abundant gut-associated lymphoid tissue, making it highly relevant for studies of microbiota-gut-brain axis. Half of the intestine samples should be designated for immunofluorescence analysis. The sample should be gently flushed with ice-cold phosphate-buffered saline (PBS, pH=7.4) to remove luminal contents without mechanical damage to the mucosa. Next, the sample should be immediately embedded in optimal cutting temperature compound. Cryosections should then be cut using a cryostat and should be stored at −80 °C until staining. Other half of the intestine samples should be designated for H&E staining. The sample should be fixed by immersion in PBS at 4covernight (12h), followed by standard dehydration through a graded ethanol series, clearing in xylene, and embedding in paraffin. Transverse sections should then be cut and mounted on glass slides. Additionally, to ensure reproducibility, three non-consistent sections per mouse, spaced at least 100 μm apart, should be selected for each staining method.

Secondly, brain samples should be prepared. The mice brain should be taken out of mice skull. First of all, the organ should be immersed in 4% PFA. This step may inhibit enzymes within the organ, prevent tissue autolysis and sustain the original states of brain cells. Then, brain should be immersed in PBS and sucrose solution, which is to formation of ice crystals during rapid freezing. Next, the brain should be embedded in OCT and rapid-frozen in isopentane and cooled with dry ice. Finally, frozen sections should be cut at 20 μm thickness using a cryostat.

3.5.5. Microscopy: hematoxylin and eosin (H&E) staining of intestine sample

H&E staining clarifies the macroscopic histology of intestine, and highlights physical variations like lengths of micro-villi. Variations of micro-villi lengths represent changes in digestion abilities of mice. This divergence between groups may indicates difference of gut development, which would possibly trigger GI problems [19-21]. By further comparing the severity of micro-villi lengths and

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development of ASD, researchers will be able to re-examine the potential causal relationship between gut microbiota and ASD.

3.5.6. Microscopy: immunofluorescence of intestine sample

Immunofluorescence staining will target proteins reflecting intestinal barrier integrity and immune status. Specifically, ZO-1 and occludin content represent tight junctions, Muc2 content stands for mucus production, Ezrin content represents micro-villus structure, and F4/80 content stands for macrophage infiltration. These markers may reveal both abundance and spatial distribution of key proteins, indicating where barrier or immune changes occur.

3.5.7. Microscopy: viral vector transduction fluorescence of brain sample

Viral vectors, in this case adeno-associated virus (AAV), are used to carry green fluorescence protein (GFP) genes. Treated AAV are injected into multiple brain areas. Neurons of all types are infected and marked with GFP [22]. Specifically, sparse labeling is used to ensure clear and exact observation of each single neuron [23]. The microscopy should be carried out about 2-4 weeks later than the injection to ensure full expression of GFP genes. Then, as quantity of spines within 10 μm*10 μm are counted, the spine density is calculated and compared.

Spine density is an important measure of ASD, and it's shown that ASD patients usually show abnormal spine densities [13]. In other words, by gaining and analyzing data of spine density from different experimental groups, the severity of ASD can be understood and compared between experimental animals of different groups.

4. Statistical analysis

Statistical analysis of open field test and marble burying test should proceed with one-way ANOVA. In the open field test, one-way ANOVA could compare the differences in ratio of the time that each mouse spends on central areas and edges. In the marble burying test, one-way ANOVA should be used in marble burying test in order to compare the differences of buried marbles between F_c, F_{E1} and F_{E2} in Figure 1. Open field test should be used to identify the anxiety level of mice by viewing the time spend on each area. This test is useful in showing the behavioral changes directly through the difference of time. Additionally, the marble burying test shows the changes of repetitve behavior, which is closely related to ASD-LIKE symptoms. Combination of the two methods could notate the increase of ASD-LIKE symptoms [24].

After sc-RNA seq, AUGUR can rank which cell types are the most responsive or severely perturbed by a biological perturbation (for instance, a variation in gut microbiome). By comparing data from experimental and control group, the predictive accuracy (AUC score) serves as a metric for how strongly that cell type has been reprogrammed. Then, with a focus on specific type of cells, Pseudotime analysis, RNA velocity, and Partition-based graph abstraction (PAGA) could help establish knowledge on the temporal development of single specific cells. Variations in the mRNA expression level and mRNA expression directions. Developmental trajectory of cells will be predicted to further understand the impact of gut microbiota on mice.

5. Result

The changes in mice behavior can be viewed directly through social behavior test and social communication tests. For the open field test, it comparing the time that the mice spend on different

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area of field to see whether the symptoms influenced by the diet. By observing the video detected by computers, the longer the time that the mice spend on the central area, the less anxiety level it get. Conversely, the longer the mice stay on the edge of the box, the anxiety level grows. For the marble burying test, the anxiety level can be detected by counting the number buried marbles. The repetitive behavior represents a higher level of ASD. Counting the marbles that buried more than 70% of its value to measure the repetitive behavior of mice [15].

To compensate shortcomings of past studies, this proposal included more measurements on the microscopic level. As for microscopy observations, H&E staining may exhibit alternations in physical structures of intestine, and therefore measure the significance of gut microbiota in triggering GI problems. This observation can be later utilized in assuring causal relationship between gut microbiota and development of ASD-LIKE symptoms. Yet H&E staining is limited to investigation of overall changes, and immunofluorescence is here to fill the gap. Immunofluorescence give insights on the content varitaions of key proteins, which often reflect intestinal barrier integrity and immune status. However, immunofluorescence may be limited to a preselected set of proteins and cannot capture global transcriptional alterations or identify the specific cell types involved.

In this case, scRNA-seq will be needed to profile the transcriptome of individual cells in an unbiased manner, uncovering cell-type-specific gene expression and novel pathways. Thus, immunofluorescence and scRNA-seq are complementary: the former validates and localizes key protein changes, while the latter dissects underlying transcriptional mechanisms and cellular heterogeneity.

While scRNA-seq predicted compositional changes in immune cell populations at the transcriptional level, flow cytometry provided a direct, quantitative count of these specific cellular subsets (protein-level validation). The consistency between the scRNA-seq predictions and the flow cytometry counts confirms that the transcriptional alterations genuinely translate into physiological changes in immune cell abundance within the gut. In the study, AUGUR provides a critical screening function that uncovers a counter-intuitive result: for instance, in previous studies, although non-neuronal cells (like glia) had the most differently expressed genes, AUGUR ranked neurons as the most affected cells [14]. Thus, it shifts the researchers' focus from mere variations in quantity of cells to the functional severity of the perturbation, guiding which cell types warrant deeper experimental investigation.

As for the effect of diet intervention, the results will be shown through three experiments. By comparing the changes in types of bacteria abundance and classification, it shows whether the KD influences the symptoms of mice through changing the bacteria types in their gut.

6. Discussion

Methodology-wise, one major limitation is the batch problem caused by scRNA-seq. Since cells are unable to go through the test within the same time period, the environmental conditions vary from batch to batch. Also, the number of cells of different cell type varies from one another, this inconsistency in sample size is ineluctable. In summary the batch effect, as mentioned, is inevitable. Fortunately, relative applications and techniques are developing and attempting to eliminate this effect, and therefore, further explorations can go through the experiment while using applications like Harmony to minimize the batch effect [25].

One major strength is the high human relevance and practical significance. Firstly, in our proposal, fecal microbiome from ASD children are extracted and transplanted to experimental subjects. This measure offers the most authentic simulation of ASD patients' gut micro-environment.

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Also, this treatment helps narrow the research scope to children, which stands 30% of ASD patients' quantity in total. Secondly, KD changes the energy consumption of using ketone bodies instead of using glycogen. So it is not only helpful in treating ASD, it is also a popular eating habit among patients who want to control body weight and cure obesity.

One argument against our proposal, which has also been against studies involving FMT is that: in experimental animals, activity mechanisms between different species of gut microbiome and between gut and its microbiome remain unclear to today scientists, and thereby being too general for specific studies. However, as both microbiome in human and experimental animals interact with its surrounding and each reach a status of dynamic equilibrium, our team believe it's possible to neglect the specific activity mechanism between different microbiome species when the research purpose is to investigate effect of certain diet intervention on a tissue level.

KD changes the energy consumption of using ketone bodies instead of using glycogen. So, it is not only helpful in treating ASD, it is a popular eating habit among patients who want to control body weight and curing obesity. Using ketogenic diet may effectively help the treatment of ASD. However, it has some negative effect to our body. 5.9% patients who were intervened with KD developed kidney stones [26]. As a result, the treatment should try to avoid that risk when giving patients appropriate treatments.

As for the future direction, effect of multiple diets, like Mediterranean diet, on ASD-LIKE symptoms should be explored. Additionally, clinical trials regarding ASD and diet intervention should be carried out, since experimental animals differ from human children.

7. Conclusion

Three main problems are investigated through the experiments. The first one is the casual relationship between gut microbiota and ASD symptoms. The second one is whether the microbiota exposure during pregnancy impact ASD symptom. The third one is whether KD can help for the treatment of ASD patients. Both macroscopic and microscopic methods are used for discovering the result and data processing. Through the experiment, the cause of ASD can be specified by viewing the relationship between brain and gut microbiota. This result will be beneficial for treatment of ASD patients as their ASD-LIKE symptoms can be noticed at an early age. Moreover, the KD can be an inspiration for ASD treatment. For the future direction, the research may focus on testing whether the change in gut microbiota is the necessary factor of causing ASD.

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