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Investigating Effects of Atorvastatin and Lactulose on ameliorating Crohn's Disease in 2,4,6-Trinitrobenzene Sulphonic Acid (TNBS)-Induced Colitis: A Research Protocol

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Abstract

Introduction: Crohn's disease (CD) is an inflammatory bowel disease (IBD) triggered by excessive activation of the gastrointestinal (GI) immune system, causing intestinal inflammation. Current therapeutic strategies use probiotics and fecal matter transplantation (FMT) to increase microbiome diversity and induce CD remission, but their efficacy is unclear. Conversely, prebiotics such as lactulose were found to increase anti-inflammatory bacteria thus reducing inflammatory markers in colitis mouse models. Additionally, atorvastatin has been found to modulate GI immune system activity by reducing chemokine expression, providing a potential treatment for CD. In this research protocol, a combination therapy using lactulose and atorvastatin is proposed as a treatment strategy for CD.

Methods: 2.5% 2,4,6-trinitrobenzene sulphonic acid (TNBS) was injected intrarectally at a dose of 100 mg/kg to induce colitis in the mouse models. In this randomized controlled trial, the mice will be equally assigned to 5 groups: untreated TNBS mice (n = 10); TNBS mice treated with lactulose (0.005 ml / g of body weight, n = 10); TNBS mice treated with atorvastatin (5 mg/kg, n = 10); TNBS mice treated with the atorvastatin and lactulose combination therapy (n = 10); and the untreated healthy mouse (n = 10) to serve as the negative control. Histopathological analysis was conducted on colon tissue along with ELISA identification of inflammatory markers and mRNA analysis.

Anticipated Results: It is expected that the TNBS group treated with the combination therapy will have the lowest levels of inflammatory cytokines. It is also expected that the treated TNBS mice will have the closest Crohn's Disease Activity Index (CDAI) score to the healthy untreated condition.

Discussion: Findings indicate that atorvastatin and lactulose combined supplementation could provide a more optimized treatment strategy for CD as opposed to atorvastatin or lactulose alone.

Conclusion: The protocol anticipates the combined treatment to significantly improve colonic health in TNBS-induced colitis mice, resulting in decreased colonic ulcerations, diarrhea, and bowel wall thickness. Future studies should explore other inflammatory markers like CRPs and include different animal models to gain insights into treatment dosage and efficacy.

Keywords: Crohn's disease; irritable bowel syndrome; prebiotics; statins

Introduction

Crohn's Disease (CD) is a chronic widespread type of inflammatory bowel disease (IBD) that affects approximately 135,000 Canadians each year [1]. Despite its prevalence, the complete pathogenesis of CD remains unknown due to its complexity. CD affects the gastrointestinal (GI) tract from any region along the mouth to the anus [2]. Common symptoms of this disorder involve abdominal pain, intestinal cramping, diarrhea, weight loss, and fatigue [2]. Based on several human studies and animal disease models, an increase in the following inflammatory markers found in the colon lamina were associated with CD: interferon-gamma (IFN-γ), tumor necrosis factor-α (TNF-α) and transforming growth factor-β (TGF-β) which are produced by T helper (Th) 1, Th2, Th3, Th17-specific cytokines, and regulatory T cells (Treg) [3, 4]. This may contribute to intestinal strictures, fistulas, abscesses, and obstructions, characterized in CD [5].

Current therapeutic strategies for CD involve an amalgamation of dietary supplementation such as pro biotics, and other lifestyle adjustments such as physical activity and psychological supports [6]. Previous studies have investigated the use of probiotics in CD-related

dysbiosis with inconclusive results. A study conducted by Bjarnason et al. found no difference in inflammatory marker fecal calprotectin (FCAL) levels in CD patients given probiotics compared with the placebo [7]. Additionally, faecal microbiota transplantation (FMT) is currently undergoing clinical trials to provide a prospective treatment for CD. FMT is the process of transferring microbiota from healthy patients to a CD patient to restore normal GI function [7]. Despite being used throughout history for the treatment of dysentery and *C. difficile*, the effects of FMT remain unclear in improving CD symptoms of abdominal tenderness, fatigue, flatulence, emesis, and diarrhea [8-10].

Prebiotics are defined as non-digestible fermented fibers that reduce inflammation in the GI tract [11]. Lactulose is a prebiotic found to decrease inflammatory macrophage numbers, thus suppressing inflammatory tumorigenesis related to CD [5].

Hydroxymethylglutaryl coenzyme A reductase inhibitors, otherwise known as statins, are commonly used for their cholesterol-reducing ability, but has been found to have additional anti-inflammatory and immunomodulatory effects. Atorvastatin is an FDA-approved medication normally used to lower cholesterol in patients with cardiovascular disease [12]. Animal model studies provide evidence that atorvastatin inhibits chronic GI inflammation by decreasing leukocyte migration [13]. Atorvastatin was found to reduce T-cell recruitment, thus decreasing the inflammatory presentation of CD by inhibiting HMG-CoA [14].

The objective of our research study examines whether the combination treatment of statins in addition to prebiotics will improve CD symptoms by reducing GI immune system-induced inflammation. We hypothesize that a combined treatment plan involving prebiotic lactulose and atorvastatin would be the most effective at decreasing inflammatory markers in CD patients compared to either treatment alone. To mimic conditions of Crohn's disease, we will be using 2,4,6-Trinitrobenzene Sulphonic Acid (TNBS) -induced colitis mice models.

Methods

Animals

Male and female adult C57BL/6J mice from Charles River Laboratories were housed in specific pathogen-free animal cages. The mice were 8-10 weeks old and weighed 20-25 g. They were maintained under a 12-hour light and 12-hour darkness cycle. A diet of chow pellets and tap water was provided over the 2 weeks unless otherwise specified [15].

Colitis Induction

Mice were acclimated to their environment, then were fasted overnight and anesthetized using the methods described by Liu T et al [15]. Experimental colitis was induced in the mice by intrarectal injection of 100 mg/kg or 4µl/g body weight of 2,4,6-trinitrobenzene sulphonic acid (TNBS). The solution was prepared by dissolving 2.5% TNBS in 50% ethanol. The mice were also placed in a head-down position to aid in recovery from the anesthetic, during and a short while after TNBS induction. The negative control was administered 2.5% ethanol only. Colitis induction was confirmed through fecal occult blood tests (using a Luminol Reaction Experiment Kit), as well as by measuring weight loss and diarrhea levels, and scored according to the Crohn's Disease Activity Index (CDAI) [16, 17].

Lactulose and Atorvastatin Preparation

The mice treated with lactulose were orally administered 0.005 ml/g of body weight of lactulose dissolved in 0.5% carboxymethylcellulose (CMC), twice a day for up to 5 days post TNBS induction [18]. While the mice treated with atorvastatin received a daily intraperitoneal injection of 5 mg/kg of atorvastatin for 5 days post TNBS induction [19].

Lactulose and Atorvastatin Treatment Protocol

Mice were randomly divided into 5 treatment groups. The first group included mice with untreated TNBS-induced colitis (n=10). The 3 interventions groups consisted of mice with TNBS-induced colitis that are treated with lactulose (n=10), mice with TNBS-induced colitis that are treated with atorvastatin (n=10), and mice with TNBS-induced colitis that are treated with a combination of atorvastatin and lactulose (n=10). A total of 10 healthy mice were used as a negative control.

Mice were monitored daily for weight loss, survival, and signs of distress (pallor of extremities, behavioural changes, feeding pattern disruptions, etc.), according to the guidelines established with the Canadian Council on Animal Care [20]. The experiment has been designed to minimize distress when possible.

All mice were euthanized a week after colitis induction by administering an overdose of xylazine and ketamine [15].

Histological Analysis

Mice were assessed for colonic damage at the macroscopic and microscopic histopathological levels. All measurements were performed double-blinded by 2 independent pathologists. First, macroscopic grading of the colon tissue was performed according to the four established parameters. The first parameter was the degree of colonic ulcerations (beginning at 0, representing a healthy, normal presentation, and ranging up to 10, as the most severe condition), followed by intestinal/peritoneal adhesions (0-2), diarrhea (0-1), and thickness (0-1) [19].

For microscopic grading, the distal region of the colon was harvested after a week. It was fixed in an overnight solution of 4% formaldehyde diluted in 1X phosphate buffer saline (PBS, pH = 7.4), dehydrated in fixed cycles of increasing alcohol and xylene, and embedded in paraffin

[15]. The colon was further sliced into 4 µm sections and stained using hematoxylin and eosin (H&E). In total, five sections were chosen across the length of the colon, while 3 areas from the cross-section were selected, all at random [15]. These sections were scored using a microscopic scoring system (scale of 0-4) beginning with the amount of inflammation (measuring the increase of inflammatory cells in the gut lumen), distribution of lesions, depth of inflammation (measuring the involvement of the submucosa and mucosal layers), and nature of mucosal changes, based on the procedure from Aharoni et al 2006 [21]. The average scores per mouse intestine were totalled for each treatment condition.

Assessment of Inflammatory Markers using ELISA

Pro-inflammatory cytokine levels and other cytokines were analyzed. These lymphocytes were isolated from the lamina propria of the colon, by a previously described method [20], and incubated for 48 hours with anti-CD3 and CD28 antibodies at 10 and 2 µg/ml, respectively, in a 96-well plate containing 10% fetal bovine serum (FBS) and 0.2 ml Roswell Park Memorial Institute (RPMI) 1650 medium [23]. The presence of TNF-α, IL-1, IL-4, IL-5, IL-6, IL-10, IL-12, IL-17, IL-23, and IFN-γ were quantified using Enzyme-Linked Immunosorbent Assay (ELISA) kits.

mRNA Analysis

The colonic mucosa was isolated from the distal region of the colon (roughly 1 cm). The RNA was extracted from the mucosa using Trizol, and the corresponding cDNA was synthesized using M-MLV reverse transcriptase enzymes alongside a series of primers for the corresponding genes of interest, *IL-1A, Il-4, IL-5, IL-6, IL-10, IL-12A, IL-12B 1L-17A, IL-23A, IFN-γ,* and *TNF-α* [19]. Real-time PCR was performed, and the mRNA transcription levels were calculated. This technique follows the methods outlined by Liu T et al [15].

Statistical Analysis

The Analysis of Variance (ANOVA) or Kruskal-Wallis Test was performed to compare data between two or more groups, depending on whether the data were normally distributed or not, respectively. All data was calculated as mean values and presented with ± standard deviation. The data was considered significant at $p < 0.05$. Calculations were performed on the GraphPad Prism software.

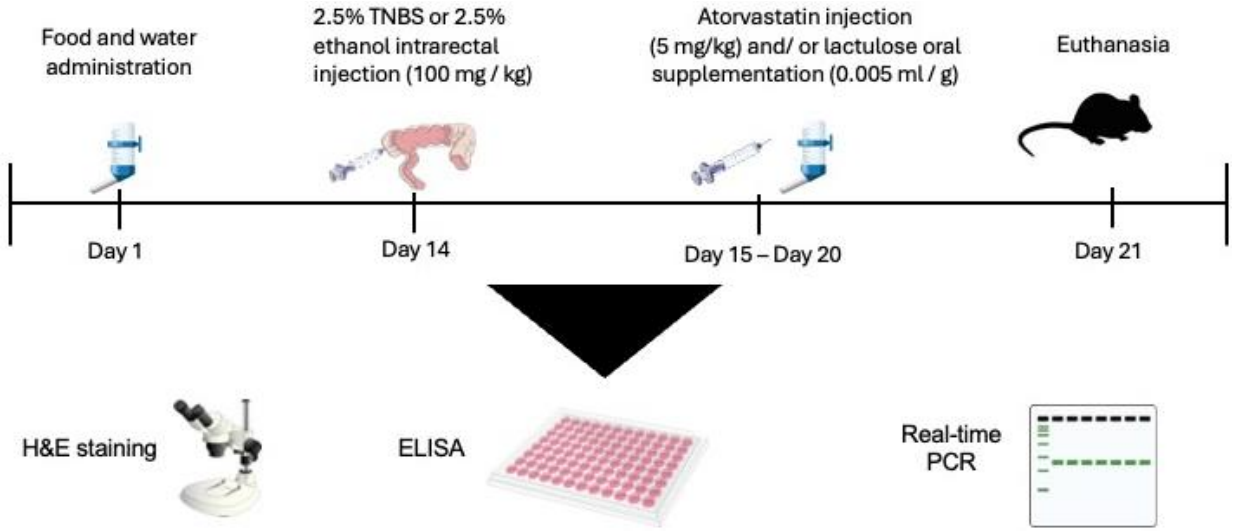

Figure 1. Schematic Diagram of the Experimental Design. Developed by the authors using Microsoft PowerPoint and all images were sourced from https://bioicons.com, under the CC by SA license.

C57BL/6J mice were randomly divided into 5 experimental groups. All mice were given food and water ad libitum on day 1. On day 14, the mice were given 2.5% TNBS (2,4,6-Trinitrobenzene sulfonic acid) (100 mg/kg) as an intrarectal injection. The untreated mice were also given 2.5% TNBS to induce colitis (n=10) at day 14. The healthy group (n=10) was used as a negative control and were given a 2.5% ethanol injection at the same time. Mice in the lactulose treatment group (n=10) were orally given a 0.005 ml / g of body weight of a lactulose supplement containing lactulose dissolved in 0.5% carboxymethylcellulose (CMC), twice a day from day 15 to day 20. On day 15, mice in the atorvastatin condition (n=10) underwent TNBS-induction of colitis and were then given a daily intraperitoneal injection of 5 mg/kg of atorvastatin until day 20. The combined treatment group (n=10) were given lactulose supplementation, TNBS-induced colitis, and atorvastatin injections along the same time frame. At the end of this experiment, at day 21 all mice were euthanized by administering an overdose of xylazine and ketamine.

Results

Colitis induction was confirmed by performing fecal occult blood tests, measuring weight loss, and monitoring the

presence of diarrhea, in accordance with the CDAI. The total scores were then rated from 0 (healthy) to 12 (severe colitis) [17]. It is expected that the treated TNBS conditions will score significantly lower than the untreated TNBS mice.

The Effect of Lactulose and Atorvastatin on Cytokines and mRNA Expression in the Colons of TNBS-Induced Mice

It is expected that the untreated TNBS mice will have the highest level of proinflammatory cytokines in the intestine, due to the increased numbers of Th1 and Th17 T cells [19]. The mRNA analysis is expected to indicate that the untreated TNBS mice will have a significant increase in the expression of TNF, IFN-γ, IL-4, IL-5, IL-6, IL-10, IL-12, and IL-17A [16]. Atorvastatin combined with lactulose is expected to significantly decrease proinflammatory mRNA (TNF-α, IL-1, IL-6, IL-10, IL-12, IL-17, IL-23, and IFN-γ), and increase anti-inflammatory cytokines (IL-4, IL-10). Previous studies [19] using atorvastatin have found that it significantly decreases the presence of IL-10, IL-17, IL-23, and IFN-γ, which suggests it may have immunomodulatory effects. Another study [24] found that lactulose significantly reduced the presence of pro-inflammatory cytokines such as TNF-α, IL-β, 1IL-6, and IL-17, which supports its role as an immune regulator. We expect that the combination treatment is more likely to induce a beneficial effect compared to atorvastatin and lactulose alone.

Discussion

In this study, we hypothesized that a combined treatment plan involving prebiotic lactulose and atorvastatin will reduce the inflammatory presentation of CD. Youssef et al. and Aktunc et al. and found that atorvastatin decreased TGF-β, TNF-α, IFN-γ, IL-6, IL-17, and IL-23 inflammatory markers in TNBS-induced colitis mice, showing potential as a treatment option for CD [4, 19].

TNBS treatment using both atorvastatin and lactulose is hypothesized to successfully improve the overall health of TNBS mice, and significant but lesser improvements are expected for mice singly treated with atorvastatin or lactulose. The combination treatment is expected to cause a decrease in colonic ulcerations, adhesion to peritoneal organs, diarrhea, and thickness of the bowel wall in comparison to the untreated colitis group. A similar study [19] found that the TNBS-injected mouse model treated with atorvastatin had a significant reduction in inflammation and adhesion to adjacent organs but did not significantly reduce bowel wall thickness and diarrhea. Previous research [24] also indicated that lactulose treatment led to lower levels of colonic ulceration and inflammation and did not cause diarrhea side effects. We anticipate that mice treated with the combination of lactulose and atorvastatin will yield the lowest score of the microscopic evaluations, such as an absence or mild levels of inflammation, lesions, or changes in the mucous membrane.

The conditions with atorvastatin and TNBS separately are expected to yield a mild to moderate amount of inflammation, indicated by the presence of focal or multifocal lesions [24, 19]. Finally, the TNBS untreated group is expected to exhibit a high degree of accumulation of inflammatory cells, diffuse lesions, and transmural inflammation which could lead to necrosis [24].

The proposed research protocol has limitations and confounding variables to consider. Firstly, administering statins to target GI inflammation may have some confounding variables as statins are commonly prescribed to reduce cholesterol levels. Future studies can compare the effects of atorvastatin on CD patients with and without hypercholesteremia to observe differences in treatment outcomes. This approach will help determine whether the anti-inflammatory effects of atorvastatin are independent of its lipid-lowering properties. Furthermore, the reliance on mRNA analysis alone does not identify the specific cells responsible for the secretion of inflammatory cytokines. Future investigations will benefit from techniques such as single cell sequencing to pinpoint cellular sources more precisely. Another limitation includes the use of mice models. The mice used in the experiment are inbred with similar genetic backgrounds whereas humans have a diverse genetic background. The intestinal microbiota of mice differs significantly from that of humans. Translating findings to a clinical context requires further exploration through human clinical trials. Furthermore, a TNBS-induced colitis model may not fully represent all characteristics of CD. Causes of CD are multifactorial and challenging to mimic perfectly in experimental models. As such, it would be interesting to investigate whether the combination therapy of lactulose and atorvastatin would be similarly beneficial in different induced colitis models. Finally, examining mouse models for UC may give us a more comprehensive understanding of how our treatment works in IBD.

Future studies should investigate different dosages of atorvastatin to determine an optimal dosage. Future directions include further investigation of the immune cell-based pathogenesis of CD. Th1 and Th17 cells are specifically affected by the combination treatment of atorvastatin and lactulose and should be studied in greater detail. Our protocol primarily focuses on T cells, but further research should be conducted on the role of C-reactive proteins (CRPs). High CRP levels are observed in patients with GI disorders like CD. Atorvastatin has been found to reduce elevated CRP levels [25]. *In vitro* studies conducted by Grip and Janciauskiene determined that atorvastatin was linked to decreased CRPs in epithelial cell culture models, but these findings have not yet been explored in IBD [25]. Future studies can focus on the effects of the combination atorvastatin and lactulose treatment on CRP levels in CD animal models.

Conclusions

Atorvastatin and lactulose are proposed as a combined treatment for Crohn's Disease. The combination treatment is expected to yield the lowest levels of inflammatory cytokines when compared to each individual treatment and the untreated group. In addition, it is expected that treatment with both atorvastatin and lactulose will have a similar Crohn's Disease Activity Index (CDAI) score to the healthy condition. It is anticipated that the combined treatment will significantly improve colonic health in TNBS-induced colitis mice, resulting in decreased colonic ulcerations, diarrhea, and bowel wall thickness. Future studies should explore other inflammatory markers like CRPs and include different animal models to gain insights into treatment dosage and efficacy.

List of Abbreviations Used

ANOVA: analysis of variance
CD: Crohn's disease
CD3 / CD28: cluster of differentiation
CDAI: Crohn's disease activity index
cDNA: complimentary DNA
CMC: carboxymethylcellulose
CRP: C-reactive protein
DNA: deoxyribonucleic acid
ELISA: enzyme linked immunosorbent assay
FBS: fetal bovine serum
FCAL: fecal calprotectin
FMT: faecal microbiota transplantation
GI: gastrointestinal
H&E: hematoxylin and eosin
IBD: inflammatory bowel disease
IFN-gamma: interferon gamma
IL: interleukin
mRNA: messenger ribonucleic acid
PBS: phosphate buffer solution
q-PCR: quantitative polymerase chain reaction
RNA: ribonucleic acid
RPMI: Roswell Park memorial institute medium
Rt-PCR: reverse transcription polymerase chain reaction
TGF-β: transforming growth factor-beta
Th: helper T cells
TNBS: 2,4,6-trinitrobenzene sulphonic acid
TNF-alpha: tumour necrosis factor-alpha
Treg: regulatory T cells
UC: ulcerative colitis

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

Ethics approval is required if the experiment is executed. The exclusion of human trials implies that participant consent is impractical.

Authors' Contributions

SK: Provided significant contribution to the research protocol design, drafted the manuscript, and gave final approval of the version to be published.
SV: Provided significant contribution to the research protocol design, drafted the manuscript, and gave final approval of the version to be published.

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