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Metformin as an Adjuvant Therapy Attenuates Dextran Sulphate Sodium-induced Acute Colitis in Rats

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Authors' contributions

This work was carried out in collaboration among all authors. Author RMM designed the study, supervised the experiments, performed the data analysis and wrote the first draft of the manuscript. Author FHA designed the study, performed the literature search, acquired the data and performed the data analysis. Author MA supervised the experiments and performed the data analysis. Author ASA designed the study, supervised the experiments, performed the data analysis and wrote the first draft of the manuscript. Author FOK supervised the experiments and performed the data analysis. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In numerous cases, patients with inflammatory bowel diseases (IBDs) are refractory to standard treatment. Sirolimus (SIR) and tacrolimus (TAC) are immunosuppressant drugs with encouraging outcomes. However, they have side effects causing limitations in their use. Metformin (MET), which is an antidiabetic drug, has promising anti-inflammatory effects. Thus, this study aimed to validate the effect of the concomitant administration of MET and SIR or TAC in the management of experimentally induced colitis.
Study Design: Dextran sulphate (DSS) induced colitis model was used.

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Methodology: Colitis was induced by administering 5% DSS in water twice daily via oral gavage for 9 days. MET 200 mg/kg alone or in combination with SIR 1 mg/kg or TAC 1 mg/kg was started on day 7 and was continuously administered for 12 days. Then, samples of distal colon tissues were collected for histopathological and immunohistochemistry staining. Then, the pro-inflammatory cytokine, tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-17A levels in tissue homogenates were measured.
Results: MET, SIR or TAC significantly attenuated the effect of DSS and the levels of all pro-inflammatory cytokines. Moreover, adding MET reinforces the effect of SIR and TAC.
Conclusion: MET had a strong anti-inflammatory effect against DSS-induced colitis. Hence, it could be a promising adjuvant therapy in the management of IBDs. The effect was mediated, in part, by inhibiting NF-κB activation. However, the results of this study must be further validated and translated to clinical implications.

Keywords: Metformin; immunosuppressant; inflammatory bowel disease; ulcerative colitis; NF-κB.

1. INTRODUCTION

Inflammatory bowel diseases (IBDs) are chronic relapsing and remitting inflammatory diseases affecting the gastrointestinal tract (GIT). IBD has two clinical types, ulcerative colitis (UC) and Crohn's disease. These conditions differ in terms of clinical manifestations [1]. However, they have a similar aetiology [2]. Moreover, both affect millions of people worldwide [3] and in Saudi Arabia [4,5].

The exact aetiology of IBD is unknown. However, its pathophysiology involves multiple factors, which include dysregulated immune response to commensal gut flora in the mucosa of genetically susceptible individuals [6].

Currently, aminosalicylates, corticosteroids, immunomodulators, antibiotics, and biologic therapies are used for the treatment of IBD [7]. However, in several cases, patients with IBDs are refractory to standard drugs. A recent study showed the efficacy of the potent immunosuppressant tacrolimus (TAC; FK506) in the management of colitis [8]. Similarly, sirolimus (SIR), also known as rapamycin, is a macrocyclic antibiotic and potent immunosuppressant. SIR was found to be an effective adjuvant pharmacotherapy for severe refractory IBD in children [9] and adults [10]. However, it has serious adverse effects, such as a depressed immune system and increased susceptibility to infection [11,12]. Therefore, more novel therapeutic targets are urgently needed to control IBD.

One of the most interesting therapeutic targets for IBD is adenosine monophosphate-activated kinase (AMPK). AMPK is an energy sensor and hemostasis regulator in several cells. AMPK dysfunction has been associated with several disorders, such as diabetes and inflammatory diseases [13]. Remarkably, AMPK enhances intestinal barrier function and gut epithelial differentiation [14].

Metformin (MET) is a well-tolerated oral antidiabetic drug. Its therapeutic indications continue to expand. It has anti-tumour, anti-inflammatory and anti-ageing effects. Interestingly, it is one of the most commonly prescribed AMPK activators [15]. Recently, MET was found to improve colitis in the rodent models [16-19]. However, its potential synergistic effect in combination with immunosuppressants on colitis has not yet been assessed. Moreover, MET may minimal adverse effects, such as TAC-induced hyperglycaemia, via dose reduction [20]. Thus, the current study aimed to investigate the therapeutic effect of the concomitant administration of MET and low-dose SIR on chemically induced UC in rats. Furthermore, the mechanism of the synergistic effect of these two drugs was explored.

2. MATERIALS AND METHODS

2.1 Materials

Dextran sulfate sodium (DSS) salt (MW Ca 40,000) was purchased from ALFA AESAR, Germany. MET and Sodium carboxymethyl cellulose (NaCMC) were purchased from Sigma Aldrich Co., USA. SIR and TAC were purchased as a white powder from Beijing Mesochem Technology Co., Ltd, Beijing, China.

2.2 Animals

In this study, 35 male Wister rats weighing 200 gm ± 25 gm were used, and the rats were obtained from our Medical Research Center

animal housing. All animals were taken care based on ethical standards.

2.3 Experimental Design

The animals were randomly divided into the following groups (5 rats in each group): control, acute colitis, MET, SIR, TAC, MET+SIR and MET+TAC groups. In a pilot study and other previous studies [21], acute colitis was induced by administering 2.5 mL of 5% (w/v) DSS solution via oral gavage twice daily for the first 9 days (Fig. 1). From days 7 to 18, the rats received 0.5% NaCMC (vehicle), MET 200 mg/kg/day [18], SIR 1 mg/kg/day [22], TAC 1 mg/kg/day (Beijing Mesochem Technology Co., Ltd., Beijing, China) [23], MET+SIR and MET+TAC. All treatments were administered orally. Then, the rats were sacrificed 24 h from the last treatment. The whole colon of the rats was taken out for gross evaluation. Then, its length was measured (cm) and recorded. Then, the distal part of the colon was further cut longitudinally into two equal sections. One segment was fixed in a 10% formaldehyde solution for histopathology, and the other was washed with cooled phosphate buffered solution (PBS) and was stored in −80°C for immunological examination to measure inflammatory mediators.

2.4 Histopathological and Immuno-histochemistry Staining and Analysis

The paraffin-embedded colon tissue was sliced into 5-µm thick sections. Then, the slides were stained using standard hematoxylin-eosin (HE) stain. Similarly, the 5-µm thick sections of the paraffin-embedded colon tissues were deparaffinised and rehydrated. Then, the sections were incubated with anti-nuclear factor-κB (NF-κB) antibodies (Optiplus, Biogenex, Milmont Lane, CA, the USA) overnight at 4°C (1:150). After washing with PBS for three times, the sections were then incubated with the streptavidin-biotin complex for 1 h. All parts were stained with HE. The slides were visualised and examined using a light microscope (Olympus, Japan) under a magnification of 400×. Finally, the optical density values of the nucleus and cytoplasmic immune-positive cells were measured using the Image J software (NIH, Maryland, the USA).

2.5 Tissue Homogenate Preparation

In preparing colon tissue homogenates, 10% (w/v) of tissue samples with ice (0.03 M sodium phosphate buffer, pH 7.4) were homogenised using TissueLyser II

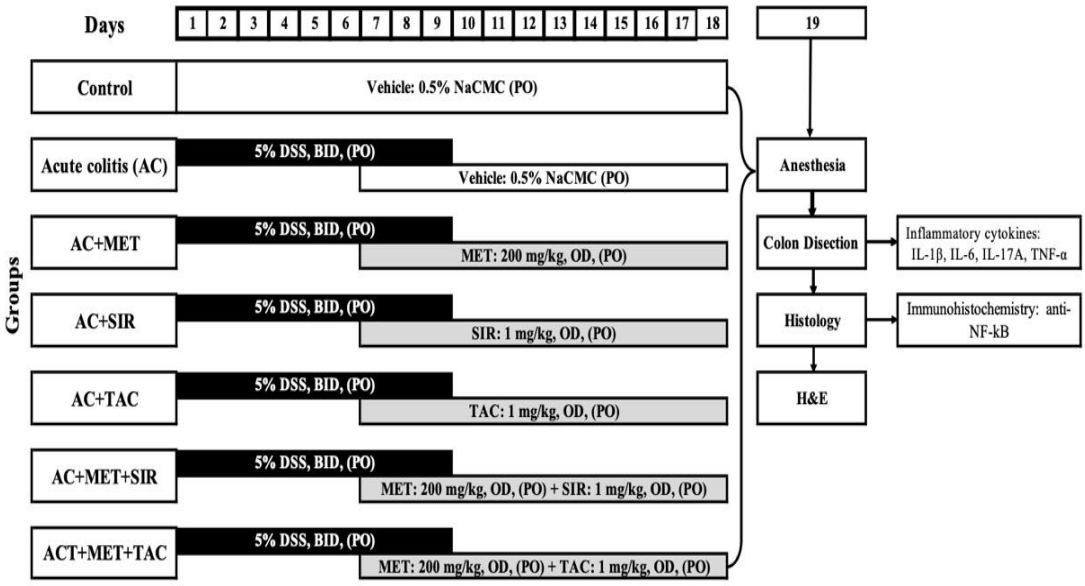

Fig. 1. Experimental design

(Qiagen Co., Germany). Then, the tissue homogenates underwent two freeze-thaw cycles to break the cell membrane. Then, the supernatant was collected. The protein concentration in the supernatants was measured using Bradford protein assay (catalogue number: MBS 355526) and was adjusted to obtain 100 mg/mL in each sample.

2.6 Analysis of Pro-inflammatory Cytokines in the Distal Colon Tissue Homogenate

Enzyme-linked immunosorbent assay (ELISA) kits for rat tumour necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6 and IL-17A (MyBioSource, the UK) were used. The procedures were conducted based on the manufacturer's instruction. Then, the absorbance of each test was read at 450 nm using ELISA microplate reader (BioTek Co., the USA). The results were expressed as pg/mL.

2.7 Data Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences software (IBM SPSS, version 23). The values in the figures were represented as mean ± standard deviation (M ± SD). One-way analysis of variance (ANOVA) analysis was used for the multiple comparisons of inflammatory parameters and numerical density estimation in immunohistochemistry analysis. A *P* value < .05 was considered statistically significant. The graphs were sketched using the GraphPad Prism software version 8 (GraphPad ® Inc., the USA).

3. RESULTS

3.1 Metformin alone or in Combination with Immunosuppressants Improved the Colon Length in Rats with DSS-induced Colitis

To investigate the potential role of MET alone or in combination with immunosuppressants on the progression of colitis, the colon length was measured after colitis was induced using DSS. The daily administration of DSS caused a significant reduction in colon length in the experimental group compared with the control group (Fig. 2; *P* < .001). However, the MET, SIR and combination groups showed a significant improvement in colon length compared with the acute colitis group (*P* = .003, *P* = .03 and *P* < .001; respectively). All groups, except the TAC group, showed improvement compared with the control group. Treatment with MET in combination with SIR resulted in a significant improvement compared with treatment with SIR or TAC alone (*P* = .05 and *P* = .03; respectively).

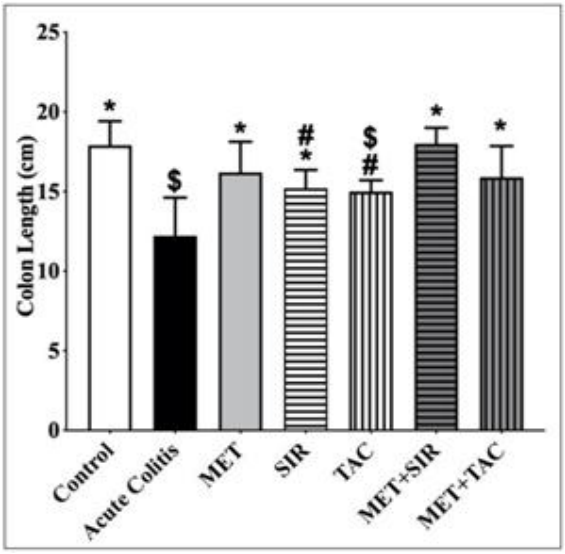

Fig. 2. Effects of metformin, sirolimus, tacrolimus and combined treatment on colon length

*MET: Metformin, SIR: Sirolimus, TAC: Tacrolimus. Data are expressed as means ± Standard deviation (SD) of n=5 rats from each group. Statistical analysis was carried out using One-Way-ANOVA test. *, #, $: statistically significant compared with the acute colitis group, MET+SIR group, and control group, respectively, P < .05*

3.2 Metformin alone or in Combination with Immunosuppressants Enhances the Histopathological Changes in the Colon in Rats with DSS-induced Colitis

To confirm the gross morphological findings in the colon, a microscopic assessment of colon sections was performed using H&E stain. The administration of 5% DSS solution twice daily for 9 days caused complete disturbance of crypt and loss of crypt surface columnar apex and basal lining of the cells with the aggregation of inflammatory cell infiltration, abscess formation and absence of normal Goblet cells and normal surface columnar cells in the experimental group (Fig. 3B) compared with the control group (Fig. 3A). The SIR-treated group presented with

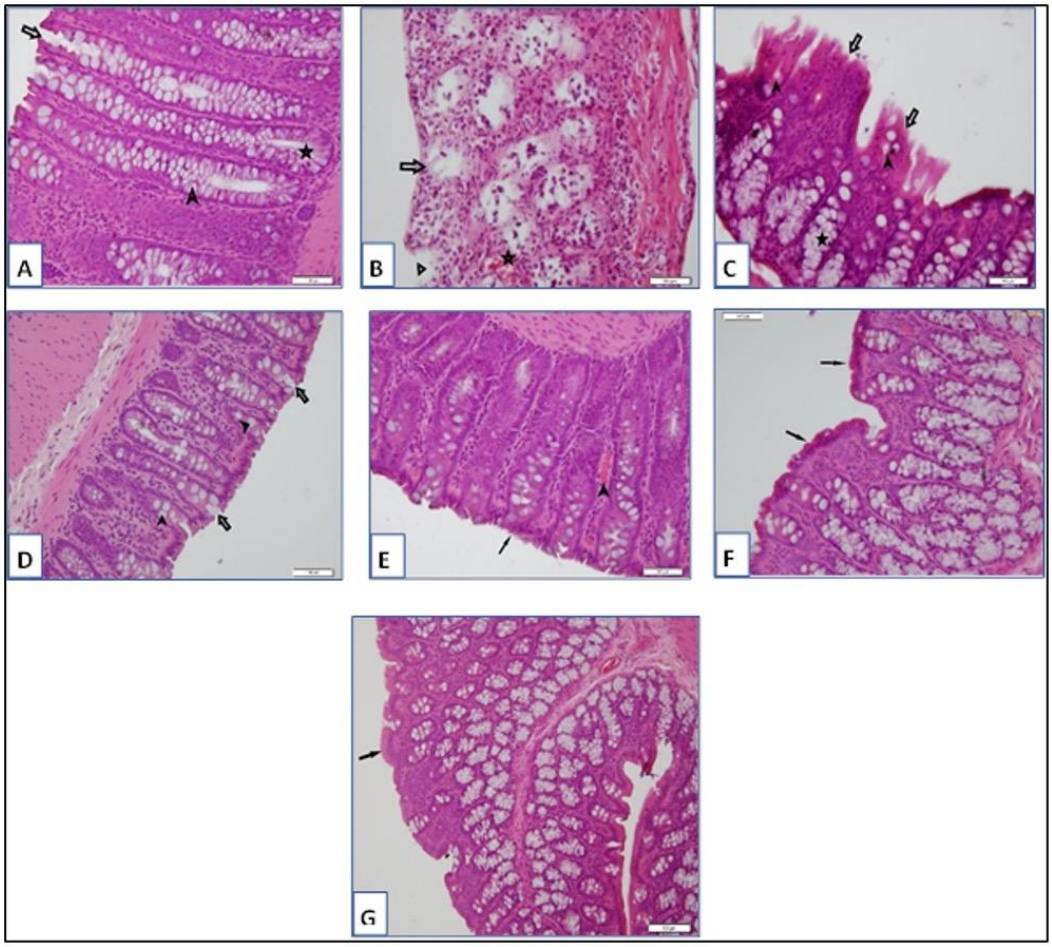

Fig. 3. Colon section stained with hematoxylin and eosin

(A) Control group shows normal crypt apical epithelial covering ex (arrow) and normal base (star), and normal Goblet cells (arrowhead). (B) Acute colitis group shows loss of crypt apex apical columnar epithelium (arrow) and base (star) with inflammatory cell infiltration, and abscess formation (triangle). (C) Sirolimus group shows regeneration of the preservation of disturbed apical crypts regions (arrow) with the reappearance of some Goblet cells (arrowhead) and normal crypt bases (star) but the surface columnar cells is still completely distorted. (D) Metformin group shows complete regeneration of the apical part crypts surface columnar epithelium (arrows) with the reappearance of many Goblet cells (arrowhead). (E) Tacrolimus group: shows incomplete regeneration of the apical part crypts epithelium with the reappearance of some Goblet cells. The lamina propria showed congested capillaries and infiltrated by inflammatory cells (arrowhead) and the surface columnar cells are distorted (arrows). (F) Sirolimus and metformin group shows complete regeneration of the apical part crypts with the reappearance of many Goblet cells, normal crypt bases and prominently appeared surface columnar cells with their striated borders (arrows). (G) Tacrolimus and metformin group: shows complete regeneration of the apical part crypts surface columnar cells (arrow), with the reappearance of Goblet cells, normal crypt bases, and prominently appeared surface columnar cells with their striated borders (arrow). Scale bar = 100 mm

regeneration of the disturbed apical crypts in the epithelium with the reappearance of some Goblet cells and normal crypt bases (Fig. 3C). However, the surface columnar cells were completely distorted. The colon section of the rats that received MET presented with complete regeneration of colonic crypts in the apical part with the reappearance of several Goblet cells and intact healthy surface columnar cells (Fig. 3D). The group treated with TAC presented with incomplete regeneration of crypts in the apical part with the reappearance of some Goblet cells. The lamina propria was infiltrated by inflammatory cells, and the surface columnar cells were still distorted (Fig. 3E). The SIR+MET group had complete regeneration of crypts in the apical part of the epithelium that looked healthy with striated brush borders along with the reappearance of several Goblet cells, normal crypt bases and prominently surface columnar cells with striated borders (Fig. 3F). Finally, the TAC+MET group presented with complete regeneration of the crypts in the apical part of the surface epithelium with the reappearance of Goblet cells and evident normal crypt bases in the surface columnar cells with striated borders (Fig. 3G).

3.3 Metformin alone or in Combination with Immunosuppressants Down-regulated NF-κB Expression in Rats with DSS-induced Colitis

The increase in the expression of NF-kB was associated with IBS[24] and the protein expression of NF-kB in the colon sections was assessed via immunohistochemistry. Results showed strong immunostaining of NF-κB in the colonic cell nuclei and cytoplasm of the colitis group treated with 5% DSS solution (Fig. 4B) compared to the control group (Fig. 4A). By contrast, the NF-κB immunoexpression was weak in the MET-, SIR-, and TAC-treated groups, as shown in (Fig. 4C, D and F, respectively). Moreover, the NF-κB immunoexpression was extremely weak in the combined treatment groups, as shown in (Fig. 4E and G, respectively).

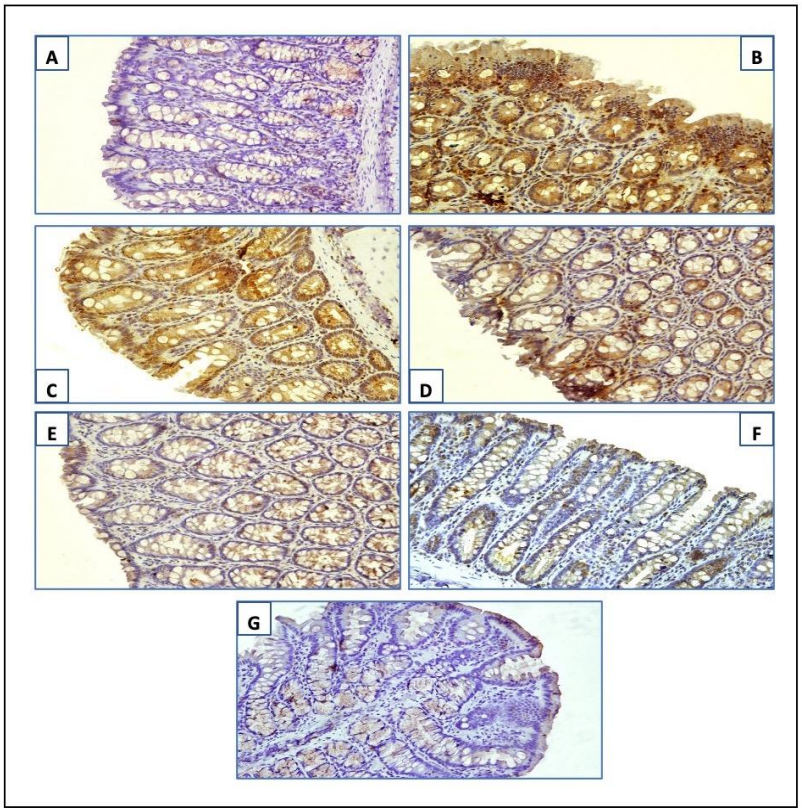

Fig. 4. Photomicrographs showing NF-κB immunoexpression in the colon of rats
(A) Control, (B) acute colitis, (C) metformin, (D) sirolimus, (E) tacrolimus, (F) sirolimus and metformin and (G) tacrolimus and metformin. Magnification power, 400x

Table 1. Effects of metformin, sirolimus, tacrolimus and combined treatment on the immunoexpression of NF-κB

	Groups						
	Control (n = 5)	**Acute colitis (n = 5)**	**MET (n = 5)**	**SIR (n = 5)**	**TAC (n = 5)**	**MET+SIR (n = 5)**	**MET+TAC (n = 5)**
Nuclei	$0.3 \times 10^{-4} \pm 0.2 \times 10^{-4}$*	$1.0 \times 10^{-4} \pm 1.2 \times 10^{-4}$	$0.6 \times 10^{-4} \pm 1.3 \times 10^{-4}$*	$0.9 \times 10^{-4} \pm 0.9 \times 10^{-4}$*	$0.7 \times 10^{-4} \pm 0.8 \times 10^{-4}$*	$0.5 \times 10^{-4} \pm 0.7 \times 10^{-4}$*	$0.4 \times 10^{-4} \pm 1.1 \times 10^{-4}$*
Cytoplasm	$1.4 \times 10^{-4} \pm 1.0 \times 10^{-4}$*	$3.6 \times 10^{-4} \pm 2.9 \times 10^{-4}$	$2.1 \times 10^{-4} \pm 1.5 \times 10^{-4}$*	$2.5 \times 10^{-4} \pm 2.8 \times 10^{-4}$*	$2.3 \times 10^{-4} \pm 2 \times 10^{-4}$*	$1.7 \times 10^{-4} \pm 1.5 \times 10^{-4}$*	$1.6 \times 10^{-4} \pm 1.2 \times 10^{-4}$*

*Data were expressed as mean ± standard deviation, n: number of rats per group. Statistical analysis was carried out using one-way ANOVA. MET: Metformin, SIR: Sirolimus, TAC: Tacrolimus. *: statistically significant in the acute colitis group, P < .05*

The protein expression of NF-κB in the nuclear area and cytoplasm in the colon was quantified in all groups, as shown in (Table 1). According to the immunohistochemistry analysis, there was a statistically significant increase in the numerical optical density values of the nucleus and cytoplasmic immune-positive (brown-stained) cells (nuclei and cytoplasm) in NF-κB in the colitis group compared with all the other groups that were treated and the control group (P = .03).

3.4 Metformin alone or in Combination with Immunosuppressants Inhibited the Levels of Inflammatory Biomarkers in the Colon Homogenate of Rats with DSS-induced Colitis

The overexpression of NF-κB increased the synthesis and release of pro-inflammatory mediators, such as TNF-α, IL-1β, IL-6 and IL-17A. Thus, the level of these mediators in the colon tissue homogenates of rats with DSS-induced acute colitis has been assessed.

DSS-induced colitis in AC group caused a three-fold increase in the TNF-α level (Fig. 5A), a four-fold increase in the IL-1β level (Fig. 5B), a three-fold increase in the IL-6 level (Fig. 5C) and a two-fold increase in the IL-17A level (Fig. 5D) in the compared with the control group (all P < .001). However, treatment with MET, SIR and TAC alone or in combination caused a significant decrease in the level of all cytokines in compared with the acute colitis group (all P < .001). Apart from TAC treatment alone, all other treatments decreased the cytokine level in the acute colitis group compared with the healthy control group (P > .05). Interestingly, the characteristics of the MET+SIR group were almost similar to those of the control group. Indeed, the combination of MET+SIR significantly decreased the TNF-α and IL-6 levels compared with TAC alone, which is the standard treatment (P = .03 and P = .01, respectively).

4. DISCUSSION

Although there is recent progress in understanding the pathophysiology of IBD, the current treatments are not extremely satisfactory, and the prolonged use of these treatments has side effects. Hence, a new and effective treatment for IBD with a better safety profile is urgently needed.

Most of the pharmacological effects of MET are caused by its ability to activate the AMPK pathway [15]. Moreover, a growing body of evidence shows the important role of AMPK in IBD pathogenesis. AMPK enhances the intestinal barrier function [14] and suppresses pro-inflammatory cytokines in the colon epithelial cells.[19] Consequently, the AMPK level in the colonic epithelial cells decreases with the progression of colitis [18]. Hence, MET could be a promising agent for the treatment of IBD.

The use of DSS in inducing UC with pathological characteristics similar to those found in the human colon is a well-established and accepted animal model [21]. The daily administration of 5% DSS-induced a significant shortening of the colon length in the experimental group compared with the control group. This finding is consistent with that of earlier studies [18,21,25,26]. Interestingly, the combination of MET and SIR had a significant additive effect, which is superior to immunosuppressants alone, on colon length. These findings underlined the anti-inflammatory effect of MET.

The shortening of the colon caused by DSS reflects the severity of inflammation. Hence, in the current study, the gross inflammatory signs in the colon segments were confirmed via microscopic histopathological evaluation. As expected, MET alone or in combination with immunosuppressants enhanced the inflammatory changes in the colon. The improvement in the histopathological changes caused by MET is correlated with the changes in colon length. This finding is in accordance with that of a recently published paper by Chen et al. [18].

The NF-kB signalling pathway is an inflammatory pathway that can be triggered and is associated with the pathogenesis of IBD [24,27]. Gan et al. reported an increase in the expression of NF-kB based on colon biopsy in patients with UC [28]. Similarly, in the current study, the administration of DSS increased the protein expression of NF-kB in the nuclei and cytoplasm of colon cells. In agreement with what published before [16], this study showed that treatment with MET decreased NF-kB expression. However, the combined treatment of MET and immunosuppressants had an addictive effect on the inhibition of NF-kB overproduction due to DSS. Therefore, MET mediates its anti-inflammatory effects on IBD by inhibiting NF-kB signalling.

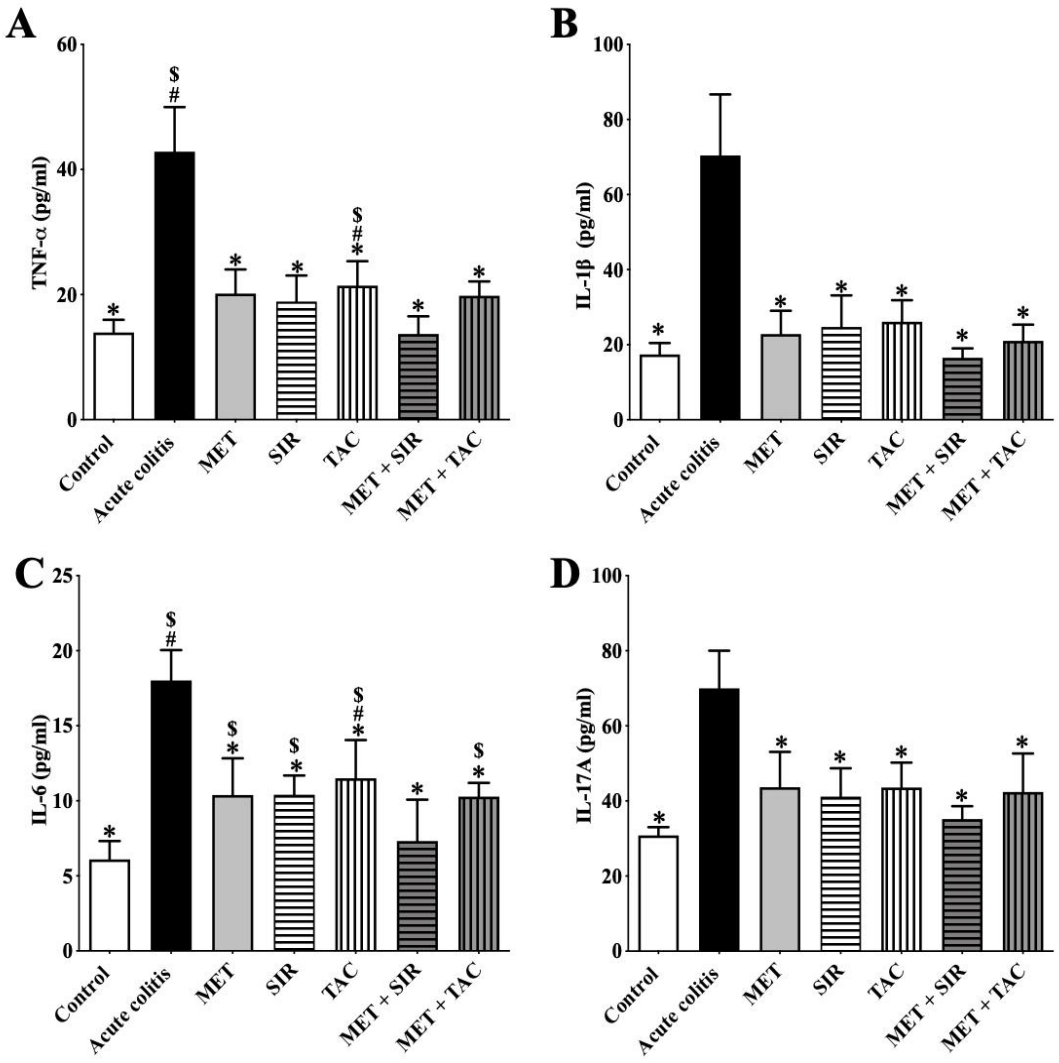

Fig. 5. Inflammatory biomarker levels in the colon homogenate tissues of rats with colitis

*MET: Metformin, SIR: Sirolimus, TAC: Tacrolimus. Data are expressed as means ± Standard deviation (SD) of n=5 rats from each group. Statistical analysis was carried out using One-Way-ANOVA test. *, #, $: statistically significant compared with the acute colitis group, MET+SIR group, and control group, respectively, P < .05*

In the physiological state, NF-kB is bound to its antagonists (kappa B [IkB] inhibitors) in the cytoplasm as an inactive NF-kB/IkB complex [29]. Upon activation of the intestinal epithelial cell caused by specific inflammatory triggers, NF-kB is released from the NF-kB/IkB complex and is translocated into the nucleus where it binds to DNA. Subsequently, it induces the transcription and synthesis of pro-inflammatory cytokines, such as TNFα, IL-1β, IL-6 and IL-12 [30,31]. Thus, these pro-inflammatory cytokines contribute to mucosal tissue damage commonly observed in IBD [27].

This study showed that the oral administration of 5% DSS solution resulted in a significant increase in the levels of TNFα, IL-1β, IL-6 and IL-17A in the colon tissues. These findings are consistent with those of earlier studies [32,33, 34]. However, SIR and TAC treatments reversed the increased levels of TNFα, IL-1β, IL-6 and IL-17A caused by DSS. Similarly, MET had similar effects. These results are in accordance with those of the study of Lee et al. [19] showing a decrease in IL-17 A, TNF-α, IL-6 and IL-1β levels in the colon tissues of the DSS-induced colitis mouse model after treatment with MET.

Interestingly, the combination of MET and SIR had an additive effect on all tested cytokine levels, which are closest to the normal range.

Taken together, these findings confirmed the efficacy of SIR and TAC for the management of DSS-induced colitis. TAC when used in the management of refractory UC had good outcomes [35]. SIR was also considered an effective therapy in children with severe IBD refractory to corticosteroids, and it resulted in clinical remission and mucosal healing [9]. Similarly, the efficacy of MET in the treatment of DSS-induced colitis was comparable to that of potent immunosuppressant drugs. Significantly, MET had an additive effect when used with SIR in the management of DSS-induced colitis, and these effects were superior among all groups.

Several studies have shown that MET exhibits immune-modulatory effects. Therefore, it should be used in the management of several autoimmune disorders [36]. Regarding IBD, studies revealed that MET may have beneficial anti-inflammatory effects, and it can be utilised as an adjunct therapy in patients with IBD [17]. The ability of MET to reduce inflammation in the DSS model was explained from an immunological point of view. MET effectively suppresses pro-inflammatory cytokines via the inhibition of NF-kB. Notably, its anti-inflammatory effect, which inhibits NF-kB, might be more effective in the treatment of colitis than in suppressing individual pro-inflammatory cytokines, including anti-TNF-α. However, the other mechanisms could contribute to the therapeutic efficacy of MET on colitis (Fig. 6). Lee *et al.* showed that MET had anti-inflammatory effects via the inhibition of signal transducer and the activation of transcription 3 (STAT3) phosphorylation in the IBD model in mice [19]. By contrast, Chen *et al.* showed that

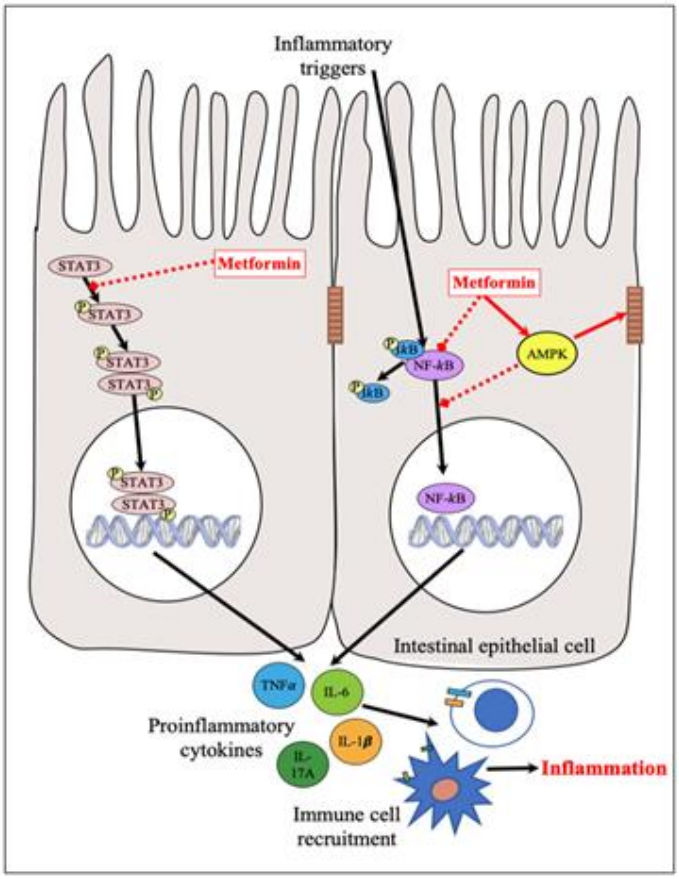

Fig. 6. Proposed mechanisms of metformin in colitis. The solid red arrows indicate activation, and the dotted arrows indicate inhibition

AMPK, adenosine 5'-monophsphate kinase; IL, interleukin; IkB, kappa B inhibitors; NF-κB, nuclear factor-κB; STAT3, signal transducer and activator of transcription 3; TNF-α, tumour necrosis factor-alpha

MET helps in preserving the tight junction in the intestinal epithelium via the activation of AMPK, thereby controlling the progression of colitis [18]. Taken together, MET regulates the different factors involved in the pathogenesis of IBD. These include immune pathways and intestinal barrier factors, making MET a more attractive alternative in the treatment of IBD.

5. CONCLUSION

In conclusion, this animal experiment provided biochemical and histological evidence of the efficacy of MET (200 mg/kg) alone or in combination with immunosuppressants in the treatment of colitis. Moreover, this study showed the ability of MET to suppress inflammatory histological changes and to decrease the expression of TNF-a, IL-1β, IL-6 and IL-17A, as induced by DSS in a mouse model of IBD. The effect is comparable to that of either SIR or TAC. Moreover, the combination of MET and SIR might have an additive effect. These effects were mediated, in part, by inhibiting NF-κB activation.

Given the magnitude of the efficacy of MET and its safety profile, this finding can be used as a basis for pilot clinical trials that aim to evaluate the effect of MET on IBD. However, the results of this study must be further validated and translated to clinical implications.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) were followed, as well as specific national laws where applicable. All experiments have been examined and approved by the research ethics committee of Faculty of Medicine at King Abdulaziz University approved the study (reference no. 127-19, date 24/02/2019).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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