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Original Research Article

Experimental repurposing of metformin for Crohn's disease in trinitrobenzene sulfonic acid induced colitis model in BALB/c mice

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ABSTRACT

Background: Inflammatory bowel disease (IBD) is characterized by repetitive episodes of inflammation of the gastrointestinal tract caused by an abnormal immune response to gut microflora. It includes two types: Ulcerative colitis (UC), which causes widespread colon inflammation, and Crohn's disease (CD). The current therapy focuses on symptom relief and provides inadequate maintenance of remission and quality of life improvement. Metformin has already been repurposed in few studies with dextran sulphate sodium (DSS) induced colitis. We aimed to evaluate the effect of metformin in TNBS induced acute on chronic colitis, to mimic the relapsing-remitting nature of CD in humans, which has not been done before.

Methods: Objectives were, phase I- evaluation of the effect of metformin in TNBS induced acute colitis in BALB/c mice. Phase II- evaluation of the effect of metformin in TNBS induced chronic colitis in BALB/c mice. Phase III- Evaluation of the effect of metformin in TNBS induced acute on chronic colitis in BALB/c mice. Materials And Methods: Trinitrobenzene sulfonic acid (TNBS) was used for inducing both acute, chronic and acute on chronic colitis in BALB/c mice. 36 BALB/c mice were divided into 4 groups i.e. normal control, disease control, positive control and test drug. Effect on DAI score, lower colon weight by length ratio, macroscopy and histopathology were assessed.

Results: Metformin showed significant improvement ($p<0.05$) in all the variables assessed i.e. reduction of (Disease Activity Index) DAI score, lower colon weight by length ratio, lower colon macroscopic score and lower histopathological score in comparison to the disease control group in all the phases i.e. acute (day 5), chronic (day 22) and acute on chronic (day 25). However, the effects were comparable to the positive control.

Conclusions: Metformin has potential to be repurposed for Crohn's disease as it showed comparable efficacy in all three phases.

Keywords: Colitis, BALB/c mice, Acute on chronic anti-inflammatory

INTRODUCTION

Inflammatory bowel diseases (IBDs) comprise chiefly of ulcerative colitis (UC) and Crohn's disease (CD). The pathogenesis of IBDs involve strong cellular immune reaction and exaggerated inflammatory response to environmental, genetic and immune factors leading to a chronic inflammatory condition of the gastrointestinal tract.[1] Crohn's disease (CD) is a chronic inflammatory condition of the gastrointestinal tract that follows a clinical course characterized by relapses and remissions. The pathogenesis of CD involves activation of Th1 cells and subsequent production of proinflammatory cytokines which eventually results in transmural colon infiltration. Despite the armoury of therapeutic agents available, 50% of patients failed to maintain remission in CD.[2] As per American College of Gastroenterology, currently mesalamine compounds like 5-Amino salicylic acid (5-ASA), sulfasalazine, corticosteroids like prednisolone and methylprednisolone, immunosuppressants like azathioprine, cyclosporine, and biologicals like infliximab, adalimumab and vedolizumab are used in the treatment of CD.[3] These therapies are not effective in all patients and have a variable response. In addition, above-mentioned agents also have serious adverse effects and are costly.[2] Hence, there is a need to develop novel compounds or repurpose drugs which are safe and can maintain prolonged remission.

Metformin, a biguanide, is used for the treatment of type 2 diabetes mellitus. Metformin has been shown to have anti-inflammatory, antifibrotic, antioxidant, anti-aging, cardioprotective and nephroprotective effects.[4] Few studies have observed anti-inflammatory activity of metformin attributable to 5' adenosine monophosphate-activated protein kinase (AMPK) activation and subsequent suppression of pro-inflammatory cytokine production. [5,6]It also has been evaluated in animal models of colitis which mimicked ulcerative colitis in humans.[7,8] In an in-vitro study, Takahara et al showed that metformin suppressed the interferon (IFN) -γ-producing LP CD4+ T cells, which were found to be regulated by activation of AMPK. In addition, metformin was found to be effective via AMPK activation in LP CD4+ T cells in an in vivo inflammatory bowel disease model.[9,10] However, no study was found in literature which evaluated the effect of metformin in an animal model of Trinitrobenzene sulfonic acid (TNBS) induced colitis in BALB/c mice resembling Crohn's disease in humans. This study also represents the first effort to utilize both acute and chronic colitis models. TNBS induced colitis model has a good construct validity.[11-13] This was the first attempt to evaluate the effect of metformin in TNBS induced acute on chronic colitis, to mimic the relapsing- remitting nature of CD in humans. Thus, the objective of phase 1 was to evaluate the effect of metformin in TNBS induced acute colitis in BALB/c mice and phase 2 was to evaluate the effect of metformin in TNBS induced chronic colitis in BALB/c mice. The objective of the phase 3 was to evaluate the effect of metformin in TNBS induced acute on chronic colitis in BALB/c mice.

METHODS

Study design

This is a preclinical research study conducted in the Central Animal House of Seth G S Medical College & KEM Hospital from December 2020 to August 2021. Institutional Animal Ethics Committee permission (IAEC/GSMC/05/2019) was taken before the commencement of the study. The study was conducted in accordance with the guidelines laid down by the Committee for Control and Supervision of Experiments on Animals (CCSEA).

Experimental animals

Male BALB/c mice, three to four weeks old and weighing 12 to 16 gm were procured from the Advanced Centre for Treatment Research and Education in Cancer (ACTREC), Navi Mumbai.

Husbandry conditions

The animals were housed in air-conditioned rooms with a temperature of 22±3° C and relative humidity of 30 – 70%. The animals were fed in the form of pellets and were provided water ad libitum. Pure drinking water through aqua guard was supplied.

Study chemicals used

Test drug

Metformin was administered at a dose of 250 mg/kg by oral gavage. The dose was extrapolated from previous studies on mice, where a dose of 250 mg/kg dose was used.

Positive control

Sulfasalazine was administered at a dose of 100 mg/kg by oral gavage.[14]

Inducing agent

2.5% 2,4,6-Trinitrobenzene sulfonic acid (TNBS) per rectum was used to induce acute colitis, whereas 1.25% TNBS was used to induce chronic colitis. All chemicals were obtained from Sigma Aldrich. The study was conducted in three phases as described below. Procedure for each phase consisted of the following steps.

Drug administration

All the study drugs were mixed in distilled water and administered orally using different feeding cannulas.

Experimental groups

36 Balb/c mice were divided into 4 groups and were administered the drugs along with the inducing agent as mentioned in Table 1 for each phase. The drugs were administered in each Phase as mentioned in Table 2.

Experimental procedure

In all phases, colitis induction was performed on day 1 with 150 µl of 2.5% TNBS for acute colitis and 1.25% TNBS for chronic colitis, except in the normal control group. In phase 3, 1.25% TNBS was used to induce chronic colitis on day 1, and 2.5% TNBS was used to induce acute colitis on day 21.

Administration of TNBS

Mice were anaesthetized with ketamine for the administration of TNBS via per rectal route. Lignocaine jelly was applied on the tip of the catheter before insertion, to avoid any injury to the mice. A 2 Fr rubber catheter was used to introduce the mixture of TNBS and ethanol in the rectum via the anal opening. The catheter was inserted 3-4 mm into the anus, and 150 µl of 2.5%/1.25% TNBS in 45% of ethanol was pushed slowly into the anal opening. The mice were then kept in an upside-down position for few minutes to avoid spillage of TNBS.[15] The animal groups were administered the study drugs for the selected duration as shown in Table 1 and 2. The groups were then observed subsequently for weight loss, stool consistency and bleeding per rectum. At the end of study period, the animals were sacrificed by cervical dislocation for assessment of variables (Colon weight by length ratio, macroscopic grading and histopathology scores). Variables assessed in all 3 phases were as follows.

Disease activity index score

The scores for weight loss, stool consistency and blood in stools on the day of assessment were considered.[16] A higher disease activity index (DAI) indicates severe colitis whereas a lower DAI indicates mild colitis. DAI scoring system is shown in Table 3.

Colon weight by length ratio

The isolated colon was rinsed with normal saline, faecal matter was removed, and then its length and weight were measured. Also, the weight by length ratio was calculated. This ratio is crucial for evaluating colitis severity, as inflammation increases weight per centimetre of length and can reduce overall colon length.

Colon macroscopic score

Following measurement of colon length and weight, the colon was longitudinally cut from distal to proximal end. Using fine-tipped forceps, the colon was opened laterally and meticulously examined for any lesions. Colitis severity was assessed macroscopically using the following scoring system given by Inokuchi et al: 0-No ulcer no inflammation, 1-local hyperemia without ulceration, 2-ulceration without hyperemia, 3-ulceration and inflammation at one site only, 4-two or more sites of ulceration and inflammation; and 5-ulceration extending >2 cm.[17]

Colon histopathologic score

After macroscopic examination, the colons were rolled using forceps in the Swiss-roll technique, then fixed in 10% formalin solution for 24 hours. After fixation, samples were sectioned, stained with Haematoxylin and Eosin, and examined for inflammation severity, crypt damage, and lymphocyte infiltration under 10x magnification. This technique allows for better longitudinal study of the colon compared to transverse sections. Grading and scoring were based on a predefined scale given by Dieleman LA, et al and is as shown in Table 4.[18]

Statistical analysis

All the results were expressed as mean±SD. Data analysis was performed using PRISM GraphPad software, Version 9 (GraphPad, San Diego, CA). Statistical comparisons were made between drug treated groups and control group. The parametric variable (colon weight by length ratio) was analysed using one-way ANOVA followed by post hoc Dunn's test. The non-parametric variable (DAI score, colon macroscopic score and colon histopathologic score) were analysed by Kruskal- Wallis test followed by post hoc Dunn's test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

The DAI scores (mean ± standard deviation) were significantly lower in the sulfasalazine group compared to the disease control in Phase I. Similarly, the metformin group showed a significant reduction in scores compared to the disease control. The DAI score in metformin group was comparable to sulfasalazine group. A similar trend of reduction in the DAI score was observed in the drug-treated groups during phases II and III, as shown in Table 5.

Table 1: Experimental groups in all phases.

Group	Description	Inducing agent (per rectal)	Dose
1	Normal control (n=6)×3	Normal saline	Equivalent volume
2	Disease control (n=10)×3	Phase I: 150 µl of 2.5% TNBS in 45% ethanol Phase II: 150 µl of 1.25% TNBS in 45% ethanol Phase III: For acute on chronic :150 µl of 1.25% and 2.5% TNBS in 45% ethanol on day 1 and 21 respectively.	Equivalent volume
3	Positive control (Sulfasalzine) (n=10)×3		100 mg/ kg (200 µl)
4	Test drug (Metformin) (n=10)×3		250 mg/ kg (200 µl)

Table 2: Duration of administration of interventional drugs.

Study phases	Administration of drugs	Day of sacrifice
Phase I	Day 2 to 4	Day 5
Phase II	Day 2 to 21	Day 22
Phase III	Day 2 to 24	Day 25

Table 3: DAI score.

Score	Weight loss	Stool consistency	Blood in stools
0	None	Normal	Negative
1	1-5%	--	--
2	6-10%	Loose stools	Positive
3	11-15%	--	--
4	>15%	Diarrhoea	Gross rectal bleeding

Table 4: Histopathological scoring of colon samples.

Feature	Score	Description
Inflammation severity	0	None
	1	Mild
	2	Moderate
	3	Severe
Inflammation extent	0	None
	1	Mucosa
	2	Mucosa and submucosa
	3	Transmural
Crypt damage	0	None
	1	1/3 of crypt damaged
	2	2/3 of crypt damaged
	3	Crypts lost; surface epithelium intact
	4	Crypts lost; surface epithelium lost
Percent area of involvement	0	0%
	1	1-25%
	2	26-50%
	3	31-75%
	4	76-100%

Table 5: DAI Score and colon weight by length ratio, macroscopic grading in phase 1,2 and 3.

Study groups	DAI Scores			Colon weight by length ratio		
	Phase I	Phase II	Phase III	Phase I	Phase II	Phase III
Normal control (n=6)	0.5±0.45	0.60 ±0.32	0.5±0.23	22 ±0.52	21±0.54	21 ±0.43
Disease control (n=6)	8.6±0.78*	7.5±0.86*	9.5± 0.94*	33±0.56*	33 ±0.47*	35±0.58*
Positive control (n=6)	4.3±0.9#	3.4±0.75#	5.6±0.76#	24 ±0.62#	23±0.64#	25±0.53#
Test group (n=6)	5.2±0.82@$	4.9±0.73@$	6.3±0.84@$	26±0.48@$	25±0.56@$	27±0.49@$

*p<0.001 vs normal control, # p<0.01 as compared to disease control, @ p<0.05 vs disease control, $: not significant vs positive control group treated with sulfasalazine; using Kruskal Wallis test followed by post hoc Dunn's test (for DAI score) and one-way ANOVA followed by post hoc Dunn's test (for colon weight by length ratio).

Table 6: Macroscopic grading and microscopic scoring in phase 1, 2 and 3.

Study groups	Macroscopic grading			Histopathological scoring		
	Phase I	Phase II	Phase III	Phase I	Phase II	Phase III
Normal control (n=6)	0.2±0.16	0.4±0.18	0.2±0.18	0.60± 0.48	0.80±0.52	0.7±0.36
Disease control (n=6)	4.2±0.75*	3.7±0.33*	4.4±0.35*	24.3±1.72*	22.4±2.26*	26.8±2.64*
Positive control (n=6)	1.3±0.62#	1.3± 0.45#	1.7± 0.62#	8.7±2.41#	8.3± 2.47#	12.9±1.96#
Test group (n=6)	2.46±0.86@$	1.82±0.65@$	2.7±0.53@$	11.04±1.90@$	10.1±1.78@$	16.8±2.72@$

*p<0.001 vs normal control, # p<0.01 as compared to disease control, @ p<0.05 vs disease control, $: not significant vs positive control group treated with sulfasalazine; using Kruskal Wallis test followed by post hoc Dunn's test.

The colon weight-to-length ratio (mean±standard deviation) was significantly higher in the disease control group compared to the normal control group across all phases. Sulfasalazine treatment significantly reduced the ratio, with results comparable to those observed with metformin in all phases, as shown in Table 5. Both macroscopic and microscopic scores were significantly lower in the sulfasalazine group compared to the disease control group across all phases. Similarly, metformin treatment resulted in a significant reduction in scores, with sulfasalazine and metformin showing comparable outcomes in all phases, as detailed in Table 6. In histopathology, the normal crypt architecture was maintained in normal control group (Figure 1A). Disease induction in disease control group was evidenced by disruption of crypt architecture and leucocytic infiltration throughout the colon (Figure 1B). The crypt architecture was restored to near normal in both sulfasalazine (Figure 1C) and metformin treated group (Figure 1D). However, a few leucocytic infiltrates were observed in both the groups. This trend was observed in all the three phases.

DISCUSSION

We observed that metformin 250 mg/kg by oral route reduced the inflammation in BALB/c mice. This was observed in all the three phases. Metformin showed a significant improvement ($p<0.05$) in all the variables in comparison to disease control group. However, the beneficial effect of metformin was comparable to sulfasalazine in all the phases. Metformin's anti-inflammatory effect has been assessed in many prior studies in which colitis was induced with DSS.[6,19-22]

Most experimental colitis studies have utilized oral DSS at varying concentrations, as it is the most cost-effective and induces colitis rapidly. It has been found that it leads to increase in markers of inflammation IL-18, IL-1β, IL-2, increase in DAI scores and histopathological changes. Chen et al, observed reduction in the various inflammatory markers with 125, 250 and 500 mg/kg of metformin.

Koh et al, found that 100 mg/kg metformin was effective in colitis associated cancer in C57BL/6 mice.[20] It significantly ameliorated the severity of colitis by reducing DAI score and histological damage, improving colon length and body weight. Pandey et al, assessed metformin in acetic acid (4%) induced colitis in Wistar rats.[21] They observed increased levels of TNF-α, MPO, nitrite and PGE2 which was reduced by metformin.

Metformin was also found to be effective in reducing SPHK1, S1P, IL-13, IL-6, STAT-3, NO, GSH, and MPO levels in oxazolone induced colitis.[21] However, El-Mahdy et al reported Metformin treated group did not show significant reduction in colon length and body weight as compared to disease control group.[22]

Similarly, Liu et al observed that metformin reduced DAI score, improved colon length and histopathological picture. But found no significant difference between levels of TNF-α, IL-17 and IFN-γ on ELISA of homogenized colon samples between disease induced mice and those treated with metformin.[19]

No researcher, till date, has assessed the effect of metformin in a model of colitis that closely mimics Crohn's disease in humans i.e., TNBS induced colitis in BALB/c mice. Metformin has been shown to have anti-inflammatory by virtue of its action on 5'-adenosine monophosphate kinase (AMPK). It causes AMPK activation which further leads to inhibition of nuclear factor κB (NFκB). This subsequently causes suppression of production of pro-inflammatory cytokines (like IL-6, TNF-α). Some investigators have combined TNBS with 45-50 percent ethanol, administered per rectum. Ethanol disrupts the superficial layer of gastrointestinal tract.[23]

This facilitates haptenization of the proteins of lamina propria due to interaction with the inducing agent i.e., TNBS. This activates and stimulates an immune response. There is, further activation of Th1 cells.[24,25] Th1 cells are responsible for production cytokines like IL-12 and TNFα.

This, in turn, leads to activation and recruitment of more Th1 cells and antibody production. This entire cascade of inflammation and cytokine production, which occurs in TNBS induced colitis, is seen with Crohn's disease in humans as well.

Hence, TNBS induced colitis is best to evaluate new drugs for use in Crohn's disease. Crohn's disease is a condition characterized by relapses and remissions throughout the life, modifying the dose of TNBS makes it possible to evaluate in both acute and chronic disease.[26]

Despite the fact that TNBS was given with 50% ethanol, no ethanol control group was used in this study. Morris et al, who first characterized this model of TNBS-induced colitis, included multiple groups of ethanol controls with rising ethanol concentrations.[26] He reported that ethanol caused dose-dependent disruption of the colon epithelium with no leucocytic infiltration or engagement of cytokines like IL-12 and TNF alpha, as found with TNBS.

A review of literature revealed preclinical studies of other drugs targeting AMPK pathway that have shown anti-inflammatory effect in TNBS model of colitis.[27,28] In a study by Arab et al, dapagliflozin was studied in TNBS induced acute colitis in Wistar rats.[27]

Arab et al, also assessed phosphorylated AMPK (p-AMPK) to total AMPK ratio which signifies AMPK activation. This ratio was significantly reduced in disease control group in comparison to normal control. P-AMPK to total AMPK ratio was significantly increased in study groups treated with dapagliflozin 1 mg/kg and 5 mg/kg in comparison to disease control group.

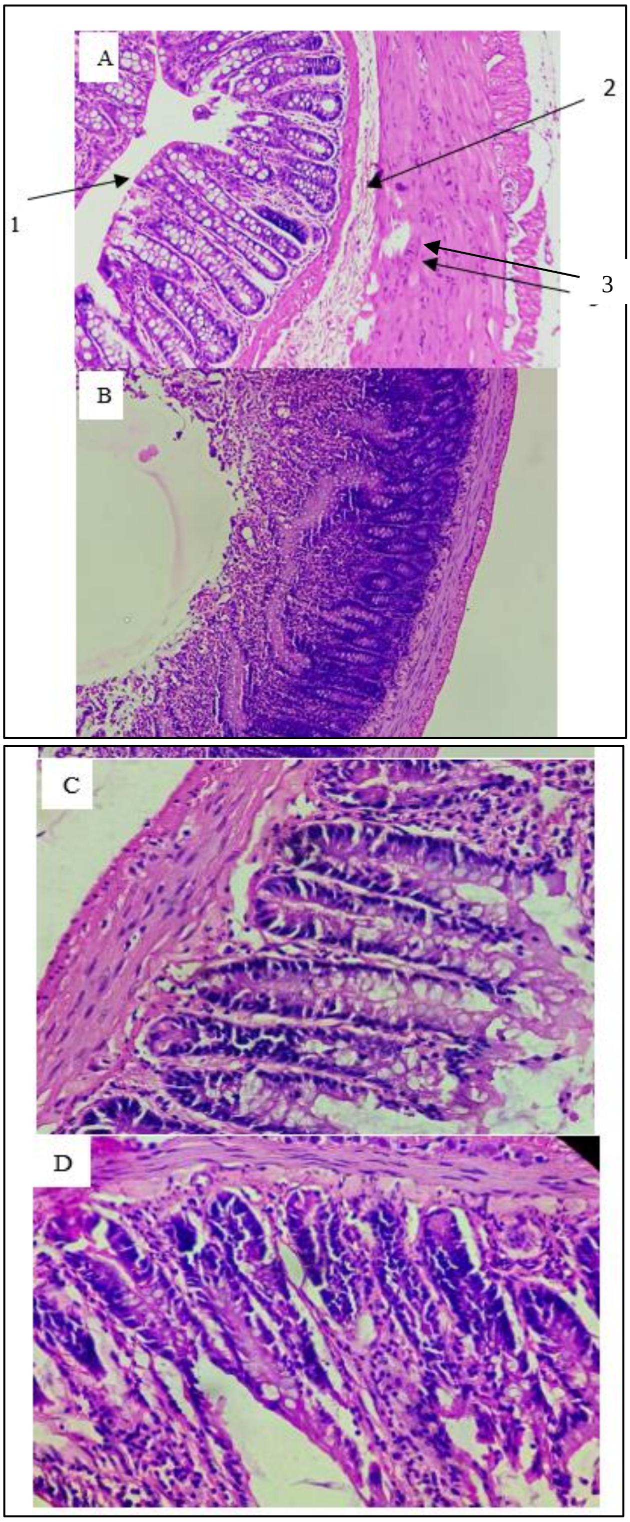

Figure 1: (A) Histopathology of colon depicting normal architecture (1. Mucosa 2. Submucosa 3. Muscle layer and serosa). (B) Colon in the disease control group showing disruption of cryptic architecture and leucocytic infiltration throughout the colon. (C) Crypt architecture with few leucocytic infiltrates in sulfasalazine group. (D) Crypt architecture with mild leucocytic infiltration in metformin treated group.

Another study by El-Rous et al, evaluated dapagliflozin in acetic acid induced colitis in Sprague Dawley rats.[28] Dapagliflozin was found to significantly reduce colon weight by length ratio, DAI score, macroscopic score and histopathologic score in comparison to disease control. It also significantly reduced pro-inflammatory markers i.e., MPO levels and IL-6 levels and significantly increased anti-inflammatory marker i.e., IL-10 levels in comparison to disease control group. Thus, Metformin's activity in improving the colitis can be attributed to its anti-inflammatory activity. TNBS induced colitis model used in this study has established, good construct validity.[11-13] The study assessed effect of metformin on inflammatory changes developed by TNBS and was backed by histopathological evaluation and macroscopic score along with DAI score. Metformin's beneficial effect may help in repurposing it for Crohn's disease.

Limitations of the study was that Metformin with a wide range of doses and addition to existing therapies can be carried out. Assessment of biomarkers can further validate Metformin's beneficial activity in this model.

CONCLUSION

The acute on chronic TNBS model mimics the relapsing remitting nature of Crohn's disease in humans, hence the efficacy of metformin may prove beneficial for all patients. To conclude, the knowledge that Metformin acts on inflammatory mediators involved in the pathogenesis of acute, chronic and acute on chronic phases opens an opportunity for metformin to be placed as an alternative to sulfasalazine for the management of relapses in Crohn's disease.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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