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ORIGINAL ARTICLE

Pioglitazone Protects Against Acetic Acid –Induced Ulcerative Colitis in Rats via Modulation of Sirt1 / Nox4 Signaling and Suppression of Macrophage M1 Polarization

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ABSTRACT

Background: PGZ, a peroxisome proliferator-activated receptor γ agonist, possesses anti-inflammatory and antioxidant actions. The current study investigated an alternative molecular target of pioglitazone (PGZ) to protect against inflammatory bowel disease development.

Methods: Rats were allocated into 4 groups: normal control (NC), acetic acid (AA), PGZ (25 mg/kg intraperitoneal (i.p) and PGZ+ EX527 (a sirtuin1 inhibitor) (5mg/kg, i.p) groups.

Results: PGZ attenuated ulcerative colitis severity evident by decreased disease activity index, colon weight/ length ratio, gross damage, histological disruption and myeloperoxidase activity. PGZ suppressed oxidative stress by inhibition of lipid peroxidation and nitrite/nitrate content and enhancing glutathione level and antioxidant enzyme activities. PGZ reduction of colon injury was accompanied by suppressed inflammatory response as evidenced by decreased pro-inflammatory cytokines and IFN-γ- inducible protein 10 (CXCL 10) with restored interleukin-10. PGZ repaired the mucosal barrier integrity evident by increased claudin1expression. PGZ upregulated mRNA and protein expressions of SIRT1 (Silent information regulator 2 homologue 1) and p-IκBα (phosphory lated inhibitor kappa B-alpha) protein expression. PGZ down regulated NOX4 (nicotinamide adenine dinucleotide phosphate oxidase 4), TLR4 (toll like receptor4), IKKβ kinase and p65 subunit mRNA expressions. Additionally, in RAW264.7 cells, PGZ suppressed protein expressions of H3K9ac (histone H3 lysine acetylation), NOX4 and inducible nitric oxide synthase, upregulated SIRT1 and arginase-1protein expressions. EX527 co-treatment reversed all effects of PGZ.

Conclusions: Our findings indicated the beneficial effects of PGZ in UC via modulation of SIRT1/NOX4 signaling besides suppression of TLR4/NF-κB cascades and M1-M2 macrophage polarization.

Keywords: Pioglitazone; Ulcerative colitis; SIRT1; NOX4; Macrophage polarization

INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory debilitating disorder affecting the colon [1]. The milestone event in UC development is disruption of the bowel epithelial barrier which elicits adaptive and innate immune responses [2]. The robust production of reactive oxygen species (ROS) mediates extensive release of pro-inflammatory chemokines and cytokines with subsequent amplification of the inflammatory responses [3]. The enhanced inflammatory cascades recruit macrophages and neutrophils with widespread loss of mucosal integrity [4]. Silent information regulator 2 homologue 1 (SIRT1), a member of sirtuins (class III histone deacetylase), exerts a pivotal role in aging, oxidative stress, and inflammatory disorders like inflammatory bowel disease (IBD) [5]. A family of membrane enzymes, named nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), is well characterized with their participation in the pathophysiology of colitis. Several studies reported the correlation of NOX family activity and UC [6]. NOX4 is an inducible enzyme and does not need cytosolic factors to be active [7]. NOX4 inhibition by SIRT1 was reported [8]. The NOX family is a principle generator of superoxide via transferring electron from NADPH to oxygen. NOX-induced superoxide generation plays a cardinal role in pathogenesis of inflammatory diseases and carcinomas [9]. Toll like receptor (TLR)-4, a transmembrane protein, has an important role in initiating the inflammatory responses. TLR4 activates myeloid differentiation primary response gene 88 that enhances the nuclear translocation of nuclear factor kappa B (NF-κB) which in turn regulates transcription of several genes controlling cellular immune and inflammatory responses [10]. Macrophage polarization participates effectively in development and progression of inflammatory diseases. Macrophage phenotype 1 (M1) is recognized as an inflammatory enhancer while macrophage phenotype 2 (M2) has anti-inflammatory effect [11]. Patients with UC demonstrate marked colonic M1 macrophages [12]. Macrophage M1 to M2 polarization is one of the principle mechanisms by which SIRT1 produce its anti-inflammatory action [13]. Pioglitazone (PGZ), a selective peroxisome proliferator activated receptor γ (PPAR-γ) agonist, is well predictable by its immunomodulatory, antioxidant and anti-inflammatory properties [14, 15]. Our work investigated if PGZ mediates its effect via modulation of SIRT1/ NOX4 signaling, inhibition of TLR4/ NF-kB pathway and suppression of macrophage M1 polarization.

METHODS

2.1 *Drugs and chemicals*

Pioglitazone (Takeda Pharmaceutical Co., Ltd, Osaka, Japan), acetic acid (Nile Co. Egypt), Lipopolysaccharide (LPS), Thiazolyl blue tetrazolium bromide (MTT) assay kit and dimethyl sulphoxide (DMSO, 20%) all purchased from (Sigma-Aldrich, St Louis, MO, USA), EX527 (SIRT1inhibitor, APExBIO Technology, Houston, TX, USA), normal saline (NaCl 0.9%, EL-Nasr Pharmaceutical Co. Egypt).

2.2 *Animals*

Adult male albino rats (9-10 weeks old, 200-210g) were purchased from the National Research Centre, Giza, Egypt and kept in a pathogen free environment at temperature of 23 °C with 60% humidity under a 12h light/dark cycle. Rats gained free access to standard chow diet with water *ad libitum*. Rats were left for 8 days for acclimatization. The study protocol was approved by the Institutional Animal Care and Use Committee, Zagazig University **(ZU-IACUC/3/F/297/2023)** and in consistent with the National Research Council′s Guide for Care and Use of Laboratory Animals (1996).

2.3. *Induction of ulcerative colitis*

Rats were fasted for 12 h then under anesthesia (ketamine 50mg/kg; xylazine 10mg/kg), acetic acid (AA) (1ml, 4%, v/v in saline 0.9%) [16] was instilled rectally via a polyethylene tube (2mm diameter, 8cm length) which inserted into the rectum to reach the colon (about 6cm distance from the anus). Rats were maintained in a head-down vertical position, and /AA was slowly instilled

rectally over 1min and the tube was kept in situ for additional 1min then removed gently. The rats were remained in the previously mentioned position for 1min extra to prevent leakage of AA. Normal control group received equal volume of physiological saline instead of AA.

2.4. *Animal grouping, drug treatment*

Rats were randomly assigned into 4 groups (n= 8). **Group 1**: normal control (NC) group; **group 2**: colitis (AA) group in which rats received saline + DMSO (20%) by intraperitoneal injection (i.p.). **Group 3**: Pioglitazone pretreated (PGZ + AA) group: rats received pioglitazone at a dose of 25 mg/kg/day i.p. [17]. **Group 4**: EX527 + pioglitazone pretreated (EX527 +PGZ +AA) group: rats received pioglitazone and EX527 at a dose of 5mg/kg/day i.p. [18] 1h before pioglitazone injection. All drugs were administered for 7 days prior to AA rectal instillation which was performed on the 8th day, rats from groups 2-4 were subjected to induction of colitis but group 1 received rectal instillation of equal volume of physiological saline. Drug treatments continued for 3 days post AA instillation. Throughout the experiment, rats were checked daily for body weight, activity, consistency of stool and presence of blood in stool, all of these criteria were required to calculate the disease activity index (DAI). Rats were fasted for 24h after the last drug treatments then anesthetized with isoflurane in order to collect blood samples from the heart, then centrifuged 3000xg for 14min to obtain clear sera for biochemical assays. After euthanization, the distal colon of each rat was dissected, weighed, its length was measured, opened longitudinally, washed with saline to eliminate wastes, and scored macroscopically for colitis. After that, each colon sample was divided into 2 portions: the first portion was kept in formalin (10%) for histological and immunohistochemical analysis while the second portion was homogenized and subjected to centrifugation 12000xg for 14 min at 4^{o}C. The aliquots were stored at -80^{o}C.

2.5. *Disease Activity Index (DAI)*

DAI values range from 0 (no pathological features) to 12 (severe colitis) as follow: body weight loss (0-4); consistency of feces (0-4) and bleeding per rectum (0-4) [19].

2.6. *Macroscopic Scoring of colon injury*

The severity of colon injury was scored based on 0-10 scale criteria: 0= normal; 1= focal hyperemia; 2= ulcer without inflammation; 3= ulcer with inflammation; 4= multiple ulcer and inflammation; 5= damaged area about 1cm along the colon length; 6-8= damaged area is more than 2cm along the colon length. Then for every 1cm increase in the area of damage, the score is increased by one[20].

2.7. *Histological assessment*

2.7.1. Microscopic scoring of colon injury

The obtained colon tissue was fixed in formalin (10%) then processed in ethanol (95%) and cleared in xylene. Following embedding in paraffin blocks, 5µm colon sections were cut. The processed sections were deparaffinized and stained with Hematoxyline and Eosin (H&E) stain and examined under light microscope. All the histological procedures were performed by a specialist blinded with the treatments. The microscopic score was done according to as follow: 0= normal; 1=Inflammation or focal mucosal ulceration; 2= Inflammation and focal/extensive mucosal and submucosal ulceration; 3= Inflammation and focal or extensive ulceration with involvement of muscularis; 4= Inflammation and focal or extensive ulceration with serosa involvement; 5= Transmural inflammation, extensive ulceration with serosa involvement[21].

2.7.2. *Immunohistochemistry*

The paraffin wax colon tissue was cut into sections which were dewaxed with alcohol and incubated with H_2O_2 (3%) for 12min. Then heating was performed in a microwave oven. Primary polyclonal antibodies forSIRT1, claudin-1 and p-IκBα (phosphorylated inhibitor kappa B alpha) (1:100 dilutions) (Santa Cruz Biotechnology, CA, USA) incubated with the sections overnight at 4^{o}C. Subsequently, the sections were incubated with anti-rabbit secondary antibodies (Ding Guo Changsheng Biotechnology Co. Ltd) for 40min at room

temperature. Finally, the sections were washed with protein buffered saline for 10min. For color development, Diaminobenzidine and H_2O_2 (0.01%) containing solution was added, then counter staining with hematoxyline was done. Under light microscope (OLYMPUS, CX43), the slides were examined for immunoreactivity. A semiquantitative score was performed depend on a scale ranged from 0-3 (for the intensity of staining) as: 0=negative, 1= faintly positive cells, 2= focal aggregates of homogeneously stained cells, 3= disseminated positively stained cells. The score extent: 1= 1- <10%, 2= 10- <50%, 3=50-100%. The entire score was attained as: the entire score= intensity x extent, give 9-point score graded from 0 (negative stain) to 9 (strong widespread staining) [22].

2.8. *Biochemical analysis*

2.8.1. Assay of the colon myeloperoxidase (MPO) activity

As an indicator for neutrophil infiltration and mucosal injury, MPO activity was determined based on Myeloperoxidase Colorimetric Activity Assay Kit (ARG82770, biolaboratories, Arigo, Taiwan) according to the manufacturer′s instructions.

2.8.2. Assay of markers of inflammation and oxidation

The colon IL-6, IL-1β, TNF-α and IL-10 levels, serum (CRP) C-reactive protein level, colon nitrite/nitrate (NOx), MDA (malondyaldehyde), SOD (superoxide dismutase), CAT (catalase) and GSH (glutathione) levels were determined by using enzyme linked immunosorbent assay (ELISA) kits (MyBioScience, USA) according to the manufacturer′s instructions.

2.8.3. Quantitative real time polymerase chain reaction (qRT-PCR)

The total RNA extraction of the colon tissue was done using UNIQ-10 TRIZOL total RNA extraction kit (Sangon Biotech, Shanghai, China). The obtained NAA was revised transcriptionally into cDNA by superscript Choice Systems (Life Technologies, USA) based on the manufacturer′s commands. The target genes were amplified by the use of My ptc-200 PCR system (Bio-Rad, CA, USA) and quantitatively assayed by SYBER green PCR Master mix (Applied Biosystems, CA, USA). The primers for the target genes were listed in table 1. PCR reactions were performed at 95°C for 12sec and finally, at 62°C for 2min (annealing and extension). The ct values of target genes and housekeeping gene were determined dependant on the fluorescence signals. The relative level of expression of target gene was identified by $2^{-\Delta\Delta ct}$ formula [23].

2.8.4. *Cell culture*

Immortal RAW 264.7 mouse macrophages cell line was purchased from American Type Collection (Manassas, VA, USA). The cells were supplemented with fetal bovine serum (FBS, 10%) and penicillin/ streptomycin (100µg/ml) (Invilrogen, CA, USA) at a temperature of 37°C in presence of CO_2 (5%) and incubated in Dulbecco′s modified Eagle medium (DMEM) (Thermo Fisher Scientific, USA). RAW264.7 cells were trypsinized (trypsin/EDTA) in phosphate buffered saline then the adherent and non-adherent cells were collected and centrifuged at 400g for 5min. The cells were incubated in six well plates ($3x10^4$ cells/ cm^2) in serum free DMEM prior to treatment. PGZ and EX527 were dissolved in DMSO (1mM), then diluted (as proper) in culture medium. The cells were grouped as follow:

1) control group: the cells were treated with the same volume of culture medium for the same duration as other groups.

2) LPS group: the cells were incubated with LPS (100ng/ml) for 24h.

3) PGZ +LPS group: the cells were treated with PGZ (10µM) for 1h and further incubated with LPS (100ng/ml) for 24h.

4) PGZ +EX527 +LPS group: the cells were treated with EX527 (10µM) [24] for 30min prior to treatment with PGZ (10µM) for 1h and then incubated with LPS (100ng/ml) for 24h.

2.8.4.1. *Cell viability assay*

RAW264.7 cells were seeded in 96 well plates at solidity of $5x10^3$ cells / well for 12h at 37°C. After that, the cells were treated with different concentrations of PGZ for 24h. Then, the cells in each well were incubated

with 10µL of MTT solution (5mg/ml) at 37°C for 4h. Finally, the supernatants were discarded and DMSO (140µL) was added to every well and incubated for 20min. The percentage of viable cells was calculated based on the absorbance at 490nm by using microplate reader (Thermo, Waltham, USA). The formula to estimate the cell viability is: % of viable cells = OD (treated group) - OD (blank group) / OD (control group) - OD (blank group) x 100%.

2.8.4.2 Western blot analysis of SIRT1, H3K9ac (histone H3 lysine acetylation), NOX4, iNOS (inducible nitric oxide synthase) and Arg-1(Arginase-1) in Immortal RAW264.7 cell line

The total proteins were extracted and separated by electrophoresis using SDS-polyacrylamide gel (10%), next, transported to PVDF membranes. Skim milk (5%) was used as a blocking agent at room temperature for 1h. The membranes were inoculated throughout night at 4°C with specific primary antibodies against SIRT1 (1:500 dilution) (E-AB32901, Elabscience, USA), H3K9ac (1:1000 dilution) (Cat. No. GTX630554, Gene Tex, USA), NOX4 (1:100 dilution) (NB110-58851, NOVUS BIOLOGICALS, Abingdon, United Kingdom), iNOS (1:200 dilution) (NB300-605, NOVUS BIOLOGICALS, Abingdon, United Kingdom), Arg-1 (1:1000 dilution) (NBP1-32731, NOVUS BIOLOGICALS, Abingdon, United Kingdom) followed by incubation with secondary antibodies for 2h at room temperature. Enhanced chemiluminescence visualization reagents (GE, Healthcare, Sweden) were used to detect the blots under imaging system (ChemiDOC™ XRS; Bio-Rad, USA) with NIH Image J software. β-actin was considered as the housekeeping protein.

Statistical analysis:

The obtained data were presented as mean ± S.D. (standard deviation) and analyzed statistically by using One Way Analysis of Variance (ANOVA), followed by Tukey-post hoc test to define comparisons between groups. Graph Pad Prism version 5.01 was used. Values with $p<0.05$ were considered of significance.

RESULTS

3.1. Pioglitazone ameliorates the signs of AA-induced colitis in rats

The signs of colitis, body weight loss, diarrhea, and presence of blood in feces, were investigated. Rats received rectal AA instillation suffered from significant loss of body weight compared with NC group (Table 2). Moreover, the rats exhibited high DAI score which includes occurrence of diarrhea and marked blood content of feces together with significant increase in the colon weight/length ratio, which is a good marker of the inflammatory process affecting the colon (Table 2). Macroscopically, the colon showed severe destruction characterized by damaged mucosa, hyperemia, and thickened bowel wall in addition to ulcerations (Table 2). PGZ attenuated these destructive features in respect to AA group (Table 2). On the other hand, combined administration of PGZ and EX527 reversed the previously mentioned effects of PGZ (Table 2).

3.2. Pioglitazone mitigates the histological derangements

The colon sections in AA group revealed significant microscopic damage scores compared with NC group as they showed mucosal and submucosal changes with loss of the mucosal epithelium and ulceration. Additionally, neutrophils infiltrated mucosa, submucosa and muscularis (Fig 1B). PGZ pretreatment significantly reduced the microscopic damage scores as shown by preservation of the colon cellular architecture with significant decrease in the inflammatory cell infiltration (Fig 1C). However, co-treatment with EX527 markedly reversed the previously mentioned histological improvement encountered with PGZ (Fig 1D).

The colonic tissue infiltration with neutrophils was determined by MPO activity which demonstrated significant increase in AA group when compared with NC group (Fig 2A). PGZ significantly decreased MPO activity in respect to AA group (Fig 2A).

However, EX527 administration afforded significant elevation in colon MPO activity in relation to PGZ group (Fig 2A).

3.3. Pioglitazone reduces the colon pro-inflammatory cytokines and chemokine

Acetic acid elicited severe inflammatory reaction evidenced by significant increase in the colon levels of proinflammatory cytokines: IL-6, TNF-α and IL-1β compared with NC group (Fig 2B, C, D). Moreover, the colon CXCL-10 mRNA expression exhibited significant increase in AA group in relation to NC group (Fig 2F). While, the colon IL-10 level showed significant decrease in AA group in respect to NC group (Fig 2E). PGZ significantly lowered the colon IL-6, TNF-α and IL-1β levels compared with AA group (Fig 2B, C, D). Also, PGZ significantly downregulated the colon mRNA expression of CXCL-10 in respect to AA group (Fig 2F). As regard the colon level of anti-inflammatory IL-10, PGZ group showed significant increase compared with AA group (Fig 2E). These observations refer to the capability of PGZ to downregulate the inflammatory cytokines and upregulate the anti-inflammatory IL-10. On contrary, co-treatment with EX527 reversed the previous effects of PGZ.

3.4. Pioglitazone lowered the serum C-reactive protein level

As shown in Fig 3A, the serum CRP levels in AA group significantly increased compared with NC. PGZ significantly lowered the serum CRP levels in relation to AA group. However, co-treatment of PGZ with EX527 diminished the effect of PGZ on serum CRP level.

3.5. Pioglitazone attenuates the colon oxidative injury and enhances colon antioxidant defense

AA triggers marked oxidative tissue reactions detected by elevated colon levels of lipid peroxidation end product, MDA, compared with NC group (Fig 3B), while, the colon level of GSH showed significant decrease (Fig 3C) as well as the colon activities of SOD and CAT (Fig 3D, E). PGZ significantly decreased the colon MDA level and increased GSH level and the activities of SOD and CAT in comparison with AA group (Fig 3D, E). In contrast, EX527 significantly reversed the observations.

3.6. Pioglitazone amended the colon Nitrite/Nitrate (NOx) content

As shown in Fig 3F, the colon NOx content in AA group significantly increased compared with NC group. PGZ revealed significant decrease in colon NOx content in respect to AA group, while, in the presence of EX527, PGZ failed to lower the colon NOx.

3.7. Pioglitazone upregulates the colon mRNA expression of SIRT1 and inhibits NOX4 mRNA expression

The colon mRNA expression of cell survival regulator SIRT1 showed significant downregulation in AA group (Fig 4A). PGZ significantly enhanced the colon SIRT1 mRNA expression. Co-treatment with EX527 significantly suppressed the colon mRNA expression of SIRT1 in relation to PGZ group (Fig 4A).

mRNA NOX4 colon expression showed significant increase in AA group (Fig 4B). PGZ significantly inhibited mRNA NOX4 colon expression compared with AA group. On contrary, combined treatment with EX527 significantly reversed the above effect of PGZ (Fig 4B).

3.8. Pioglitazone suppresses the colon mRNA expressions of TLR4, IKK_{β} (the inhibitor of NF-κB kinase) and NF-κB-p65

In AA group, the colon mRNA expressions of TLR4, IKK_{β} and NF-κB-p65 showed significant increase compared with NC group (Fig 4C, D, E). PGZ group revealed significant decrease in mRNA expressions of the previously mentioned genes in respect to the AA group. However, EX527 significantly reversed the downregulatory effect of PGZ on TLR4, IKK_{β} and NF-κB-p65 mRNA expressions (Fig 4C, D, E).

3.9. Pioglitazone reduces the colon level of p-$I\kappa B_{\alpha}$, enhances SIRT1, and improves the intestinal barrier function

An inflammatory colonic reaction was elicited by acetic acid leading to enhanced phosphorylation of $I\kappa B_{\alpha}$ with subsequent extensive immunohistochemical expression of p-$I\kappa B_{\alpha}$ compared with NC group (Fig 5B). PGZ significantly downregulated p-$I\kappa B_{\alpha}$ immunohistochemical expression in respect to AA group (Fig 5C). As regard colon SIRT1 protein expression, it showed significant

decrease in AA group compared with NC rats (Fig 5G). PGZ afforded significant increase in colon tissue SIRT1 protein expression level in relation to AA group (Fig 5H). On contrary, Ex527 reversed PGZ effects on p-IκB$_\alpha$ and SIRT1 protein immunohistochemical expression levels (Fig 5D, I).

The protein expression level of claudin1, a tight junction protein that indicates the integrity of mucosal barrier, was significantly decreased in AA group when compared with the NC group (Fig 5L). Furthermore, PGZ treated group exhibited significant upregulation in claudin 1 expression level in respect to AA group (Fig 5M). On contrast, EX527 significantly diminished the colonic expression level of claudin1 (Fig 5N).

3.10. Effect of pioglitazone on the cell viability

RAW264.7 cell viability was determined colorimetrically by MTT assay with multiple concentrations of PGZ (2.5, 5, 10, 20, 40μM). The percentage of cell viability exhibited significant reduction at PGZ concentration of 20μM compared with the control group (Fig 6A). So, PGZ at a concentration of 10μM was selected in the following experiments to avoid occurrence of cytotoxicity.

3.11. Pioglitazone suppresses M1 macrophage polarization in RAW264.7 cell line via modulating SIRT1/NOX4 signaling

LPS challenged macrophages showed significant enhancement in iNOS protein expression level (M1 macrophage marker). PGZ significantly reduced LPS induced iNOS protein expression level and effectively elevated Arg-1 protein expression (M2 macrophage marker) (Fig 6B, F, G). Furthermore, SIRT1 expression was significantly enhanced (Fig 6B, C) while, H3K9ac (an upstream positive regulator for NOX4) and NOX4 proteins expression levels showed significant reduction in PGZ treated cells when compared with LPS challenged cells (Fig 6B, D, E). These findings suggested that PGZ exerts anti-inflammatory activity, in part, by suppression of M1 macrophage polarization and stimulating M2 macrophage polarization. Next, in order to clarify the involvement of SIRT1 in M1 macrophage polarizing inhibitory effect of PGZ, the cells were treated with SIRT1 inhibitor, EX527, prior to treatment with PGZ and before incubation with LPS. Interestingly, protein expression level of iNOS showed significant elevation while, Arg-1 protein expression level was significantly reduced when compared with PGZ treated cells (Fig 6).

Table 1: primers required for qRT-PCR

Name	Primer sequence
SIRT1	F: 5'-TCATTCCTGTGAAAGTGATGACGA-3' R:5'-CTGCCCTAGTGTCATATCATCCAA-3'
NOX4	F: 5'-TTCTGGACCTTTGTGCCTATAC-3' R:5'-ATCTGAGGGATGATTGATTACTG-3'
TLR4	F: 5′-AGGACTGGGTAAGGAATGAGC-3′ R:5′-ATCACCTTTCGGCTTTTATGG-3′
NFκB-p65	F: 5′-ACCTGGAGCAAGCCATTAGC- 3′ R: 5′- CCGCATTCAAGTCATAGTCCC-3′
CXCL-10	F:5′-GGGATCCCTCTCGCAAGAA-3′ R:5′-CTCAGCGTCTGTTCATGGAAGT-3′
GAPDH	F:5'-TGCCACTCAGAAGACT -3', R: 5'-TTCAGCTCTGGGATGACCTT-3'.

Table 2: Effect of pioglitazone and co-treatment with EX527 on body weight, % decrease in body weight, colon/weight length ratio, macroscopic score, and disease activity index of rats with AA-induced colitis

Groups	B.W (gm)	%↓in B.W	colon/wt length ratio (mg/cm)	Macroscopic score	DAI
NC	201.9± 2.9	0.00	99.88± 3.64	0.18 ±0.08	o.25± 0.14
AA	170.9 ±2.9*	16.92*	152.10 ±3.90*	7.12 ±0.83*	7.37 ±0.78*
PGZ+AA	188.8± 1.6$^{*\#}$	6.48$^{*\#}$	119.50 ±4.59$^{*\#}$	3.0 ±0.75$^{*\#}$	3.22 ±0.57$^{*\#}$
PGZ+EX527 +AA	169.0 ±3.07$^{*\$}$	15.35$^{*\$}$	140.9 ±5.66$^{*\$}$	6.75 ±0.70$^{*\$}$	6.20 ±0.71$^{*\$}$

B.W: body weight, DAI: disease activity index, NC: normal control, AA: acetic acid, PGZ: pioglitazone. Data represent mean ± SD. (n=8), *p < 0.05 significant vs. NC group, $^{\#}$p < 0.05 significant vs. AA group, $^{\$}$p< 0.05 significant vs. PGZ+AA group.

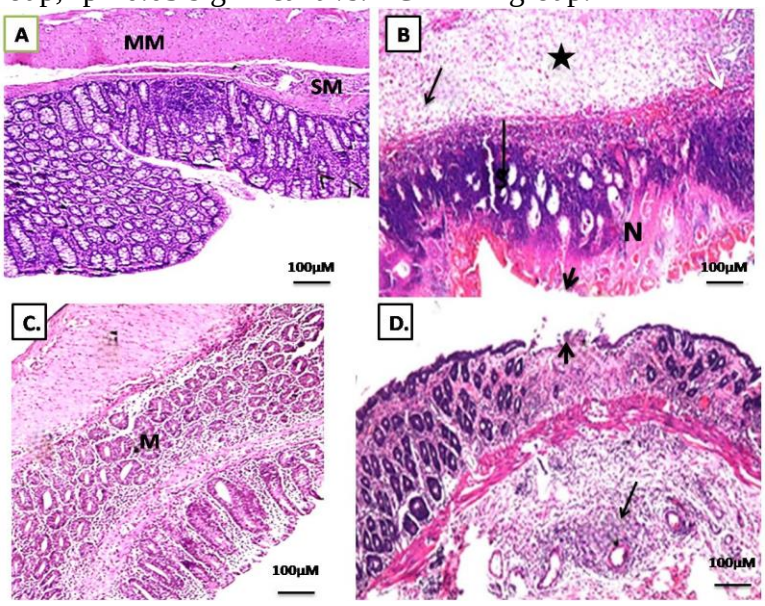

Fig.1: Colon sections stained with Hematoxyline and Eosin (magnification x40). (A) NC group. (B) AA group showed inflammatory cell infiltration (black arrow), mucosal edema (star), hemorrhage (white arrow), (N) necrosis and ulceration (black arrow head), and (C) PGZ group showed improved colon histological appearance. (D) PGZ+EX527 group showed reversal of PGZ effect on AA-induced colon histological damage. (n=8), M: mucosa; SM: submucosa; MM: muscularis mucosa; NC: normal control; AA: acetic acid; PGZ: pioglitazone.

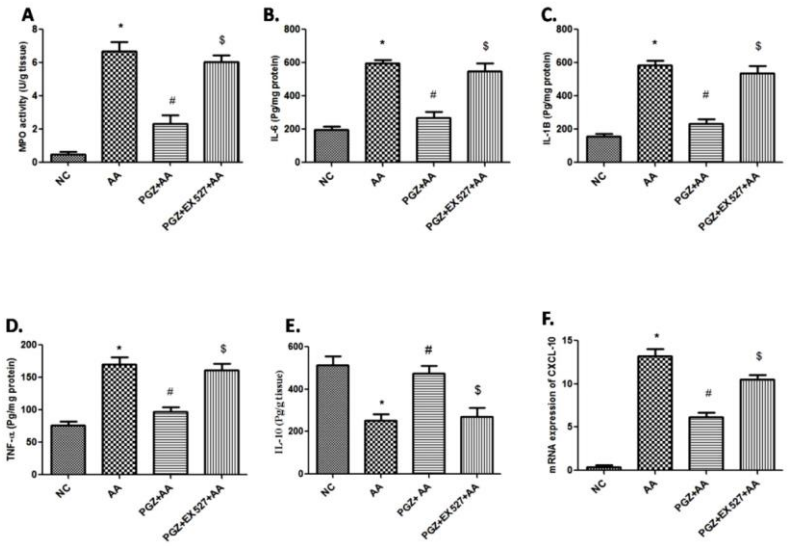

Fig.2: Effect of PGZ and co-treatment with EX527 on the colon (A) MPO activity, (B-E) levels of IL-6, IL-1β, TNF-α and IL-10, (F) mRNA CXCL10 expression. NC: normal control, AA: acetic acid, PGZ: pioglitazone. MPO: myeloperoxidase activity; IL-6: interleukin-6; IL-1β: interleukin 1beta; TNF-α: tumor necrosis factor alpha; IL-10: interleukin-10; CXCL 10: IFN-γ- inducible protein 10. Data represent mean ± S.D., (n=8),*p < 0.05 significant vs. NC group, $^{\#}$p < 0.05 significant vs. AA group, $^{\$}$p< 0.05 significant vs. PGZ+AA group.

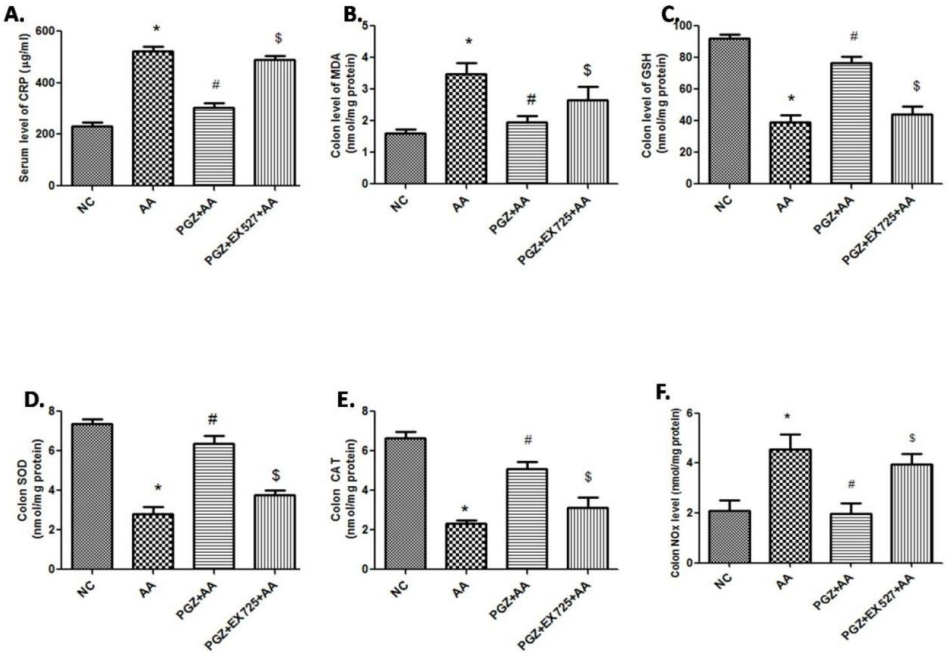

Fig. 3: Effect of PGZ and co-treatment with EX527 on the colon levels of (A-C) CRP, MDA and GSH. (D, E) SOD and CAT activities. (F) NOx content. NC: normal control, AA: acetic acid, PGZ: pioglitazone. CRP: C-reactive protein; MDA: malondyaldehyde; GSH: glutathione; SOD: superoxidedismutase; CAT: catalase; NOx: nitrite/nitrate. Data represent mean ± S.D., (n=8),*p < 0.05 significant vs. NC group, $^{\#}$p < 0.05 significant vs. AA group, $^{\$}$p< 0.05 significant vs. PGZ+AA group.

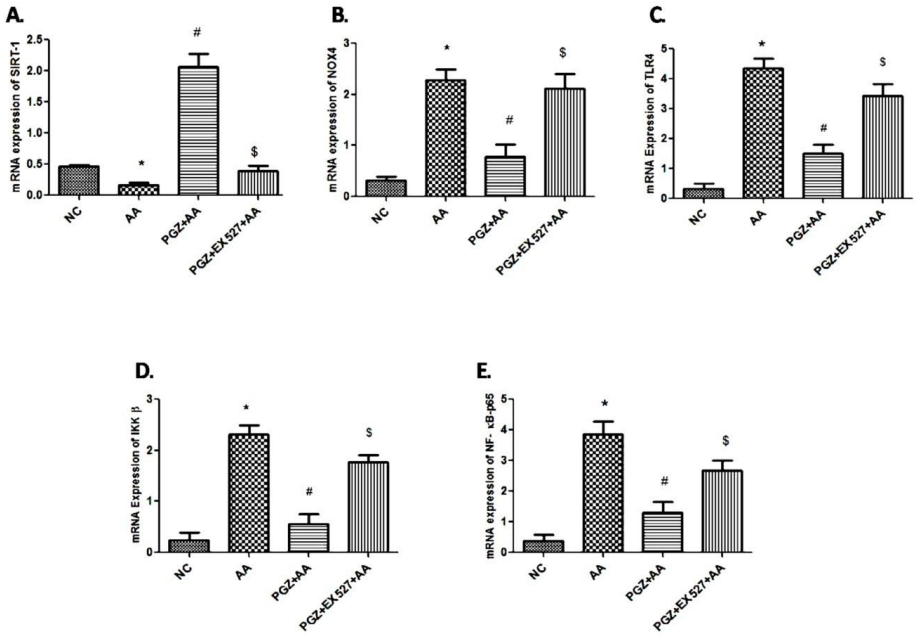

Fig. 4: Effect of PGZ and co-treatment with EX527 on the colon mRNA expression levels of (A-E) SIRT1, NOX4, TLR4, IKK$_{\beta}$ and NF-κB. NC: normal control, AA: acetic acid, PGZ: pioglitazone. SIRT1: Silent information regulator 2 homologue 1; NOX4: nicotinamide adenine dinucleatide

phosphate oxidase 4; TLR4: toll like receptor 4; IKK$_\beta$: the inhibitor of NF-κB kinase; NF-κB-p65: nuclear factor kappa beta- p65 subunit. Data represent mean ± S.D., (n=8), *p < 0.05 significant vs NC group, $^{\#}$p < 0.05 significant vs AA group, $^{\$}$p< 0.05 significant vs PGZ+AA group.

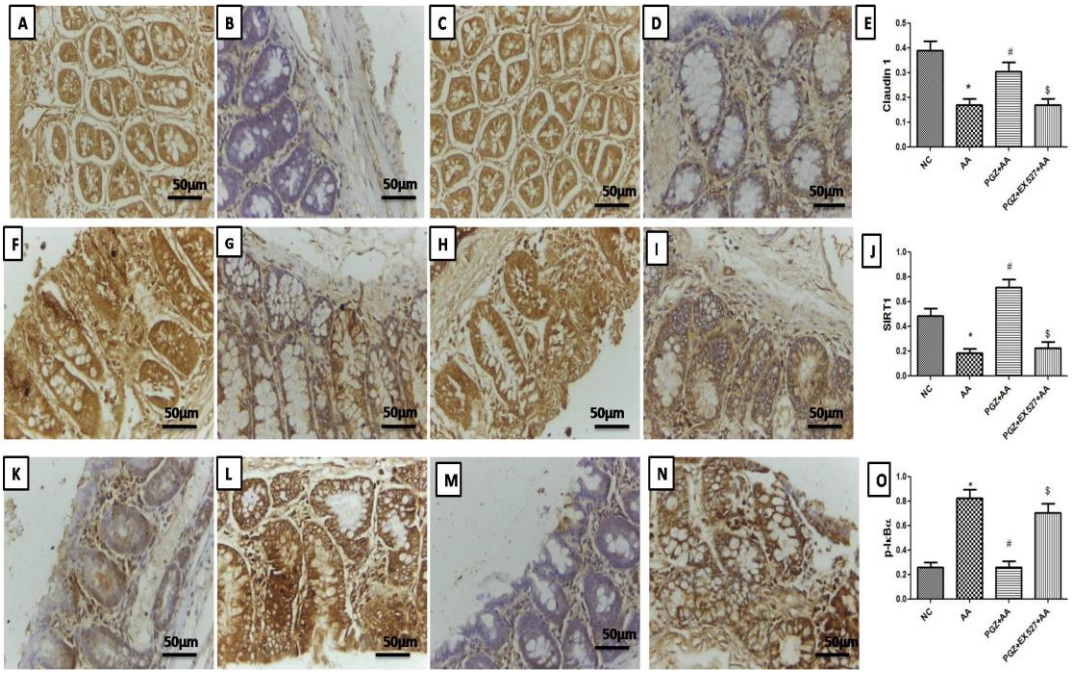

Fig.5: Immunostained colon sections (magnification x400) (A-D) for p-IκBα: (A) NC group. (B) AA group showed strong positive reactivity. (C) PGZ group showed marked reduction in stain reactivity. (D) PGZ+EX527 group showed reversal of PGZ effects. (F-I& K-N) for SIRT1and Claudin 1 respectively: (F, K) NC group. (G, L) AA group showed negative reactivity. (H, M) PGZ group showed marked positive reactivity. (I, N) PGZ+EX527 group showed reversal of PGZ effects. (E, J, o) the data were quantified. NC: normal control; AA: acetic acid; PGZ: pioglitazone. The data represented as mean ± S.D. (n=8), *p < 0.05 significant vs NC group, $^{\#}$p < 0.05 significant vs AA group, $^{\$}$p< 0.05 significant vs PGZ+AA group.

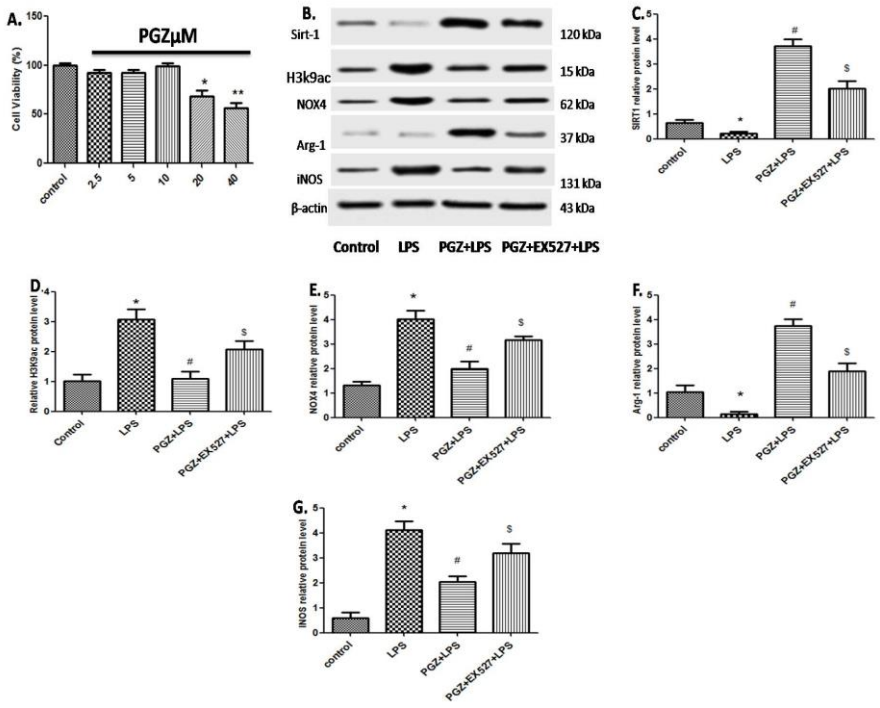

Fig.6: Effect of PGZ on expression levels of macrophage M1 and M2 bio-indicators in RAW264.7

cells. (A) Cytotoxicity of PGZ. **P< 0.05 (PGZ vs Control). (B) Effects of PGZ and co-treatment with EX527 on expression of SIRT1, H3K9ac, NOX4, Arg-1and iNOS in LPS-challenged RAW264.7 cells. The cellular proteins levels were estimated by Western blotting. (C-G): quantification of the cellular proteins expression. PGZ: pioglitazone; LPS: lipopolysaccharide; SIRT1: Silent information regulator 2 homologue 1; H3K9ac: histone H3 lysine acetylation; NOX4: nicotinamide adenine dinucleatide phosphate oxidase 4; Arg-1: arginase-1; iNOS: inducible nitric oxide synthase. The experiment was performed twice. * P<0.05 significance vs untreated control, # P< 0.05 significance vs LPS-treated cells, $ P<0.05 significance vs PGZ+LPS treated cells

DISCUSSION

PGZ efficiency in our investigation is derived from suppression of the colon oxidative stress and inflammation via modulation of SIRT1 / NOX4 signaling alongside restraining TLR4 / NF-κB pathway with suppression of M1 macrophage polarization. In the present work, the colonic tissue injury was attenuated by PGZ treatment which parallel with earlier studies that reported the protective effects of PGZ on UC [25, 26]. Oxidative stress is a critical contributor in UC pathogenesis. The robust production of ROS by stimulated phagocytes causes extensive lipid peroxidation and colonic tissue insults [27]. The depletion of the antioxidant defenses with predominance of oxidative reactions results in disturbed colon redox milieu. The current study revealed that AA-induced UC was associated with obvious increase in the colon MDA level with reduction in the antioxidant defenses which coincided with a previous study done by **Goe et al** [28]. PGZ decreased lipid peroxidation and enhanced the antioxidant machinery. In the same context, PGZ mitigated the renal ischemia reperfusion injury and suppressed ROS generation together with inhibition of pro-inflammatory cytokine release [29]. SIRT1 is a key cytoprotective agent that regulates the cell survival and homeostasis. Inhibition of SIRT1 activity is highly involved in the pathogenesis of the inflammatory bowel disease. Clearly, modulation of the colonic SIRT1 /NF-κB has been reported in UC experimental models leading to suppression of the oxidative stress with improvement in the mucosal injury [30]. In our study, reversal of PGZ effect on the colon SIRT1 expression and protein level by EX527 indicating the stimulatory effect of PGZ on SIRT1. These findings concur with previous studies which viewed PGZ as a SIRT1 agonist [31, 32]. In harmony with the current results, **Zhang** and co-workers revealed that PGZ reduced cisplatin –induced renal injury in mice via activation of SIRT1 signaling[33]. NOX4 exerts a central role in the inflammatory disorders. NOX family members are principal contributors in ROS production in the alimentary tract [34]. NOX4 is highly expressed in the cellular structures like mitochondria, plasma membranes, and endoplasmic reticulum as well as in vascular smooth muscle cells. Several studies have reported the involvement of NOX4 in the pathophysiology of inflammatory diseases of the bowel [35,36]. NOX4 constitutively produces superoxide and hence, its activity is a mirror of its expression level. In the current study, the colonic NOX4 mRNA expression was elevated in AA group in association with upregulated tissue lipid peroxidation, diminished tissue antioxidative defenses, upregulated inflammatory cytokines [IL-6, TNF-α], in addition to disturbed intestinal epithelial barrier integrity evident by reduced colon claudin1 expression level and elevated NOx level. Interestingly, PGZ treatment effectively amended all the previously mentioned conditions. The current findings reflect a positive relationship between NOX4 expression and colitis and so, curbing NOX4 expression by PGZ constitutes a promising mechanism of its gainful effects on UC. In the same context, **Pan** and co-workers stated that inhibition of NOX4 induced ROS generation by a bioactive danshensu derivative could suppress NF-κB/ NLRP3 inflammasome activity in disodium sulfate induced UC[6]. Thereafter, targeting NOX4 signaling provides a candidate therapeutic alternative for treatment of inflammatory bowel disease. SIRT1 mediates a negative regulatory effect

on NOX4 activity as reported by **Zhang et al** who found that SIRT1 suppressed NOX4 expression in vascular endothelium and prevent NOX4 mediated vascular dysfunction in rats′ offspring[8]. They also reported that SIRT1inhibited H3K9ac, a positive regulator for NOX4, leading to transcriptional suppression of NOX4 in endothelial cells which coincided with the current study. In parallel with the previous data, the current study showed that EX527 blocked the inhibitory effect of PGZ on the colon NOX4 mRNA expression and protein levels of H3K9ac and NOX4 in RAW264.7 cells. Mechanistically, diminishing the inhibitory effect of PGZ on the aforementioned proteins by EX527 indicating the role of SIRT1 in PGZ effects. NOX4 interacts with TLR4 with robust generation of ROS. Interestingly, the current results demonstrated that PGZ lowered the colon NOX4 as well as upstream effects of NOX4, TLR4. So, PGZ mitigated AA-induced oxidative and inflammatory responses, in part, due to suppression of TLR4-induced NOX4 production. **Dasgupta** and co-authors stated that the down regulation of SIRT1 in pancreatic carcinoma bearing mice enhanced NOX4, FOXO (Fork head box o) transcription family as well as induced NF-κB cascade mediating skeletal muscle wasting[37]. The NOX4 COOH terminal site interacts with TIR site of TLR4 in response to lipopolysaccharide pro-inflammatory signal activation in human aortic vascular smooth muscle cells. Moreover, NOX4 mediates superoxide burst which transformed into H_2O_2 enhancing NF-κB transcription activity. In the current study, mRNA expression of TLR4, NOX4 and NF-κB in the colonic tissue were significantly enhanced by AA indicating the interrelation among these proteins. In the same context, a previous study demonstrated that NOX4 promotes fatty acid oxidation in macrophages due to its interaction with TLR4 with the resultant enhanced NF-κB activity that promotes IL-6 and NLRP3 inflammasome transcription [38]. In HEK293T cell line treated by LPS, NOX4 knockdown abolished the interaction with TLR4, ROS generation and nuclear translocation of NF-κB providing evidence that NOX4 is a relevant regulator of TLR4 cascade [31]. NF-κB, a transcription factor, induces transcription of several genes that control inflammatory and innate responses. NF-κB is trapped in the cytoplasm by binding to IκBα which blocks its nuclear translocation. IκB kinase (IKK_β) phosphory lates and degrades IκBα leading to nuclear localization of NF-κB [1]. Coincided with the previous data, the current study demonstrated that PGZ effectively enhanced the colon mRNA expression of IKK_β and p- IκBα as detected by immunohistochemistry in association with reduced the colon mRNA expression of NF-κB-p65. The aforementioned findings indicated that PGZ exerts its anti-inflammatory effect via inhibition of NF-κB activity. In accordance with the current work, **Deng** and co-workersreported that PGZ exerts neuroprotective effects against traumatic brain insult in rats via inhibition of NF-κB signaling. The present results exhibited that EX527 reversed PGZ inhibitory effect on NF-κB expression indicating the participation of SIRT1 in PGZ anti-inflammatory action [39]. Activation of NF-κB depends principally on acetylation of p65 subunit at lysine 310. **Han et al** [40] reported that SIRT1 deacetylates NF-κB-p65 subunit at lysine 310 diminishing the genes transcription effects of NF-κB which supports our results. Tissue macrophages are central players in the defense mechanisms exerted by the body in inflammatory conditions. Intestinal macrophages greatly affect the innate immune system and being highly sensitive to inflammatory stimuli. Upon activation, macrophages either polarized to phenotype M1 (pro-inflammatory) or phenotype M2 (anti-inflammatory) [14]. In the present work, PGZ reduced markers for M1 phenotype and elevated IL-10 level as a marker for M2 phenotype indicating M1-M2 macrophage polarizing effect. Since M1 macrophage polarization participates in inflammation, the current study further established the impact of PGZ on macrophage polarization in RAW264.7 cell line.

CONCLUSIONS

PGZ possesses a protective activity on AA-induced UC through modulation of SIRT1

/NOX4 signaling and inhibition of TLR4/ NF-κB cascades. Additionally, PGZ enhanced M1-M2 macrophage polarization. Thereafter, the current study suggests that targeting NOX4 signaling is of a therapeutic value in treatment of inflammatory bowel disease.

Declaration of interest

The authors report no conflicts of interest.

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